The Anti-Cancer Potential of Polyphenols in the Treatment of Leukaemia

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Dedication

To Mum and Dad,

It is impossible to thank you adequately for everything you have done, your prayers, love, support, encouragement, and advice which have been a guiding light to illuminate my path.

To my beloved Sisters and Brothers,

Amal, Reem, Mohamad and Ibrahim

Thank you for being there during the good and bad times.

To my Nephews and Nieces

Raad, Yazan, Emran and Ghazal

Thank you for being my inspirations.

And finally to the Soul of the King of Saudi Arabia

Abdullah bin Abdulaziz Al Saud

For giving this opportunity of study abroad, and help my dreams become reality.

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This research process has been the most challenging, but most rewarding experience of my life. I have put so much of myself into this work, but numerous other people have assisted me along my journey.

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Abstract

Background: Leukaemia is a complex disease affecting all blood cell lineages. It affects millions of people worldwide each year and mortality rates are high, despite considerable improvements in treatment. Thus, new therapies for leukaemia are urgently needed to improve leukaemia patients' health and survival. Since polyphenols exert pro-apoptotic effects in solid tumours, our study investigated the effects of polyphenols in haematological malignancies.

Methods: The effects of eight polyphenols (quercetin, chrysin, apigenin, emodin, aloe-emodin, rhein, cis-stilbene and trans-stilbene) was studied on cellular proliferation, the induction of apoptosis and cell cycle progression in four lymphoid (JURKAT, MOLT-3, CCRF-CEM and U937) and four myeloid (HL-60, THP-1, K562 and KG-1a) leukaemia cells lines, together with normal haematopoietic control cells ($CD34^+$ HSC and $CD133^+$ HSC) from cord blood. Further to this, an investigation was made of the effects of the most promising polyphenols used in combination with nine standard chemotherapeutic agents (etoposide, doxorubicin, cyclophosphamide, chlorambucil, cisplatin, methotrexate, 6-mercaptopurine, 5-fluorouracil and imatinib). For this polyphenol and chemotherapy combination work four leukaemia cells lines were used: the two most sensitive (JURKAT and CCRFM-CEM) and two most resistant (KG1a and THP-1) to polyphenol treatment. Subsequently, an investigation was undertaken to identify potential mechanisms of action of these polyphenol when used alone and in combination with chemotherapeutics. The extrinsic and intrinsic apoptotic pathways were investigated together with effects on glutathione levels and DNA damage.

Results: Emodin, quercetin, and cis-stilbene were the most effective polyphenols at decreasing cell viability and inducing apoptosis. Lymphoid cell lines were normally more sensitive to polyphenol treatment compared to myeloid cell lines; however those myeloid (KG-1a and K562) cell lines which were most polyphenol resistant; were however affected by emodin and quercetin at micromolar treatment doses. Non-tumour cells were less sensitive to all polyphenols compared to the leukaemia cells. Mechanistically, most polyphenols alone depleted glutathione (GSH) levels associated with a direct activation in caspase 8 and/or caspase 9 in leukaemia cell lines at 24 h. Polyphenols also had differential capacities to induce DNA damage in the leukaemia cell lines. Polyphenols acted synergistically in lymphoid cell lines and differently in myeloid cell lines producing either synergistic, additive, competitive antagonistic or antagonistic effects; when they were combined with toposiomerase inhibitor agents (etoposide and doxorubicin) and alkylating agents (cyclophosphamide and chlorambucil, cisplatin). In contrast, they worked antagonistically with anti-metabolites agents (methotrexate and 6-mercaptopurine) in both lymphoid and myeloid leukaemia cell lines. Mechanistically the synergistic induction of apoptosis observed following the combination of polyphenols with

chemotherapeutic agents was caused by the direct activation of intrinsic or /and extrinsic apoptotic pathway through the up-regulation of caspase 8 or caspase 9 within the lymphoid and myeloid leukaemia cell line. Furthermore, it has been shown the synergistic effects observed when polyphenols and chemotherapy agents were combined was correlated with down regulation of GSH levels and an induction of DNA damage which drove apoptosis. Alternatively where there was an antagonist effect there was an up-regulation of GSH levels, a reduction in DNA damage and the level of apoptosis.

Conclusions: These findings demonstrate that polyphenols induce apoptosis and arrest cell cycle in leukaemia cell lines which could translate to anti-cancer activities in leukaemia, although the effects were dependant on polyphenol type and origin of the cell line investigated. Importantly, the differential sensitivity of emodin, quercetin, and cis-stilbene between leukaemia and normal cells suggests that polyphenols are potential therapeutic agents for leukaemia. Furthermore, this study concluded that the efficacy of standard chemotherapeutic agents were differentially modulated by polyphenols, producing either synergistic, additive or competitive antagonistic/antagonistic effects, which was dependent on type of polyphenol, chemotherapy agent and cell line. Interestingly the study showed that synergistic or antagonistic effects observed following the combination treatments were strongly dependent on the modulation of glutathione levels in association with the formation of γ-H2AX nuclear foci and DNA damage in leukaemia cell lines.

Dissemination

Published Papers

Mahbub AA, Le Maitre CL, Haywood-Small SL, McDougall GJ, Cross NA, Jordan-Mahy N[1] (2013). Differential effects of polyphenols on proliferation and apoptosis in human myeloid and lymphoid leukemia cell lines. *Journal of Anticancer Agents Medicinal Chemistry*, 13(10):1601-13.

Papers in Preparation

Mahbub AA, Le Maitre CL, Haywood-Small SL, Cross NA, Jordan-Mahy N[1]. Differential modulation of the efficacies of toposiomerase inhibitor agents: etoposide and doxorubicin in lymhpoide and myloide leukemia cells by polyphenols.

Mahbub AA, Le Maitre CL, Haywood-Small SL, Cross NA, Jordan-Mahy N[1]. Differential modulation of the efficacies of alkylating agents: cyclophosphamide and chlorambucil and cisplatin in lymhpoide and myloide leukemia cells by polyphenols.

Mahbub AA, Le Maitre CL, Haywood-Small SL, Cross NA, Jordan-Mahy N[1]. Antagonism effects of polyphenols combined with anti-metabolites agents: methotrexate and 6-mercaptopurine on cellular proliferation and apoptosis in human myeloid and lymphoid leukaemia cell lines.

Mahbub AA, Le Maitre CL, Haywood-Small SL, Cross NA, Jordan-Mahy N[1]. The potential mechanisms of action of polyphenols in the induction of apoptosis in human haematological malignancies.

Conference Presentations

4th International Congress on Leukemia Lymphoma Myeloma: 22nd -25th May (2013). Title of presented work: (1) Anti-proliferative and Pro-apoptotic Effects of Polyphenols on Human Myeloid and Lymphoid Leukaemia, and (2) Quercetin and Emodin Synergistically Enhance Chemotherapy Activity in Human Leukaemia Cell Lines. Istanbul, Turkey.

National Cancer Research Institution (NCRI): 5th November (2012). Title of presented Work: Anti-proliferative and Pro-apoptotic Effects of Polyphenols on Human Myeloid and Lymphoid Leukaemia. Liverpool, UK.

BMRC Summer Conference: 9th July (2012). Title of presented Work: Polyphenols Inhibit the Cell Proliferation and Induce the Apoptosis in Human Myeloid and Lymphoid Leukaemia Cell Lines. Sheffield, UK.

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The 5th International Conference on Polyphenols and Health (ICPH): 17th-20th October (2011). Title of presented Work: The Anti-Cancerous Potential of Polyphenols in the Treatment of Leukaemia. Barcelona, Spain.

Multiple Sclerosis and cancer user group at Sheffield Hallam University: Jun (2011). Title of presented Work: The Anti-Cancerous Potential of Polyphenols in the Treatment of Leukaemia. Sheffield, UK.

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Prizes and Awards

The Sir Alastair Currie Poster Prize was awarded for best poster and presentation entitled: "The Anti-cancerous Potential of Polyphenols in the Treatment of Human Myeloid and Lymphoid Leukaemia"; out of 88 posters from clinicians, pathologists and scientists. At Summer Meeting (202nd Scientific Meeting) of the Pathological Society of Great Britain and Ireland: 3rd -5th July (2012). Sheffield, UK. This abstract was also published in Journal of Pathology.

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Abbreviations

6-MP	6-Mercaptopurine
5-FLU	5-Fluorouracil
AML	Acute Myeloid Leukaemia
ALL	Acute Lymphoblastic Leukaemia
ATLL	Adult T-cell Leukaemia/Lymphoma
ARF	Alternative Reading Frame
ATP	Adenosine-5'-Triphosphate
Apaf-1	Apoptotic Protease Activating Factor-1
AP	Apigenin
ADD	Additive
ATM	Ataxia Telangiectasia Mutated
ATR	ATM-Rad3-Related
BM	Bone Marrow
BMNC	Bone Marrow Mononuclear
Bcl-2	B-Cell Lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Bad	Bcl-2-Associated Death Promoter
Bak	Bcl-2-Analgised/Killer
Bax	Bcl-2- Associated X Protein

HTLV-I	Human T-cell Lymphotropic Virus-1
HIF	Hypoxia-Inducible Factor
HER2	Human EGF (Epidermal Growth Factor) Receptor 2
HDAC	Histone Deacetylase
HGPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
Hoe	Hoechst 33342
INK4	Inhibitors of CDK4
IAPs	Inhibitor of Apoptosis Proteins
I^{CAD}	Inhibitor of Caspase Activated DNAse
IL	Interleukin
IC_{50}	Inhibitory Concentration of 50%
IM	Imatinib
INF-α	Interferon-alpha
JAK	Janus Kinase
JNK	Jun NH2-Terminal Kinase
LSCs	Leukemic Steam Cells
LSD	Lowest Significant Dose
MPT	Mitochondrial Permeability Transition
MMP	Mitochondrial Membrane

BH-3	Bcl-2 Homology Domain3
Bid	BH3-Interacting-Domain Death Agonist
Bik	Bik, Bcl-2-interacting killer
BRG1	Brahma-Related Gene-1
BIR	Baculovirus IAP Repeat
CLL	Chronic Lymphoid Leukaemia
CML	Chronic Myeloid Leukaemia
CDKs	Cyclin-Dependent Kinases
CDKIs	CDK Dependent Kinase Inhibitors
CAD	Caspase-Activated Deoxyribonuclease
c-FLIP	Cellular FLICE-Like Inhibiting Protein
C3	Caspase 3
CO_2	Carbon Dioxide
CIS	*Cis*-Stilbene
CYCLO	Cyclophosphamide
CLB	Chlorambucil
CSP	Cisplatin
CV	Co-efficient of Variation
CNS	Central Nervous System
CR	Complete Remission
DNA	Deoxyribonucleic Acid
DSBs	DNA Double Strand Breaks
DD	Death Domain
DED	Death Effector Domain
DISC	Death-Inducing Signalling Complex
Diablo	Direct IAP Binding Protein
DOX	Doxorubicin
DHFR	Dihydrofolate Reductase
ERK	Extracellular Regulated Kinase
E2F	E2 Promoter Binding Factor
EGFR	Epidermal Growth Factor Receptor
EMO	Emodin
ETP	Etoposide
FLT-3	Fms-Like Tyrosine Kinase 3
Fas	First Apoptosis Signal
FADD	Fas-Associating Death Domain Protein
FBS	Fetal Bovine Serum
FDA	French American Death Domain
FSC	Forward Scatter
G_1	Gap 1
G_2	Gap 2
GADD45	Growth Arrest and DNA Damage
GLUT1	Glucose Transporter 1
GSH	Glutathione
GST	Glutathione S-Transferase
GCS	Glutamylcysteine Synthetase
GS	Glutathione Synthetase
GR	GSH Reductase
GSS	GSH Synthetase
GTT	Gamma Glutamyltransferase
HSCs	Hematopoietic Stem Cells
HOX	Homeobox Gene

	Permeabilisation
MDM2	Murine Double Minute 2
M	Mitotic
MYC	Myelocytomatosis
MAPK	Mitogen-Activated Protein Kinase
MMPR	Methylmercaptopurine Ribonucleotide
MNCs	Mononuclear Cells
Mg^{2+}	Magnesium
MDR	Multidrug Resistance
MTX	Methotrexate
NK	Natural Killer
NF-kB	Nuclear Factor-kappa B
NER	Nucleotide Excision Repair
NSD	Non Significant Dose
OIS	Oncogene-Induced Senescence
O_2	Oxygen
PI3K	Phosphoinositol-3-Kinase
PKB	Protein Kinase B
PARP	Poly (ADP-ribose) Polymerase
PCD	Programmed Cell Death
PI	Propidium Iodide
PDGF	Platelet-Derived Growth Factor
PKC	Protein kinase C
PTEN	Phosphatase and Tensin Homolog
P-gp	P-glycoprotein
QUE	Quercetin
R	Restriction
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
Rb	Retinoblastoma Protein
RAS	Rat of Sarcoma
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT	Room Temperature
RH	Rhein
S	DNA Synthesis Phase
Smac	Second Mitochondria-Derived Activator Caspase
SSC	Side Scatter
STD	Standard Deviations
SHP-2	Src Homology 2 Domain Containing non-Transmembrane PTP
SF3B1	Splicing Factor 3B Subunit 1
SIPS	Subsequent Stress Induced Premature Senescence
TAC	Tricarboxylic Acid Cycle
TGF-α	Transforming Growth Factor-alpha
TGF-β	Transforming Growth Factor-beta
TNFR	Tumour Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRADD	Tumour Necrosis Factor Receptor 1-Associated Death Domain Protein
TKI	Tyrosine Kinase Inhibitor
TdT	Terminal Deoxynucleotidyl Transferase
WHO	World Health Organisation
Wt	Wild type
XIAP	X-linked Inhibitor of Apoptosis Protein

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Chapter 1

Introduction.

1.1 Introduction to Cancer

Cancer is considered as a leading cause of disease worldwide. In 2012, there were an estimated 14.1 million cancer cases and 8.2 million cancer deaths in the world (American Cancer society, 2014; Cancer Research UK, 2014). Approximately 7.4 million cases (53%) and 4.7 million deaths (57%) were estimated in males; and 6.7 million cases (47%) and 3.5 million deaths (43%) were estimated in females, giving a male: female ratio of 10:9 of cancer cases and 10:8 of cancer mortality (Cancer Research UK, 2014). There are more than 200 different types of cancer, but the top four most common cancers worldwide are lung cancer, breast cancer, colorectal cancer and prostate cancer, together accounting for 53% of all cancer cases and 46% of all cancer deaths. Furthermore the ten most common cancers in men and women based on the estimation of UK in 2011 (Cancer Research UK, 2014) and USA in 2014 (American Cancer society, 2014) are summarised and showed in Table 1.1.

Cancer is caused by both external (chemicals, radiation tobacco and infectious) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism) (Cancer Research UK, 2014). These factors may act together or in sequence to initiate or promote the development of cancer (Cancer Research UK, 2014). Cancer is defined as a group of diseases characterised by the uncontrolled growth and spread of abnormal cells. In fact, the abnormal growth of cancer cells is usually derived from a single abnormal cell that has lost normal control mechanisms. When cancer cells abnormally grow and multiply, they form a mass of cancerous cells called a tumour. Most tumours appeared as a solid mass, and can be classified as either benign (non-cancerous) or malignant (cancerous) tumours (Cancer Research UK, 2014).

The benign tumours are not cancer and non-life threatening. They can be removed in most cases, and usaully do not return. Most importantly, cells from benign tumours do not invade neighbouring tissues, or spread throughout the body. However, they can sometimes continue to grow and cause a problem by pressing on the surrounding organs or tissues. Papilloma (a projecting mass on the skin), adenoma (a tumour that grows in and around the glands), lipoma (a tumour in fatty tissue), osteoma (a tumour originating in the bones), myoma (a tumour of muscle tissue), angioma (a tumour composed of small blood or lymph vessels) and nevus (a skin tumour); are the most common examples of benign tumours (Cancer Research UK, 2014).

In contrast, malignant tumours are cancer and life threatening. They have the capacity to spread by either invasion to adjacent tissues; or by the formation of metastasis which spread the cancer throughout the body via the lymphatic and blood systems giving rise to secondary cancerous tumours. Malignancies or cancers can be divided based on the tissue type and cell type that are affected. They include: sarcomas (derived from connective tissues), carcinomas

(derived from epithelial tissues), lymphoma and leukaemia (which arising from hematopoietic blood cells), germ cell tumours (derived from pluripotent cells of the testis and ovaries), and blastoma (derived from immature precursor cells; or embryonic tissue) (American Cancer society, 2014). Each type of cancer can behave very differently, because they grow at different rates and respond differently to treatment (American Cancer society, 2014).

A Summary of Ten Most Common Cancers for Men and Women in UK (2011) and USA (2014) in Rank Order Based on Case Number.			
Men		Women	
UK (2011)	USA (2014)	UK (2011)	USA (2014)
Prostate (25% cases; 13% deaths)	**Prostate** (26% cases; 6% deaths)	**Breast** (30% cases; 15% deaths)	**Breast** (29% cases; 15% deaths)
Lung (14% cases; 23% deaths)	**Lung** (14% cases; 28% deaths)	**Lung** (12% cases; 21% deaths)	**Lung** (13% cases; 28% deaths)
Colon and Rectum (14% cases; 10% deaths)	**Colon and Rectum** (8% cases; 8% deaths)	**Colon and Rectum** (11% cases; 10% deaths)	**Colon and Rectum** (8% cases; 9% deaths)
Urinary Bladder (4% cases; 4% deaths)	**Urinary Bladder** (7% cases; 4% deaths)	**Uterus** (5% cases; 3% deaths)	**Uterine corpus** (6% cases; 3% deaths)
Malignant Melanoma (4% cases)	**Malignant Melanoma** (5% cases)	**Ovary** (4% cases; 5% deaths)	**Thyroid** (6% cases)
Kidney and Renal pelvis (4% cases; 3% deaths)	**Kidney and Renal pelvis** (5% cases; 3% deaths)	**Non-Hodgkin Lymphoma** (4% cases; 3% deaths)	**Non-Hodgkin Lymphoma** (4% cases; 3% cases)
Non-Hodgkin Lymphoma (4% cases; 3% deaths)	**Non-Hodgkin lymphoma** (4% cases; 3% deaths)	**Malignant Melanoma** (4% cases)	**Malignant Melanoma** (4% cases)
Oesophagus (3% cases; 6% deaths)	**Oesophagus** (4% cases; 4% deaths)	**Brain** (3% cases; 3% deaths)	**Kidney and Renal pelvis** (3% cases; 4% deaths),
Leukaemia (3% cases; 3% deaths)	**Leukaemia** (4% cases; 5% deaths)	**Pancreas** (3% cases; 6% deaths)	**Pancreas** (3% cases; 7% deaths)
Brain (3% cases; 3% deaths)	**Liver and Intrahepatic Bile duct** (3% cases; 5% deaths)	**Leukaemia** (2% cases; 3% deaths)	**Leukaemia** (3% cases; 4% deaths)

Table 1.1: A Summary for Ten Most Common Cancers with their Percentage Estimated Cases and Deaths For Men and Women in UK (2011) and USA (2014) from the Latest Data Available. Summarised from American Cancer society, 2014; Cancer Research UK, 2014.

1.2 Normal and Leukaemia Haematopoiesis

1.2.1 Normal Haematopoiesis

Hematopoietic stem cells (HSCs) give rise to all blood cells during the process of haematopoiesis (Sachs, 1996; Passegué *et al*, 2003; Kondo, 2010). Blood cell production is highly regulated; insuring homeostatic maintenance of the blood cell population (Sachs, 1996; Passegué *et al*, 2003; Kondo, 2010). HSCs are mostly found in bone marrow (Passegué *et al*, 2003). HSCs are capable of self-renewal with the ability to divide, produce daughter cells with identical characteristics and retain all of their proliferative and developmental potential. HSCs can differentiate into all classes of blood cells (Figure 1.1) (Passegué *et al*, 2003).

During self-renewal, HSCs have the ability to form two sub-populations: (i) Long-term haematopoietic stem cells, which have prolonged self-renewal ability; and (ii) short term haematopoietic stem cells, which have limited self-renewal ability (Figure 1.1) (Passegué *et al*, 2003). Self-renewal ensures that the HSC pool is protected and not used up. HSCs develop into multipotent progenitors cells which subsequently divide and differentiate into either common myeloid or common lymphoid progenitor cells (Figure 1.1) (Passegué *et al*, 2003). Each cell type can then go on to differentiate into one or more specific blood cell types (Figure 1.1) (Passegué *et al*, 2003).

The common myeloid progenitors give rise to erythrocytes, megakaryocytes and some classifications of white blood cell or granulocytes (neutrophils, eosinophils, basophils), monocytes which in turn form macrophages and dendritic cells (Figure 1.1) (Passegué *et al*, 2003; Kondo, 2010). The common lymphoid progenitors give rise to T-and B-lymphocytes and Natural Killer (NK) cells (Figure 1.1) (Passegué *et al*, 2003). B-cells are commonly synthesised in bone marrow and subsequently differentiate into plasma cells within the lymph nodes. Once formed the plasma cells do not divide further (Passegué *et al*, 2003).

1.2.1 Aberrant Haematopoiesis and Leukaemia Development

Normally, during haematopoiesis there is a regulation of cellular proliferation, differentiation and release of cells from the bone marrow into blood (Passegue′ *et al*, 2003). This ensures that there is the correct compliment of each blood cell type within blood and that a state of homeostasis and optimal function is maintained (Passegue′ *et al*, 2003). Therefore, aberrant production of blood cells is associated with the onset of leukaemia (Jones *et al*, 2008). Typically, aberrant haematopoiesis in leukaemia results in an enormous overproduction of a sub-group of blood cells or alternatively the production of immature cells that fail to differentiate into their mature state (Jones *et al*, 2008).

Leukaemia often develops from accumulation of mutations in HSCs. This leads to the production of non-functional immature HSCs or multi-potent progenitors cells. These immature cells are often referred to as leukaemia stem cells (Figure 1.2) (Jones *et al*, 2008). These stem cells retain some of the properties of normal HSCs: their capacity for self-renewal, proliferation and prolonged survival, but fail to terminally differentiate (Figure 1.2) (Jones *et al*, 2008). This increase in proliferation and/or survival is believed to be caused by gene mutations in the RAS family members; which is the name specified to a family of related proteins that is ubiquitously expressed in all cell lineages and organs; and the tyrosine kinase receptors that drive growth: c-Kit (tyrosine-protein kinase Kit) and FLT-3 (Fms-like tyrosine kinase 3) amongst others (Jones *et al*, 2008).

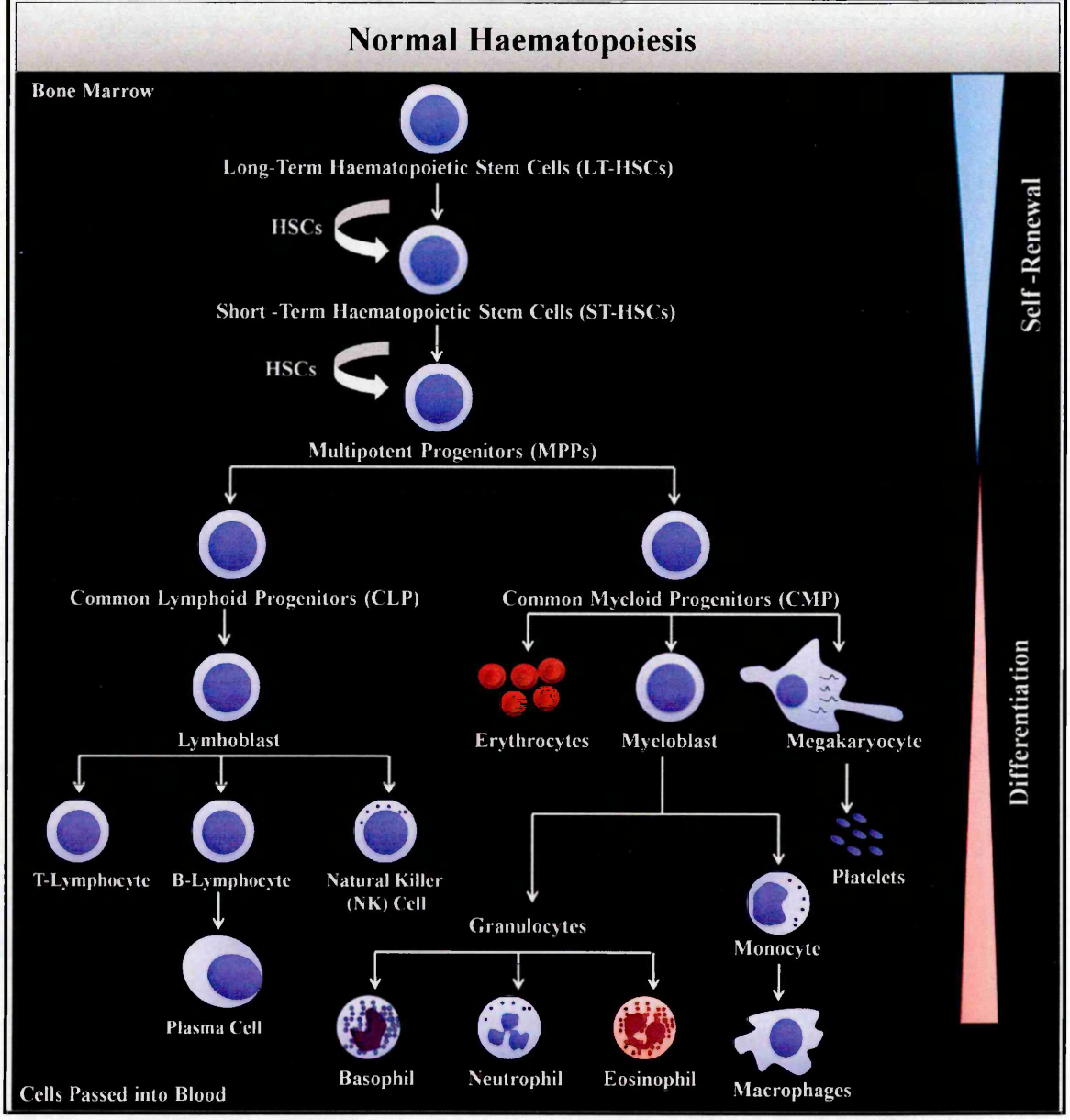


Figure 1.1: Schematic representation of the haematopoietic hierarchy. This figure represents the generation of mature myeloid and lymphoid haematopoietic cells from long-term haematopoietic stem cells. Modified from Passegué *et al*, 2003.

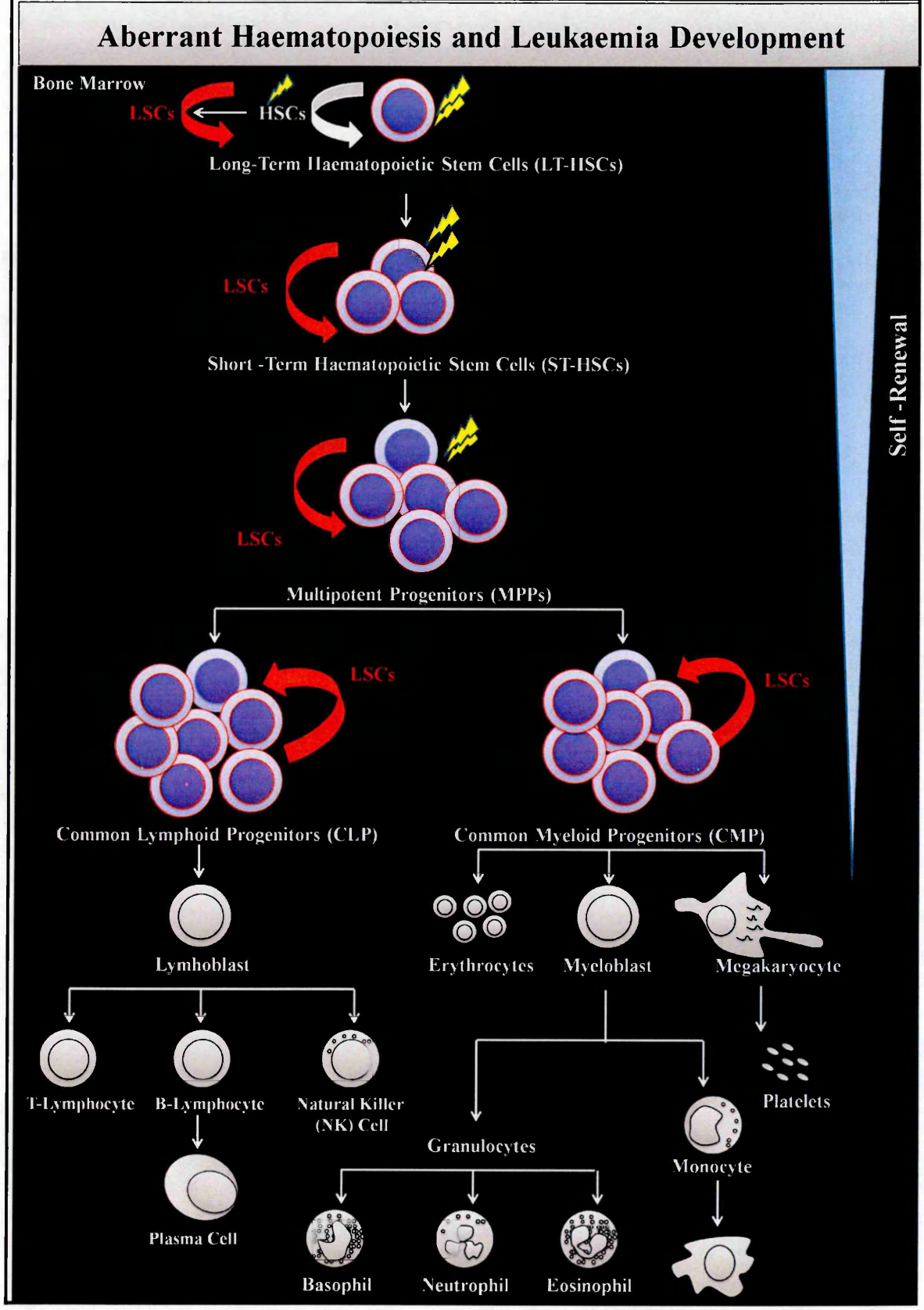


Figure 1.2: Schematic representation of the aberrant haematopoietic and initiation of leukaemia stem cells (LSCs) that caused extensive self-renewal. Modified from Passegué *et al*, 2003.

1.3 Leukaemia

Leukaemia is a malignant neoplasm affecting the hematopoietic system. It is defined as a cancer of the myeloid or lymphoid blood cells (Passegue′ *et al*, 2003; Pokhare, 2012; Wagner *et al*, 2012). Leukaemia is generally characterised by: i) increased cell survival caused by the up-regulation of Bcl-2, Bcl-x and down-regulation of Fas-receptor signalling pathways (FAS); ii) increased proliferation capacity caused by aberrant c-Myc, Cyclin D1, and BCR-ABL gene expression amongst others; and iii) increased self-renewal capacity as a result of up-regulation of HOX, WNT and NOTCH pathways; genomic instability, deregulated signalling pathways and impaired differentiation (Passegue′ *et al*, 2003).

1.3.1 Classic Classification of Leukaemia

Leukaemia is a complicated disease. Initially, it was classified according to stage of disease progression as either acute or chronic. Acute leukaemia is characterised by the large number of immature blood cells or blast cells, the disease progression is rapid and the prognosis is generally poor. Chronic leukaemia is characterised by a large number of precursor cells that are more differentiated than blast cells, the disease progression rate is relatively slow and is fatal in months to years if untreated (Passegue′ *et al*, 2003; Pokhare, 2012). Acute and chronic leukaemias are further sub-divided according to lineage as either myeloid or lymphoid. Four main categories of leukaemia can be identified based on differentiation and lineage: acute myeloid leukaemia (AML); chronic myeloid leukaemia (CML); acute lymphoid leukaemia (ALL) and chronic lymphoid leukaemia (CLL) (Table 1.2). The first three of these types arise from haematopoietic progenitor cells, whilst CLL is derived from mature lymphocytes (Passegue′ *et al*, 2003; Pokhare, 2012). This classification system has been updated by the World Health Organisation and the current classification system involves a number of specific subdivisions which take into account the cell lineage, stage of disease and presence of cytogenetic abnormalities which has led to tailored treatment regimens for specific leukaemia subclasses (Harris *et al*, 2000; Tefferi and Vardiman, 2008; Vardiman *et al*, 2009; Claudia *et al*, 2011).

Rate of Disease Progression	Cell of Origin	
	Myeloid	**Lymphoid**
Acute	**Acute Myeloid Leukaemia (AML)**	**Acute Lymphocytic Leukaemia (ALL)**
Chronic	**Chronic Myeloid Leukaemia (CML)**	**Chronic Lymphocytic Leukaemia (CLL)**

Table 1.2: Leukaemia Classification According to the rate of disease progression, and due to their HSC lineage.

1.3.2 Use of Leukaemia Cells in the Laboratory

A number of cell lines have been developed from leukaemia patients that can be essential for initial investigations into new treatments and understanding leukaemia pathogenesis in the laboratory. Cell lines representing the different types of leukaemia are commercially available and used routinely in the laboratory (Table 1.3).

General Cell Type	Myeloid leukaemia				Lymphoid leukaemia			Lymphoma
Cell Line Name	HL60	THP-1	K562	KG-1a	Jurkat	Molt-3	CCRF-CEM	U937
Description	Acute promyelocytic leukaemia	Acute monocytic leukaemia	Chronic myelogenous leukaemia	Acute myelogenous leukaemia	Acute T cell leukaemia	Acute lymphoblastic leukaemia	Acute lymphoblastic leukaemia	Human leukaemia monocyte lymphoma cell line
Year of Establishment	1976	1978	1970	1977	1976	1971	1964	1974
Cell phenotype	Promyeloblast	Monocytes	Lymphoblast	Myeloblast	T Lymphoblast	T Lymphoblast	T Lymphoblast	Monocytes
Patient Disease	Initially AML M3, later changed to AML M2	AML M5	CML	Erythroleukemia to AML	ALL	ALL with receiving multidrug chemotherapy	Lymphosarcom to ALL	Diffuse histiocytic lymphoma
Disease Status	At diagnosis	At relapse	At blast crisis	At relapse	At 1ST relapse	At relapse	At relapse	Refractory
Patent Data	36 year old - female	1 year old- male	53 year old female	59 year old - male	14 year old - male	19 year old - male	3 year old - female	37 year old - male
Source of cell	Peripheral blood	Peripheral blood	Pleural effusion	Bone marrow	Peripheral blood	Peripheral blood	Peripheral blood	Pleural effusion
Doubling Time in culture	25-40 h	35-50 h	24-30 h	40-50 h	25-35 h	40 h	24-30 h	30-40 h
Primary References	Collins *et al*, 1977	Tsuchiya *et al*, 1980	Lozzio[1] and Lozzio[2], 1975;	Koeffler and Golde, 1978	Schneider, 1977	Minowada, 1972	Foley, 1965	Sundström and Nilsson, 1976
p53 statues	Null	Null	Null	Null	Null	Wild type	Mutant	Null
p53 statues References	An *et al*, 2000; Durland and Resiman, 2002	Durland and Resiman, 2002	Durland and Resiman, 2002	Durland and Resiman, 2002	An *et al*, 2000	Cai *et al*, 2001	Geley *et al*, 1997; Anagnostopulos *et al*, 2005	An *et al*, 2000

Table 1.3: Summary of eight leukaemia cell lines, including their history, clinical data, p53 status. All leukaemia cell lines except MOLT-3 are p53-deficient, being either null, or containing mutant p53. MOLT-3 cells express wild type p53.

1.3.3 Epidemiology of Leukaemia

1.3.3.1 Incidence

In 2012 approximately 352,000 people were diagnosed with leukaemia worldwide (Cancer Research UK, 2014). The highest incidence rates were in Australia and New Zealand, and the lowest rates are in Western Africa (Cancer Research UK, 2014). From the latest data available, the diagnosis of new cases of leukaemia were around 8,616 cases in the UK in 2011, 82,300

cases in Europe in 2012 (Cancer Research UK, 2014), and 52,380 cases in the USA in 2014 (Leukaemia & lymphoma Research, 2014). Both the UK (2011) (Cancer Research UK, 2014) and USA (2014) Leukaemia & lymphoma Research, 2014) reports showed that the incidence rates of leukaemia were higher in males than females. In the UK there were around 5,014 (58%) male cases and 30,100 (57%) female cases (Cancer Research UK, 2014). In the USA there were around 3,602 (42%) male cases and 22,280 (43%) female cases (Leukaemia & lymphoma Research, 2014). This produced a male: female ratio of around 14:10 (Cancer Research UK, 2014). In the UK in 2011, leukaemia incidence was the 9th most common cancer in males and the 10th most common cancer in females (Cancer Research UK, 2014). In the UK, 8% of all leukaemias were ALL, 34% AML, 38% CLL and 8% CML (Cancer Research UK, 2014). Thus in the UK CLL was the most common subtype of leukaemia and ALL the least common (Cancer Research UK, 2014).

Leukaemia is considered to be largely a disease of adulthood which increases in incidence with age (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Age-specific incidence rates increase sharply from 45-49 years onwards, with the highest rates of occurrence being after 75 years (Cancer Research UK, 2014). However when considering the four major classification of leukaemia it can be seen that there is not always an increase in disease occurrence with ages (Cancer Research UK, 2014). Indeed when considering ALL, it can be seen that diagnosis normally occurs during early adulthood, in individuals under 20 years old (Table 1.4) (Cancer Research UK, 2014). AML has a more bi-modular rate of incidence; it is either diagnosed in early adulthood in individuals between 25 to 29 years or in adults between 50 and 75 years or over (Table 1.4) (Cancer Research UK, 2014). Both CLL and CML are adult diseases being once again mostly diagnosed in adults between 55 and 75 years, or older (Table 1.4) (Cancer Research UK, 2014).

Some racial variation in the occurrence of leukaemia has been observed in the USA. The highest incidence rates for leukaemia are found in the non-Hispanic white populations, whilst the lowest rates are found in American-Asian, American-Indian and Alaska Native populations (Leukaemia & lymphoma Research, 2014).

13.3.2 Relative Survival Rate

Commonly, relative survival rates of leukaemia depend on the type of leukaemia, the patient's age, gender and treatment regime (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). From the latest data available, the overall survival rates 5 years post-diagnosis for leukaemia were 44.3%; in the UK (between 2005 and 2009) and 59.2% in the USA (between 2003 and 2009) (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

In the USA (between 2003 and 2009) the 5-year relative survival rates for each major type of leukaemia were: 58.6% for all ages in CML patients; 83.1% for all ages in CLL patients; 24.9% for adults and 64.8% for children and adolescents (younger than 15 years) in AML patients; and 68.8% for adults, 91.7% for children and adolescents (younger than 15 years), and 92.6% for children younger than 5 years in ALL patients (Leukaemia & lymphoma Research, 2014).

1.3.3.3 Mortality

Worldwide, leukaemia is the 10th most common cause of cancer deaths with more than 265,000 deaths in 2012 (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Mortality of leukaemia was around 53,800 deaths in the Europe in 2012, 4,807 deaths in the UK in 2011 (Cancer Research UK, 2014); and 24,090 deaths in the USA during 2014 (Leukaemia & lymphoma Research, 2014).

In the UK in 2012, leukaemia was the 9th most common cause of cancer death among males and females (Cancer Research UK, 2014). Whilst in the USA (between 2010 until 2014), leukaemia was the 5th most common cause of cancer deaths in males and the 6th most common in females (Leukaemia & lymphoma Research, 2014). A comparison of the major types of leukaemia (AML, ALL CLL and CML) showed that AML caused the greatest number of deaths, both in the UK (2011) and the USA (2014) (Table 1.5) (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

The leukaemia mortality is strongly correlated with increasing age, in both males and females (Cancer Research UK, 2014). In the UK between 2010 and 2012, 56% of deaths from leukaemia were in individuals aged 75 or over, whilst only 4% were in those aged 35 years or under (Cancer Research UK, 2014). However in both the UK and the USA, leukaemia was responsible for more deaths than any other type of cancers in children, adolescents and young adults less than 20 years old (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). In the USA between 2006 and 2010, it was estimated that the rate of deaths from leukaemia was 7.4 per 100,000 in the non-Hispanic whites population; 7.3 per 100,000 in the whites at population and 6.1 per 100,000 in the black population (Leukaemia & lymphoma Research, 2014).

Leukaemia Types	Number Cases of Leukaemia in UK (2011)	Number Cases of Leukaemia in the USA (2014)	Most Common Age of Diagnosis
ALL	**650 cases**	**6, 020 cases**	**Children and young adults aged less than 20yrs**
AML	**2,900 cases**	**15,720 cases**	**Aged between 25 and 29; or 55 and 75, or older**
CLL	**3,200 cases**	**18,860 cases**	**Older adults aged between 55 and 75, or over**
CML	**680 cases**	**5,980 cases**	**Older adults aged between 55 and 75, or over**

Table 1.4: The major types of leukaemia and rates of occurrence in the UK and USA; along with the typical ages of diagnosis. (Cancer Research UK, 2014 and Leukaemia & lymphoma Research, 2014).

Leukaemia types	Number of Deaths for Leukaemia in UK (2011)	Number of Deaths for Leukaemia in the USA (2014)	Common Age at Which Death Occurred
ALL	**218 deaths**	**1,440 deaths**	**Children and young adults aged less than 20**
AML	**2,310 deaths**	**10,460 deaths**	**Older adults aged 75 and over**
CLL	**1,136 deaths**	**4,600 deaths**	**Older adults aged 75 and over**
CML	**248 deaths**	**810 deaths**	**Older adults aged 75 and over**

Table 1.5: A summary of the number of death caused by major types of leukaemia in the UK and USA; along with the common ages at which death occurred. This is summarised from the Cancer Research UK, 2014 and Leukaemia & Lymphoma Research, 2014.

1.3.4 Aetiology of Leukaemia

The exact cause of leukaemia in most patients remains unknown, however, there are a number of factors associated with increased risk of disease (Buffler and Kwan, 2005; Belson *et al*, 2007; Kumar, 2011; Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Leukaemia has described as having a multifactorial aetiology resulting from: a) general factors (e.g. age, gender and ethnicity); b) environmental factors (e.g. exposure to chemical agents such as benzene or smoking related carcinogen ionising radiation, chemotherapy and viral infection); and/or c) genetic factors (e.g. congenital disease such as Down Syndrome, or genetic abnormality due to specific chromosomal mutations e.g. mutation of chromosome 22, (the Philadelphia chromosome) is linked to CML; or a family history of

leukaemia) (Buffler and Kwan, 2005; Belson *et al*, 2007; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

1.3.4.1 Environmental Factors

A number of environmental factors have been implicated in the aetiology of leukaemia: smoking, exposure to high levels of radiation, exposure to chemical agents such as benzene, prior chemotherapy or radiotherapy treatment or viral infection (Buffler and Kwan, 2005; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Smoking has a strong association with leukaemia. It has been shown to increase the risk of AML by 50%; although it may not be a direct cause of the disease (Cancer Research UK, 2014). Furthermore, smoking in leukaemia patients has been linked to higher relapse rates and an increased genetic instability of leukaemia cells (Chelghoum *et al*, 2002). Exposure to ionisation radiation and long term exposure to the chemicals such as benzene are considered to be a potential risk factors for most types of leukaemia (ALL, AML, CLL, CML) (Belson *et al*, 2007). Indeed, exposure to ionizing radiation has been significantly linked with both ALL and AML; but not CLL and CML (Belson *et al*, 2007). The use of chemotherapy agents for treatment of a previous primary cancer have been also linked to an increased occurrence of leukaemia. There is a subset of AML called secondary AML or therapy related myeloid leukaemia which occurs following cancer treatment with chemotherapy agents, such as the alkylating agents (cyclophosphamide, chlorambucil and cisplatin), and topoisomerase II inhibitor agents (etoposide and doxorubicin) (Pokhare, 2012). Unfortunately, the prognosis for secondary AML is poor; when compared to that of primary AML (Pokhare, 2012).

Viral infection has also been linked to some forms of leukaemia. For example, infection with human T-cell lymphotropic virus-1 (HTLV-I) is associated to the development of Adult T-cell leukaemia/lymphoma (ATLL), a cancer of activated mature T lymphocytes. The occurrences of ATLL however are largely restricted geographically to Japan, the Caribbean basin and parts of South America and Africa (Pokhare, 2012). Most cases of chronic leukaemia (CLL and CML) have no clear cause and there is no way to prevent them (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). The risk of developing CLL and CML does not seem to be associated with smoking, exposure to chemicals, or indeed as a result of an infection (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). However, it is important to note that the exposure to some environmental agents may be linked with relatively specific clonal chromosome aberrations; which in turn may lead to chronic leukaemias. For example; an epidemiological study has reported that prolonged exposure to organic solvents is linked with Philadelphia chromosome positive CML (Cancer Research UK, 2014).

1.3.4.2 Genetic Factors: Genetic Risk Verses Genetic Aberrations in Leukaemia

1.3.4.2.1 Hereditary Genetic Factors

Most leukaemias have an abnormal genetic component which contributes to the disease state. These genetic abnormalities are however not generally inherited (Leukaemia & lymphoma Research, 2014). Only very occasionally, does CLL occur within the same family group and may be present in a parent and child, or in two siblings (Kumar, 2011). Likewise in genetically identical twins, if one is diagnosed with AML or ALL there is a greater risk of disease in the second twin, however this is related to congenital abnormalities, rather than a shared predisposition to sporadic cancer (Kumar, 2011). Genetically, Down syndrome is the most common genetic mutation that is significantly associated with the development of acute leukaemias, especially AML (Xavier *et al,* 2009).

1.3.4.2.2 Acquired Genetic Factors

Leukaemia can also be caused by other genetic mutations which result in the inactivation of tumour suppressor genes, activation of oncogenes, or general genomic instability. This in turn causes inappropriate cellular proliferation, and differentiation which disrupts cell death pathways leading to the development of cancer. The many genetic abnormalities and risk factors associated with the incidence of leukaemia are shown in Table 1.6 (Johansson *et al*, 2002; Shet *et al*, 2002; Akagi *et al*, 2009; Meijerink *et al*, 2009; Kumar, 2011; Leukaemia & lymphoma Research, 2014; Puiggros *et al*, 2014). Most genetic abnormalities and mutations occur spontaneously, or as a result of exposure to carcinogens or radiation; rather than being inherited (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). In fact, identification of these genetic abnormalities and mutations helps in the understanding of the pathophysiology and classification of the leukaemia; and hence the identification of the most appropriate treatment (Table 1.6) (Pokhare, 2012).

(A) Genetic Abnormalities and Mutations in Leukaemia

Generally, leukaemia is characterised by multiple random cytogenetic abnormalities including: chromosomal abnormalities such as translocations, deletions, inversions, duplication or substitutions, and/or gene mutations through the activation of oncogenes and/or deactivation of tumour suppressor genes, all of which contributed directly to malignant transformation (Johansson *et al*, 2002; Shet *et al*, 2002; Akagi *et al*, 2009; Meijerink *et al*, 2009; Kumar, 2011; Leukaemia & lymphoma Research, 2014; Puiggros *et al*, 2014). Most types of leukaemia (ALL, AML, CLL, AML) are mainly associated with chromosomal translocations (Table 1.6) (Johansson *et al*, 2002; Shet *et al*, 2002; Akagi *et al*, 2009; Meijerink *et al*, 2009; Kumar, 2011; Leukaemia & lymphoma Research, 2014; Puiggros *et al*, 2014). However, it is

possible to have a variety of alternative genetic mutation which can result in leukaemia (Puiggros *et al*, 2014).

Leukaemia Type	The Most Common Associated Chromosomal Abnormalities and Genetic Mutations with Classical Type of Leukaemia	References
ALL	➢ Translocation between chromosome 1 and 19. ➢ Translocation between chromosome 4 and 11. ➢ Translocation between chromosome 11 and 19. ➢ Translocation between chromosome 8 and 14. ➢ Translocation between chromosome 22 and 9. (Philadelphia chromosome). ➢ Hyperdiploid (> 46 chromosomes). ➢ Hypodiploid (< 46 chromosomes). ➢ Most common mutations occur in FLT3, SHP-2 and RAS/MAPK genes. ➢ Other mutations also can be occurred in NOTCH1, JAK1 and HOX11 genes which are commonly seen in T-ALL; and mutations in the JAK2 gene are occurred commonly in B-ALL.	Meijerink *et al*, 2009; Leukaemia & lymphoma Research, 2014.
AML	➢ Translocation between chromosomes 8 and 21. Translocation between chromosomes 15 and 17 ➢ Inversion in chromosome 16. ➢ Trisomy of chromosome 8 or 21. ➢ Monosomy in chromosome 7 or 21 and/or a loss of an X or Y chromosome. ➢ Mutations in FLT3, NPM1, c- KIT and/or RAS genes.	Akagi *et al*, 2009; Kumar, 2011; Leukaemia & lymphoma Research, 2014.
CLL	➢ Deletion in chromosome 13 ➢ Mutation in chromosome 11 and 17, ➢ Trisomy of chromosome 12 ➢ Mutations in P53, NOTCH1 and SF3B1 genes	Dierlamm *et al*, 1997; Döhner *et al*, 2000; Leukaemia & lymphoma Research, 2014; Puiggros *et al*, 2014.
CML	➢ Translocation between chromosome 22 and 9. (Philadelphia chromosome). The Ph chromosome gives rise to a constitutively active fusion gene between the BCR gene on chromosome 22 and the ABL1 locus on chromosome 9. ➢ Mutations in the tumour suppressor genes RB1, and P53.	Johansson *et al*, 2002; Shet *et al*, 2002; Leukaemia & lymphoma Research, 2014.

Table 1.6: The most common chromosomal abnormalities and genetic mutations that is associated with the classical type of leukaemia. This information is summarised from Johansson *et al*, 2002; Shet *et al*, 2002; Akagi *et al*, 2009; Meijerink *et al*, 2009; Kumar, 2011; Leukaemia & lymphoma Research, 2014 and Puiggros *et al*, 2014.

1.3.4.3 Epigenetic Alterations in Leukaemia

Recent studies have demonstrated that the complexity of leukaemogenesis can not be accounted by chromosomal aberrations and gene mutations alone, but also involves epigenetic modifications in processes such as DNA methylation and histone modifications, which normally regulate gene transcription (Chen *et al*, 2010; Polakova *et al*, 2013). Changes in DNA methylation state and histone modification pattern lead to permanent changes in the genes expression that regulate the leukemogenic phenotype (Chen *et al*, 2010; Polakova *et al*, 2013).

(A) Alterations in DNA Methylation in Leukaemia

DNA methylation is usually mediated by DNA methyltransferases (DNMTs) which catalyse the conversion of cytosine residues that preceed guanosine (CpG) to 5-methylcytosine, by the covalent addition of a methyl (CH3) group at the carbon 5 position of the cytosine (Ostrosky *et al*, 2007; Chen *et al*, 2010). DNMT is commonly responsible for maintaining DNA methylation patterns. DNMTs are usually over expressed and contribute to the pathogenesis of leukaemia by inducing aberrant regional hypermethylation (Ostrosky *et al*, 2007). Furthermore, leukaemia might be characterised by a general deregulation of CpG island methylation (Ostrosky *et al*, 2007). In normal cells, CpG residues cluster particularly in promoter regions of genes, and this is described as a CpG island. These CpGs, are commonly unmethylated, and are linked with hyperacetylated histones, and have an open chromatin configuration; which assist accessibility to transcription factors and transcriptional activation (Chen *et al*, 2010). However if the CpG islands are methylated in the promoter regions of genes this normally led to transcriptional repression, gene silencing and a condensed chromatin state (Chen *et al*, 2010).

Compared to the normal and leukaemic cells; show that the later exhibit either DNA hypermethylation, hypomethylation or aberrant methylation of CpG islands within gene promoters regions. Subsequently, this leads to the inactivation of tumour suppressor genes and/or activation of oncogenes (Chen *et al*, 2010).

Aberrant DNA hypermethylation in some genes has been shown to play a critical role in leukaemogenesis. (Ostrosky *et al*, 2007). For example, AML is associated with aberrant hypermethylation of tumour suppressor genes such as cyclin-dependent kinase inhibitor 2B (CDKN2B) gene, which encodes for $p15^{INK4b}$. This gene tended to express higher levels in the presence of DNMT (REF). This phenomenon has been described well, and it is consequently associated with transcriptional silencing, and the recruitment of methyl-binding proteins and HDACs to regions surrounding the transcriptional initiation sites (Chen *et al*, 2010). Consequently, DNA hypermethylation has been suggested as one of the more important therapeutic targets in AML (Ostrosky *et al*, 2007). Furthermore, 95% of AML and ALL cases are associated with hypermethylation of the calcitonin gene, which is associated with an unfavourable clinical outcome specifically in ALL (Ostrosky *et al*, 2007). Likewise, CML is significantly linked with hypermethylation in the ABL1 promoter of the BCR-ABL fusion gene (Ostrosky *et al*, 2007). In constrast, CLL transformation is commonly associated with a massive hypomethylation phenomenon frequently affecting the enhancer regions (Martin *et al*, 2013).

(B) Histones Modifications in Leukaemia

Leukaemia is not only associated with methylation of DNA, but also changes in histone modifications (Polakova *et al*, 2013). Histone modifications are an integral part of gene regulation and transcription as well as other nuclear processes (Polakova *et al*, 2013). Histone modifications involve acetylation, methylation, phosphorylation and other post-translational modifications of chromatin accessibility, DNA integrity and repair, transcription and replication (Polakova *et al*, 2013). The acetylation state of the histone tail is reversibly and regulated by two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Ostrosky *et al*, 2007). Leukaemia is often associated with a decreased of histone acetylation by increased histone deacetylase activity (HDACs), which can also lead to epigenetic silencing of tumour suppressor gene (Ostrosky *et al*, 2007). For instance, hypoacetylation of the $p21^{wafl/cifl}$ promoter results in its silencing in acute leukaemia, and can be reversed by HDAC inhibitors (Ostrosky *et al*, 2007). Histone acetylation is normally linked to transcriptional activation, while histone deacetylation is linked to transcriptional silencing. It is reported that there is a strong link between aberrant histone acetylation and carcinogenesis (Ostrosky *et al*, 2007). In particular, histone hypoacetylation is commonly observed and linked to the initiation and/or progression of leukaemia mainly in AML (Ostrosky *et al*, 2007). Thus inhibition of histone deacetylation represents a new interesting concept in the treatment of haematological malignancies (Ostrosky *et al*, 2007).

Furthermore, leukaemia is often associated with chromatin changes in histone methyltransferase (HMTs) (Peters *et al*, 2011). The best known example of alterations in HMTs is called the mixed lineage leukaemia *(MLL)* gene, which is a frequent partner for recurrent translocations mainly in acute leukaemias (AML and ALL), and is a H3 lysine 4 (H3K4) methyltransferase (Peters *et al*, 2011). In normal cells, MLL positively regulates gene expression of many genes, including *HOX* genes (Peters *et al*, 2011). However in leukaemia, the MLL1 fusion proteins act as constitutively active chimeric transcription factors (Peters *et al*, 2011). This fusion proteins in leukemic cells have lost the H3K4 methyltransferase activity, resulting in up-regulation of downstream homeobox (HOX) genes and activation of several leukaemogenic pathways such as RAS and fms-related tyrosine kinase 3 (FLT3), and transformation into leukaemic stem cells (Peters *et al*, 2011). MLL leukaemias is used as a unique transcriptional signature and associated usually with a poor prognosis (Peters *et al*, 2011).

1.4 Hallmarks of Cancer

In 2011, Hanahan and Weinberg defined ten essential hallmarks of cancer these included: 1) reprogramming energy metabolism and abnormal metabolic pathways; 2) sustaining proliferative signalling; 3) evading growth suppressors; 4) resisting cell death (apoptosis); 5) enabling replicative immortality; 6) evading the immune system; 7) genome instability and mutation; 8) tumour-promoting inflammation; 9) inducing angiogenesis and 10) activating invasion and metastasis (Figure 1.3) (Hanahan and Weinberg, 2011). A discussion is made here of the first seven of these hallmarks; excluding those which related only to solid malignancies; rather than haematological malignancies. Those excluded are inflammation, angiogenesis, invasion and metastasis.

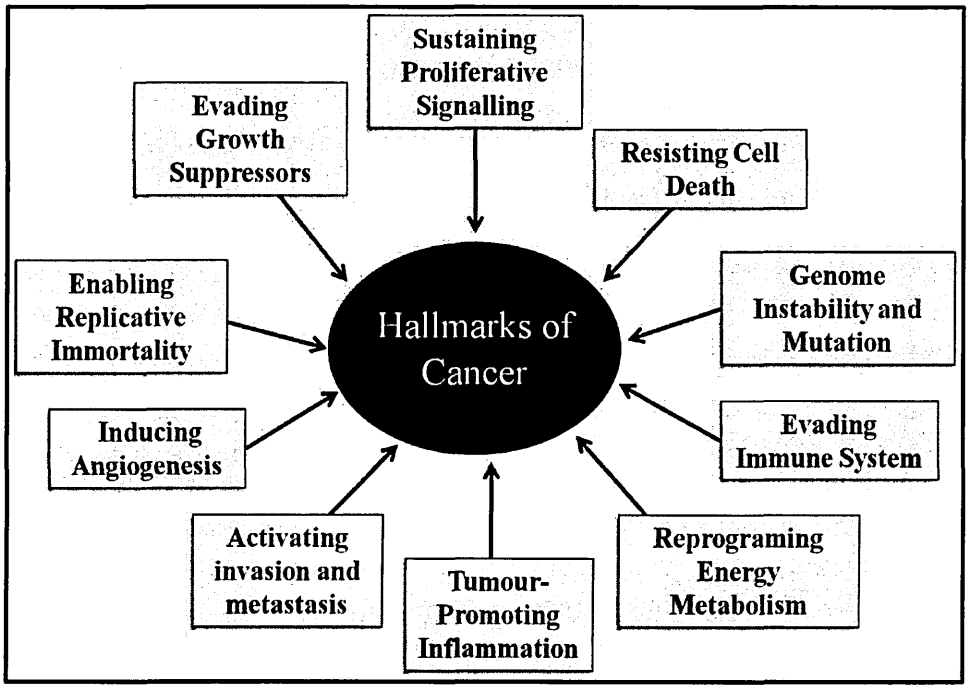


Figure 1.3: Hallmarks of Cancer. Modified from Hanahan and Weinberg, 2011.

1.4.1 Reprogramming Energy Metabolism and Abnormal Metabolic Pathways

Cellular energy metabolism is one of the major processes affected during the transition from normal to cancer cells, and it is an essential determinant of cell proliferation or cell death (Hanahan and Weinberg, 2011; Ward and Thompson, 2012; Jang *et al*, 2013). As a support for rapid proliferation, cancer cells choose to use glycolysis even in the presence of oxygen, this phenomenon, known as Warburg effect. Cancer cells use fuel macromolecules to synthesis nucleotides, fatty acids, and amino acids for accelerated mitosis, rather than to fuel the tricarboxylic acid cycle (TAC) and oxidative phosphorylation (Jang *et al*, 2013). This is described as ''aerobic glycolysis''. This phenomena was first identified by Warburg in 1956 (Jang *et al*, 2013). Preferential aerobic glycolysis in cancer cells is associated with, and caused by several mechanisms including: 1) mitochondrial defects; 2) adaptation to hypoxic

environment in cancer tissues via activating the hypoxia-inducible factor (HIF); 3) activation of oncogenic signals such as PI3K, MYC, RAS; 4) loss or mutation of tumour suppressors such as p53 and 6) abnormal expression of metabolic enzymes (Cairns *et al*, 2011).

Usually, ATP generation via glycolysis is far less efficient (two ATP per glucose) than through oxidative phosphorylation (36 ATP per glucose) (Pelicano *et al*, 2003). Cancer cells consume far more glucose than normal cells to provide and maintain sufficient ATP supply for their active metabolism and proliferation (Pelicano *et al*, 2003). As such, maintaining very high level of glycolytic activity is crucial for cancer cells to survive and grow (Pelicano *et al*, 2003). In this respect, inhibition of glycolytic capacity may contribute to an anti-cancer effect on cancer cells (Jang *et al*, 2013). As a result, cancer cell metabolism is generally characterised by an enhanced uptake and utilisation of glucose, which has been reported in many human cancers such as leukaemia and glioma (Jang *et al*, 2013). Additionally, cancerous cells have higher metabolic rates than normal cells, allowing them to sustain higher proliferative rates (Hanahan and Weinberg, 2011). Therefore, cancer cells need more nutrients and excrete more waste products than normal tissues. This leads to an accumulation of metabolites inside the cell, and develops a more hostile environment around the cancer cells (Hanahan and Weinberg, 2011). Subsequently, cells require more lipids, proteins, nucleotides and energy (in the form of ATP) for their bioenergetics and anabolic process to divide more, increase their size, and replicate DNA (Hanahan and Weinberg, 2011). These metabolic alterations create a phenotype that is essential for cancerous cell development and survival (Hanahan and Weinberg, 2011). As a result, dysregulated metabolic pathways have become attractive targets for cancer therapeutics (Hanahan and Weinberg, 2011; Jang *et al*, 2013). For this reason the reprogramming of energy metabolism and abnormal metabolic pathway is emerging as an important molecular hallmark of cancer cells (Hanahan and Weinberg, 2011).

1.4.2 Sustaining Proliferative Signalling

In normal cells, proliferative signalling is a tightly regulated process; this ensures cellular homeostasis. Normal tissues regulate production and release of growth promoting signals, which control entry and progression of the cell cycle (Hanahan and Weinberg, 2011). Normally, growth promoting signals are carried by growth factors binding to cell surface receptors which classically contain intracellular tyrosine kinase domains (Hanahan and Weinberg, 2011). The tyrosine kinase domain conveys signals by downstream signalling pathways to regulate normal cellular homeostasis (Hanahan and Weinberg, 2011). However, in cancer cells there is dysregulation of these proliferation signals resulting in sustained unregulated proliferation (Hanahan and Weinberg, 2011). This is achieved through four mechanisms: 1) production of growth factors and autocrine stimulation such as transforming

growth factor-alpha (TGF-α) and platelet-derived growth factor (PDGF); 2) promotion of stromal cells proliferation; 3) increased growth factor receptor expression on cell surface; 4) altered receptors signalling mechanisms such as constitutive activation (Hanahan and Weinberg, 2011).

For example the epidermal growth factor receptor (EGFR) is often over-expressed in gastrointestinal and lung tumours and the HER2/neu receptor is commonly over-expressed in breast cancer (Hanahan and Weinberg, 2011). Both receptors activate the Ras–Raf–MAP kinase pathway, enhancing cell proliferation (Hanahan and Weinberg, 2011). Interleukin 1 (IL-1) has also been identified as an autocrine growth factor which is over-expressed in acute myeloid leukaemia cells, and is responsible for increased cell proliferation (Cozzolino *et al*, 1989). Similarly, it is well known that tyrosine kinase receptors are over-expressed in CML; this is associated with up regulation and activation of several major downstream effectors such as Ras, PI3K and protein kinase B (AKT), all of which promote an increase in cell proliferation (Bacco *et al*, 2000).

1.4.3 Evading Growth Suppressors

In normal cells, p53 and pRb (Sections 1.5.2) are important part of large network proteins that regulate cell proliferation, cell cycle process and apoptosis (Hanahan and Weinberg, 2011). In cancer cells, these proteins are commonly dysregulated, inactivated or mutated, leading to uncontrolled growth and proliferation (Hanahan and Weinberg, 2011). For example, Zen *et al*, (2010) reported that a p53 mutation in patients with CLL was associated with poor prognosis (Zen *et al*, 2010). In addition, inactivation of p53 was extensively linked with the initiation of aggressive AML, which was resistant to chemotherapy agents, and associated with p53 role in limiting aberrant self-renewal of myeloid progenitor cells (Zen et al, 2010).

1.4.4 Resisting Cell Death: Apoptosis

Resisting programmed cell death (Section 1.6) plays a vital role in cancer cell survival (Hanahan and Weinberg, 2011). In particular, cancer cells can develop resistance to apoptosis through multiple mechanisms by: 1) modulating or loss the function of p53 tumour suppressor protein (Bouillet and Strasser, 2002; Junttila *et al,* 2009; Hanahan and Weinberg, 2011); 2) increased expression of anti-apoptotic regulators such as Bcl-2, and Bcl-xl; 3) decreased expression of pro-apoptotic regulators such as Bax, Bim and Puma; 4) increased expression of survival signals such as IL-3 and IL-1 (within leukaemia cells) finally by interrupting the extrinsic apoptotic pathway (Hanahan and Weinberg, 2011). In addition, cancer cells resist cell death by changing normal cellular autophagy and necrosis (Hanahan and Weinberg, 2011). Autophagy is a self-digestive process that ensures the lysosomal degradation of superfluous or

damaged organelles and mis-folded proteins. Normally, autophagy helps to maintain homeostasis by contributing to protein and organelle turnover. In cancer, the role of autophagy is quite complicated and still controversial; it appears to be tumour suppressive during cancer development, while it can also contribute to tumour cell survival during cancer progression. In addition, cancer cells can use autophagy to resist anti-cancer therapies (Hanahan and Weinberg, 2011). Finally, necrotic cell death results in rupture of the cell membrane which releases intracellular contents which results in pro-inflammatory signals to the surrounding microenvironment, which results in inflammation, which promotes tumour growth (Hanahan and Weinberg, 2011).

1.4.5 Enabling Replicative Immortality: Telomeres and Telomerase

Normal cells have a limited lifespan, which go through a limited number of cell divisions before they undergo senescence (Section 1.5.2.5) and subsequent crisis and cell death (Shay and Wright, 2001). During replication in normal cells, telomeres shorten with each cell division eventually resulting in replicative senescence (Shay and Wright, 2001). However, cancer cells can re-express embryonic telomerase, which is a cellular ribonucleoprotein reverse transcriptase that stabilises the telomere length by adding hexameric telomere repeats (TTAGGG) to the telomeric ends of the chromosomes. This permits continued cell division; thus preventing senescence and hence makes the cells 'immortal' (Shay and Wright, 2001). About 90% of immortalised cells in human cancers are capable of increasing their telomere length due to over expression of telomerase (Hahn, 2003). A number of leukaemia cell lines such as HL-60 and U937 have higher levels of telomerase activity compared to normal bone marrow mononuclear cells (Seol *et al,* 1998). This suggests that understanding the manipulation of telomeres and telomerase biology can lead to clinically significant applications in the diagnosis, prevention, and treatment of cancer (Hanahan and Weinberg, 2011).

1.4.6 Evading the Immune System

The immune system functions as a vital barrier to tumour formation and progression, and the capability to evade and escape from immunity, which is a hallmark of cancer development (Hanahan and Weinberg, 2011). Pre-clinical studies have reported that an active immune system continuously identifies and eliminates most cancer cells before they establish themselves and form a tumour (Cavallo *et al*, 2011). However, cancer cells can evade the immune system through three key phases: elimination; equilibrium and escape (Cavallo *et al*, 2011). During the elimination phase the immune system recognises and eliminates cancer cells (Cavallo *et al*, 2011). Those cells that avoid the elimination, progress to the equilibrium phase, in which the immune system regulates cancer cell growth; but does not totally eliminate the transformed cells (Cavallo *et al*, 2011). Tumour cells not susceptible to the immune

destruction, progress to the escape phase, in this phase the "escaped" tumour clones are not effectively recognised and destroyed by the immune system (Cavallo *et al*, 2011). Cancer cells that successfully cross these phases acquire the capability to evade the immune system.

Cancer cells usually shed surface antigens into circulation; this triggers CD8+ cytotoxic T lymphocytes, natural killer cells and macrophage production (Cavallo *et al*, 2011). The immune system is believed to provide continuous surveillance, with subsequent elimination of cells that undergo malignant transformation (Cavallo *et al*, 2011). However, deficiencies in the action or development of CD4+ Th1 helper T cells, CD8+ cytotoxic T lymphocyte, or natural killer cells can each lead to a significant increase in cancer incidence (Hanahan and Weinberg, 2011). Furthermore, highly immunogenic cancer cells may evade immune destruction via inactivating components of the immune system (Hanahan and Weinberg, 2011). This occurs through recruitment of inflammatory cells, including regulatory T cells and myeloid-derived suppressor cells, that both actively suppress the actions of cytotoxic lymphocytes (Cavallo *et al*, 2011; Hanahan and Weinberg, 2011). As a result, there are three emerging immune hallmarks for cancer cells which have the capability to: increase the chronically inflamed microenvironment; evade immune recognition and suppress immune reactivity (Cavallo et al, 2011).

1.4.7 Genome Instability and Mutation

Random genetic mutations occur very rarely throughout all the cells in the body; these confer a selective advantage on single cells, permitting overgrowth and dominance in local tissue environments (Negrini *et al*, 2010). Multistep carcinogenesis is a consequence of successive clonal expansions of pre-malignant cells, each expansion being induced by acquisition of normally random facilitating genetic mutations (Negrini *et al*, 2010). In normal cells, cellular DNA repair mechanisms are highly effective, and almost all spontaneous mutations are repaired (Negrini *et al*, 2010). The process of DNA repair is very important to maintain genome integrity (Negrini *et al*, 2010; Shen *et al*, 2011). There are four mechanisms that maintain genomic stability during normal cell division: 1) A high fidelity of DNA, which must be replicated in S-phase, condensed, and separated during each cell division; 2) cells must respond too and repair both endogenous and exogenous DNA damage; 3) precise distribution of chromosomes among daughter cells during mitosis, and 4) control of the cell cycle progression, checkpoints and cell fate, including apoptosis and senescence (Shen *et al*, 2011). Any dysregulation in these mechanisms can create genomic instability and mutations, and contribute to the development of cancer (Shen *et al*, 2011).

In cancer cells, the accumulation of mutations can be enhanced and accelerated by compromising the surveillance systems that usually monitor genomic integrity and pushes

genetically damaged cells into either senescence or apoptosis (Negrini *et al*, 2010; Shen *et al*, 2011). Typically, genetic instability can occur due to: a) deficiencies in one of three types of DNA repair systems (mismatch repair, nucleotide excision repair or recombination repair); b) a loss or gain of chromosomes or c) large scale chromosomal reorganisation (Shen *et al*, 2011). Accumulation of the genomic instability leads to multiple mutations in chromosomes, genes or within the nucleotide sequences; and can cause dysregulation of cell division, imbalance between cells proliferate and death, which can led to cancer (Shen *et al*, 2011). Genomic instability is often associated with a vast majority of cancers (Negrini *et al*, 2010). Furthermore, it is not only considered as a hallmark of cancer, but it could be considered as an indicator to how tumours develop (Hanahan and Weinberg, 2011). Many studies suggest that genomic instability and its molecular pathways and components, are good therapeutic targets for the treatment of cancer (Negrini *et al*, 2010; Shen et al, 2011; Hanahan and Weinberg, 2011; Muvarak *et al*, 2012).

Tumour cells can progress from a less malignant to a more malignant state according to the inherent genomic instability (Muvarak *et al*, 2012). For example, CML is characterised by changes in their DNA damage responses, DNA repair and cell-cycle checkpoints, all of which are crucial for cell survival, progression, and resistance to genomic instability (Muvarak *et al*, 2012). Consequently, this genomic instability leads to genomic deletions, rearrangements, and point mutations, as well as the gain or loss of whole chromosomes (aneuploidy), which are commonly observed in CML (Muvarak *et al*, 2012). For this reason, identification and targeting of particular factors that cause genomic instability is an attractive therapeutic approach, with particular relevance to CML in which tyrosine kinase inhibitor (TKI) therapy has failed (Muvarak *et al*, 2012). It is important to mention that aneuploidy mutation is one of the most common types of genomic instability seen in leukaemia, is likely to be linked with loss of function of the mitotic checkpoints, centrosome dysfunction, or both (Muvarak *et al*, 2012). Generally, cellular DNA undergoes continuous damage and re-synthesis. DNA damage could result from environmental sources and the environmental agents that damage DNA and have been demonstrated to be mutagens and most are carcinogens (Hanahan and Weinberg, 2011). The association between environmental factors and genetic alterations and how these mutations could be acquired in leukaemia are discussed in Sections 13.4.2.

1.5 Cell Cycle

1.5.1 Overview of the Cell Cycle

The cell cycle is a sequence of events that occur in a cell leading to its growth, DNA replication and cell division resulting in duplication (Garrett, 2001; Schwartz and Shah, 2005). Fundamentally, the cell cycle occurs in two distinct phases: interphase, during which the cell grows and replicates its DNA, followed by mitosis in which the cell divides to generate two

daughter cells (Figure 1.4) (Schwartz and Shah, 2005). Interphase can be further sub-divided into: Gap 0 (G_0), Gap 1 (G_1); Synthesis (S) and Gap 2 (G_2) phases (Figure 1.4), during interphase cells actively prepare for mitosis by collecting nutrients, amplifying organelles, proteins and other cellular contents and are metabolically active to mitotic phase (M); which is a relatively brief period of nuclear and cellular division (O'Connor, 2008).

1.5.2 Cell Cycle Regulation

The cell cycle is coordinated and regulated by a number of molecular mechanisms that depend on a cascade of protein phosphorylations, which include: (1) cyclins and cyclin-dependent kinases (CDKs), (2) cyclin-dependent kinase inhibitors (CDKIs), (3) checkpoints and (4) tumour suppressor genes (Garrett, 2001; Vermeulen *et al,* 2003). CDKs are controlled positively by cyclins and controlled negatively by naturally occurring CDK dependent kinase inhibitors (CDKIs) (Figure 1.4) (Vermeulen *et al,* 2003). Each protein is carefully controlled as any mutations in the components can lead to tumour development (Vermeulen *et al,* 2003).

1.5.2.1 Cyclins and Cyclin-Dependent Kinases (CDKs)

The cyclin-dependent kinases (CDKs) are a family of serine/threonine rich kinases which have a central role in cell cycle (Malumbres and Barbacid, 2009). They require the co-localisation of a regulatory cyclin subunit and a catalytic kinase subunit (Figure 1.4 and Table 1.7) (Malumbres and Barbacid, 2009). The progression of cells through cell cycle is controlled by the binding of cyclins with CDKs, which are activated at specific points of the cell cycle (Figure 1.4 and Table 1.7) (Garrett, 2001). There are four major regulatory cyclin-CDK complexes each defined by the phases of cell cycle: (1) Cyclins D1, D2, D3 bind to CDK4 and CDK6, these complexes are important for cellular entry in G_1 phase, (2) Cyclin E bind to CDK2 at the end of G_1 phase and the beginning of S phase, and this complex is regulates progression through the R point from G_1 into S phase and commits the cell to DNA synthesis, (3) Cyclin A bind to CDK2, which regulates S phase, (4) Cyclin A binds to CDK1 in late G2 phase and early M phase to promote entry into mitosis, (5) Cyclin B binds to CDK1 and this complex is required to initiate mitosis (Figure 1.4 and Table 1.7) (Malumbres and Barbacid, 2009).

1.5.2.2 Cyclin Dependent Kinases Inhibitors (CDKIs)

CDKs activity can be counteracted by cell cycle inhibitory proteins, called cyclin dependent kinase inhibitors (CDKIs). These can bind to CDK alone or to the CDK-cyclin complex and prevents cell cycle progression (Malumbres and Barbacid, 2009). There are two main protein families that can bind to and inhibit CDKs (Garrett, 2001). The first are inhibitors of CDK4 (INK4) and alternative reading frame (ARF), which include about five family members:

$p16^{Ink4a}$, $p15^{Ink4b}$, $p14^{Arf}$, $p18^{Ink4c}$, and p19 $^{Ink4d/Arf}$. These bind to and inhibit the activity of CDK4 (cyclin D/CDK4) and CDK6 (cyclin D/CDK 6) complexes, leading to G_1 phase cell cycle arrest (Figure 1.4 and Table 1.8) (Malumbres and Barbacid, 2009). The second family of CDK inhibitors are the CDK Interacting Protein/Kinase Inhibitory Protein (CIP/KIP), which include three family members: $p21^{waf1/cip1}$, $p27^{kip1}$, and $p57^{kip}$. These bind to and inhibit CDKs alone or in combination with cyclins; this leads to cell cycle arrest phases, particularly in S phase (Figure 1.4 and Table 1.8) (Malumbres and Barbacid, 2009).

The progression of cell cycle can be arrested by the inhibition of cell specific mechanisms in each phase of the cell cycle. G_1 phase arrest occurs through the inhibition of Cyclin D/CDK4 and Cyclin D/CDK6 complexes. S phase arrest is triggered by DNA damage and a reduction in expression of Cyclin A/CDK2 and Cyclin E/CDK2. Indeed, DNA damages causes up-regulation of p53 oncogenes and Transforming Growth Factor (TGF- β). These in turn cause the upregualtion of $p21^{waf1/cip1}$ and $p27^{kip1}$ which reduce Cyclin A/CDK2 and Cyclin E/CDK2 and causes S phase arrest. G_2/M phase arrest is caused by the inhibition of Cyclin B/CDK1 kinase activity through normal activation of p53 (Malumbres and Barbacid, 2009).

1.5.2.3 Checkpoints

The movement through each phase of the cell cycle and transition from one phase to the next is tightly controlled at a number of checkpoints (Garrett, 2001). These checkpoints in the cell cycle are very important to minimize errors (Garrett, 2001). Typically, checkpoints act as control mechanisms for cell cycle progression to ensure that chromosomes are intact, and that each phase of the cell cycle is completed, before the next phase is started (Malumbres and Barbacid, 2007). Checkpoints can inhibit cell cycle progression at specific points, permitting confirmation of necessary phase process and repair DNA damage via sensor mechanisms. The cells cannot proceed to the next phase until checkpoint requirements have been met. Briefly, when checkpoints are activated, for example by DNA damage, a signal is transmitted to the cell cycle-progression machinery (Garrett, 2001). This causes a slow progression in cycle until the risk of mutation has been removed or repaired (Garrett, 2001). Failure to repair the DNA damage results in cell cycle arrest (Schwartz and Shah, 2005).

There are three major checkpoints responsible for regulation the cells transition from phase to phase: (1) G_1 Checkpoint (Restriction (R) point), (2) G_1/S Checkpoint, (3) S Checkpoint, (4) G_2 Checkpoint and (5) M Checkpoint (metaphase to anaphase checkpoint) (Figure 1.4) (Foster *et al*, 2011). These checkpoints are designed to ensure that the appropriate environment to support cell growth exists and that damaged or incomplete DNA is not passed on to daughter cells. The G_1 Checkpoint (R point) is defined as a point of no return in G1, following which the cell is committed to enter the cell cycle (Figure 1.4) (Foster *et al*, 2011). The second

checkpoint is located at the end of G_1 phase and the beginning of S phase, and this checkpoint ensures the correct environment exists and adequate growth factors are provided (Figure 1.4) (Foster *et al*, 2011). The third checkpoint is located in the middle of S phase repairing any damage to DNA before entering to the G_2 phase (Figure 1.4) (Foster *et al*, 2011). The fourth checkpoint is located at G_2 phase which monitors the fidelity of DNA replication. It ensures all the chromosomes have been replicated and that the replicated DNA is not damaged before the cell enters mitosis. If DNA damage is detected the cell will attempt repair using its DNA repair mechanisms, and will return the cell to S phase to re-synthesis the DNA. If this fails controlled cell death (apoptosis) will be triggered (Figure 1.4). A fifth checkpoint is located during M phase, where it checks that mitotic spindle has formed correctly (Malumbres and Barbacid, 2007). These checkpoints have a key role to monitor the integrity of the genome throughout the cell cycle (Malumbres and Barbacid, 2007). These checkpoints are controlled by two major tumour-suppressor proteins p53 and pRb (Gollas et al, 2004).

1.5.2.4 Tumour Suppressor Genes: p53 and Rb

(A) p53

p53 is a transcription factor that plays a crucial role in cell cycle regulation and apoptosis. p53 is a phosphoprotein made of 393 amino acids, containing four domains: a domain to activate transcription factors; a domain to recognise specific DNA sequences; a core domain responsible for tetramerization of proteins and finally a domain which recognises DNA damage (Zilfou and Lowe, 2009). p53 is described as "the guardian of the genome", preserving genome stability, and preventing mutations (Ryan *et al*, 2001). Defective p53 permits abnormal cells to proliferate and the formation of cancer; as such p53 is described as a tumour-suppressor gene. In fact p53 is the most frequently mutated gene in cancer seen in more than 50% of all human cancers (Sherr *et al*, 2002). In normal cells, the p53 protein is unstable and short lived. However if DNA is damaged, p53 becomes stabilised increasing its activity, resulting in growth arrest, DNA repair or apoptosis (Sherr *et al*, 2002). The action of p53 is normally inactivated by the Mouse Double Minute 2 (MDM2) gene, which is responsible for encoding a protein known as negative regulator for p53 (Mendoza-Rodríguez and Cerbón, 2001).

p53 regulates cell cycle via the activation of the $p21^{waf1/cip1}$ genes that inhibits most CDKs if DNA damage is detected (Figure 1.5) (Gollas *et al*, 2004). If DNA damage is detected which cannot be repaired, p53 triggers apoptosis (Section 1.6.3.4) (Gollas *et al*, 2004). Tumour cells lacking normal p53 have a reduced ability to regulate cell cycle following DNA damage (Sherr *et al*, 2002).

(B) *Retinoblastoma (Rb)*

Retinoblastoma (RB1) is a tumour suppressor gene producing Retinoblastoma protein (pRb). This plays a vital role in the control of the cell cycle and tumour progression (Genovese *et al*, 2006; Giacinti and Giordano, 2006). One critical function of pRb is the control of the G_1-to-S phase checkpoint of the cell cycle (R point) (Figure 1.6) (Giacinti and Giordano, 2006). Whilst pRb is hypophosphorylated it sequesters E2F transcription factors (E2Fs); E2Fs binding sites are found in the promoters of many genes that are necessary for cell cycle progression, thus the sequestration by pRb inhibits transcription of cell cycle-promoting genes; preventing progression of the cell cycle from G_1 to S phase (Figure 1.6) (Giacinti and Giordano, 2006). During G_1 phase if environmental conditions are suitable, Cyclin D/CDK4, Cyclin D/CDK6 phosphorylate pRb, which is further Phosphorylated by Cyclin E/CDK2, once pRb becomes hyperphosphorylated pRb dissociates from E2Fs, permitting their action and induces S phase (Figure 1.6) (Genovese *et al*, 2006).

In addition, pRb suppress transcription by remodelling chromatin structure through interaction with proteins such as histone deacetylase 1 (HDAC1), brahma-related gene-1 (BRG1), and histone-lysine N-methyltransferase SUV39H1; which are involved in histone acetylation/deacetylation, nucleosome remodelling and methylation, respectively (Giacinti and Giordano, 2006). Loss of regulation of pRb can induce cell cycle deregulation and subsequently lead to malignant phenotypes (Giacinti and Giordano, 2006). Gene inactivation of pRb through chromosomal mutations is one of the major reasons for retinoblastoma tumour development, as well as, other human cancers including; lung, brain, liver cancers and leukaemia (Giacinti and Giordano, 2006). For example, a study in 35 patients with CLL of B-cell origin (B-CLL) showed a significantly higher frequency of the RB1 deletion (Stilgenbauer *et al,* 1993). In addition, it has been found that RB1 inactivation is more frequently seen in AML than in ALL (Tang *et al*, 1992).

1.5.2.5 Irreversible Cell Cycle Arrest: Senescence

Cellular senescence is persistent and irreversible cell cycle arrest, its function is to control the lifecycle of mammalian cells and inhibits unlimited cellular proliferation (Campisi, 2000). It is for this reason that cellular senescence is also known as replicative senescence (Campisi, 2000). Normal cells have a limited lifespan, which go through a limited number of cell divisions (known as Hayflick limit) before they undergo senescence (Collado and Serrano, 2010). Senescence is important as it prevents the continual proliferation of cells which could result in DNA damage due to shortening of DNA during each cell replication resulting in telomere shortening. Aging is not the only cause of senescence, it can also be triggered by

DNA double strand breaks (DSB), and premature telomere shortening during oxidative stress (Blasco, 2005).

Telomeres are repeating nucleotide sequences (TTAGGGG) at the ends of chromosomes which protect the coding DNA, which are shortened during each replication of DNA. This leads to a loss of telomeric repeats which once the telomeres reach a critical size, cellular senescence is initiated (Collado and Serrano, 2010). Similarly, if telomeres were completely lost this would result in loss of genes at the end of a chromosome or abnormal chromosomal replication. This would lead to DNA damage and consequent activation of pRb and p53 (Section 1.5.2.4 and 1.6.3.4) pathways (Ferbeyre *et al,* 2002); which would initiate senescence. Likewise reactive oxygen species (ROS) generated by oxidative stress can cause also DNA damage (Ferbeyre *et al,* 2002) and subsequent stress induced premature senescence (SIPS) (Collado and Serrano, 2010).

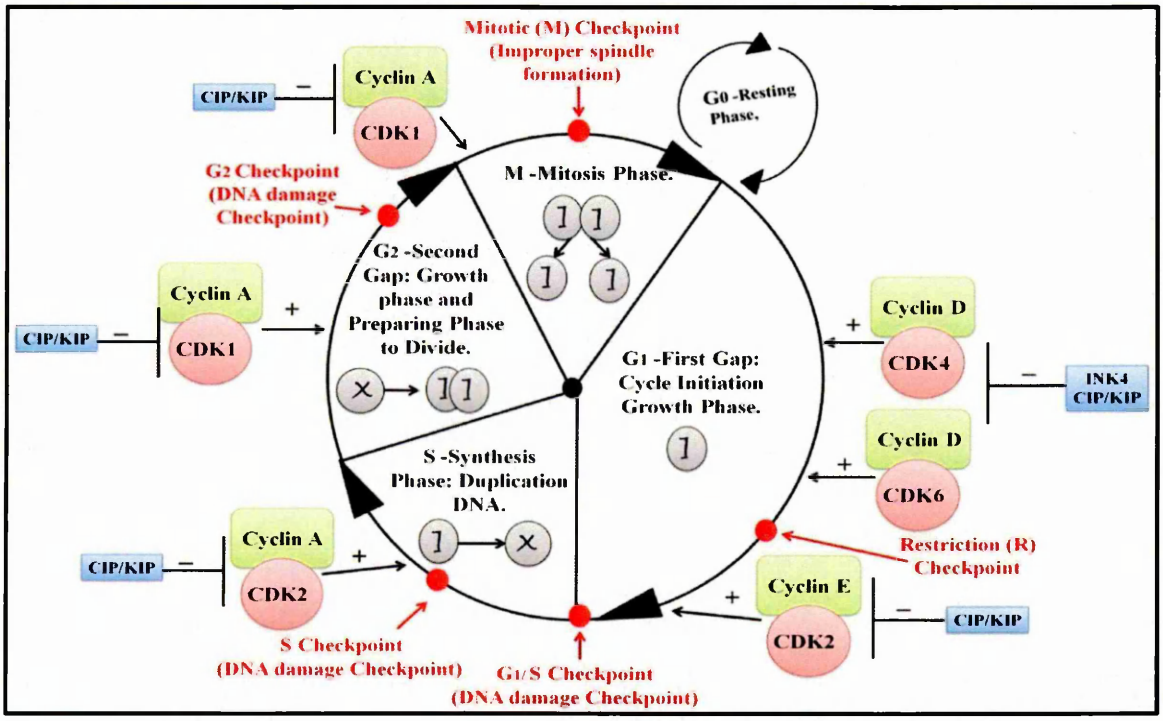


Figure 1.4: Cell Cycle Phases and Regulators. Cell cycle has four basic phases: G_1 phase (growth), S phase (replication), G_2 phase (preparation for division) and M phase (mitosis) The progression of cell cycle is regulated by a cyclins and cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitor (CDKIs) and checkpoints. Modified from Vermeulen *et al.,* 2003; and Malumbres and Barbacid, 2007.

Cell Cycle Phases	Cyclins	CDKs
G_1 phase	Cyclin D1, D2, D3	CDK4
G_1 phase	Cyclin D1, D2, D3	CDK6
G_1/S phase transition	Cyclin E	CDK2
S-phase	Cyclin A	CDK2
G_2/M phase transition	Cyclin A	CDK1 (cdc2)
M phase	Cyclin B	CDK1 (cdc2)

Table 1.7: The summary of the cyclins and cyclin dependent kinases (CDKs) activated in each phase of the cell cycle. Modified from Vermeulen *et al,* 2003.

CKI Family	Family Members	Major Function
INK4 family	$p16^{Ink4a}$	Inactivate Cyclin D/CDK4 and Cyclin D/CDK6. G1 phase inhibitors.
	$p15^{Ink4b}$	
	$p18^{Ink4c}$	
	$p19^{Ink4d/Arf}$	
CIP/KIP family	$p21^{waf1/cip1}$	Inactivate or inhibit all the CDKs alone or Cyclin/CDKs complexes. S phase inhibitors.
	$p27^{kip1}$	
	$p57^{kip}$	

Table 1.8: Cyclin dependent kinases inhibitors (CKIs) bind to Cyclin Dependent Kinases (CDKs) alone; or to the CDK-cyclin complex and regulate CDK activity. Modified from Vermeulen *et al,* 2003.

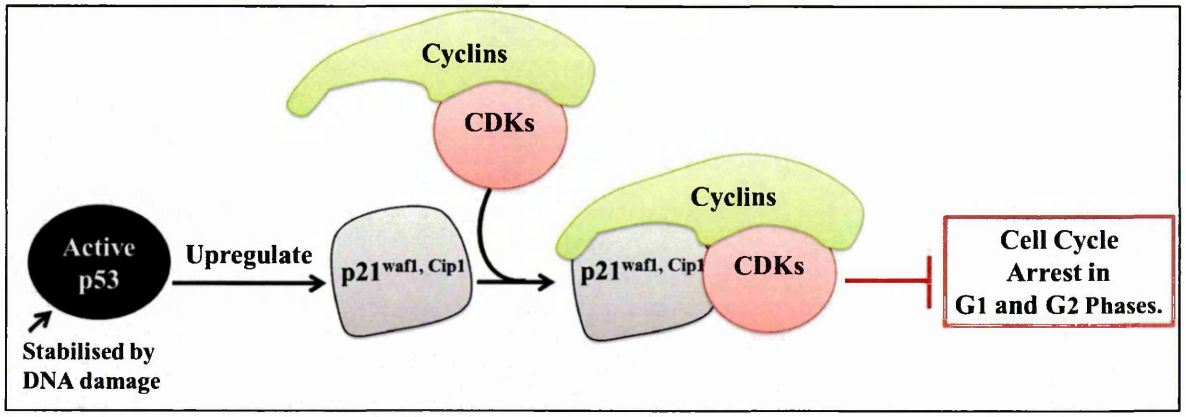


Figure 1.5: The major role of p53 in cell cycle regulation. p53-induced cell-cycle arrest in response to DNA damage. The normally unstable p53 protein is stabilized by damaged DNA, so its concentration increases. Acting as a transcription factor active p53 upregulates the expression level of $p21^{waf1/cip1}$ and a cyclin-kinase inhibitor that inhibits all Cyclins/CDKs complexes (Figure 1.4). Binding of $p21^{cip}$ to these Cyclins/CDKs complexes leads to cell cycle arrest in G_1 and G_2 phases, allowing DNA repair prior to replicate and cell division. Modified from Gollas *et al*, 2004, and Zilfou and Lowe, 2009.

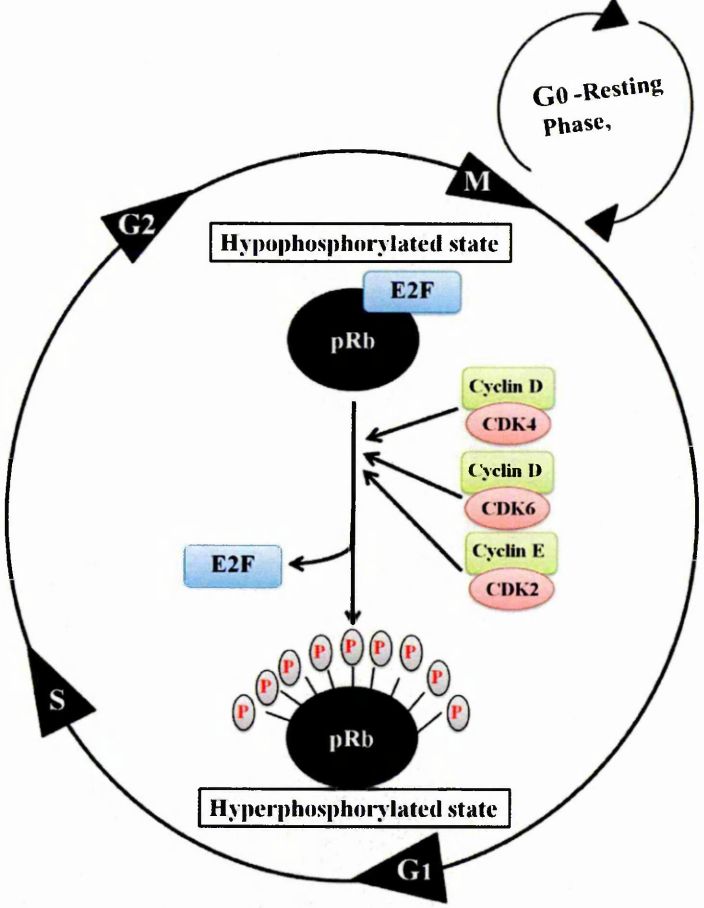


Figure 1.6: Major role of pRb in cell cycle regulation. pRb exists in two states of phosphorylation: hypophosphorylated, and hyperphosphorylated, their activity is regulated by specific phosphorylations in response to DNA damage. In G_0 and early G_1, the hypophosphorylated state occurs when pRb binds interacts with the E2F family transcription factors such that E2F. Rb/E2F complex normally induces expression of genes that required for DNA synthesis. So, pRb becomes hyperphosphorylated after the activation of cyclins and CDK complexes, at this point in late G_1, E2F dissociates from pRb and can initiate the transcription of genes required for progression into S phase. Modified from Stone *et al*, 2011.

1.6 Apoptosis

Apoptosis is described as type I programmed cell death (PCD). It is vital for cellular self-destruction essential to biological processes ranging from embryogenesis to ageing, from normal tissue homeostasis to many human diseases (Elmore, 2007). The name apoptosis was first coined by Kerr *et al*, in 1972 to describe a morphologically distinct form of cell death (Kerr *et al*, 1972). Normally, apoptosis occurs as a homeostatic mechanism to maintain cell populations throughout development and aging (Elmore, 2007). Furthermore, apoptosis takes place as a protection process when cells are damaged by disease or immune reactions (Elmore, 2007). There are several physiological and pathological stimuli or conditions that can initiate apoptosis, however, not all cells can undergo apoptosis in response these stimuli (Elmore, 2007; Wong, 2011).

1.6.1 Mechanisms of Apoptosis

The mechanisms of apoptosis are sophisticated and highly complex, involving an energy dependent cascade of molecular events (Figure 1.7). There are two main apoptotic pathways: 1) the extrinsic or death receptor pathway and 2) the intrinsic or mitochondrial pathway (Elmore, 2007). These two pathways converge on the same point and trigger the execution pathway (Figure 1.7) (Elmore, 2007). The intrinsic and extrinsic pathways can be distinguished by the differences in adapters and initiator caspases involved in each pathway (Figure 1.7) (David *et al*, 2013). Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners that are responsible for destruction of the cell (Figure 1.7) (Elmore, 2007).

1.6.1.1 The Extrinsic (or Death Receptor) Pathways of Type I and Type II Apoptosis

The extrinsic pathway is initiated by the activation of death receptors, which are cell surface receptors including: tumour necrosis factor receptor (TNFR), first apoptosis signal (Fas) also known as CD95 or APO-1 receptor, and death receptors (DRs 3, 4 or 5). The death receptors belong to the tumour necrosis factor receptor (TNFR) superfamily, each has a cysteine rich extracellular subdomain which permits them to recognise and specifically bind to ligands (TNF-α, FasL, TRAIL, respectively). Following ligand binding trimerisation of receptors occur resulting in its activation (Figure 1.7). The cytoplasmic part of the death receptor, known as the death domain (DD) binds to adaptor proteins such as Fas-associating Death Domain Protein (FADD) or Tumour Necrosis Factor Receptor 1-associated Death Domain Protein (TRADD), which each have their own death domains (DD) and death effector domains (DED) (Figure 1.7). This results in dimerization of the death effector domains (DED) with either procaspase 8 or 10 and the formation a death-inducing signalling complex (DISC). This DISC formation results in autocatalysis of procaspase 8 or 10 releasing active caspase 8 or 10 into the cytoplasm (Figure 1.7). Once caspase 8 or 10 are activated, apoptosis has begun and hence they are known as initiator caspases. Caspase 8 or 10 stimulate the execution phase of apoptosis through cleavage and activation of effector caspases 3, 6 and 7 (Figure 1.7). Cleavage of caspase substrates ultimately leads to the characteristic morphological and biochemical features of apoptosis (Figure 1.7) (Elmore, 2007; Tait and Green, 2010).

The extrinsic (or death receptor mediated) apoptosis can be negatively regulated by Cellular FLICE-Like Inhibiting Protein (c-FLIP) (Section 1.6.3.1) which can bind to adaptor proteins such as FADD or TRADD, or bind to caspase 8 to inhibit the extrinsic pathway and hence apoptosis (Figure 1.7) (Elmore, 2007). In some cell systems, this direct activation of caspase activity is sufficient to elicit apoptosis on its own. This is described as Type 1 apoptotic signalling (Figure 1.7). However, in other systems the signal coming from the DISC is

insufficient for execution of cell death on its own. In this case, the signal requires amplification of the signal by proteolytic activation of B-cell lymphoma-2 (Bcl-2) family member Bid, which is cleaved by caspase 8 to its truncated form tBid. This is conveyed to the mitochondria; where under the action of pro-apoptotic Bcl-2 family members such as Bax and Bak induces the release of cytochrome c and other mitochondrial pro-apoptotic factors into the cytosol. This interacts with the intrinsic apoptotic pathway (Section 1.6.1.2); this is called Type 2 apoptotic signalling (Figure 1.7). Now the extrinsic and intrinsic pathways converge at the level of the effector (executioner) caspases, and cross talk can occur between the two pathways (Figure 1.7) (Tait and Green, 2010).

1.6.1.2 The Intrinsic (or Mitochondrial) Apoptotic Pathway

The intrinsic or mitochondrial apoptotic pathway is triggered by multiple stimuli including: viral infections; DNA damage; oxidative stress; heat shock proteins; ischemia; hypoxia; UV radiation. Irradiation or anti-cancer drugs commonly results in DNA damage in cells, which can lead to apoptotic death through a *p53*-dependent pathway (Figure 1.7). After receiving the initial stimuli, there is the production of pro-apoptotic proteins such as Bax, Bad and Bid (Section 1.6.3.3) within the cytoplasm. These can then bind to the outer membrane of the mitochondria changing it permeability by the opening of the mitochondrial permeability transition (MPT) pores. This causes a loss in the mitochondrial transmembrane potential, which subsequently releases cytochrome c, Smac/Diablo, and Omi/HtrA2 from the mitochondria into the cytoplasm (Figure 1.7). These proteins activate the caspase dependent mitochondrial pathway. Cytochrome c binds to adaptor protein called apoptotic protease activating factor-1 (Apaf-1). Apaf-1 along with adenosine triphosphate (dATP/ATP) induces oligomerization of cytochrome-c and the formation of a heptametric structure resembling a wheel, whose core contains seven N-terminal CARDs called "apoptosome complex"; which is now capable of caspase activation (Figure 1.7). The formation of the apoptosome complex allows interaction of pro-caspase 9 with Apaf-1, leading to the cleavage of procaspase 9 to its activated caspase 9 form (Figure 1.7). Active caspase 9 now becomes an initiator caspase causing the activation of the effector (or executioner) caspases: caspase 3 and 7; this now completes the execution pathway of apoptosis (Figure 1.7) (Tait and Green, 2010; Ouyang *et al*, 2012; Rajguru *et al,* 2012).

The intrinsic pathway can be negatively regulated by inhibitor of apoptosis proteins (IAPs) (Section 1.6.3.2) which cause the neutralisation of activated caspase 9 and 3 (Figure 1.7). Furthermore, Smac/Diablo and Omi/HtrA2 are also reported to promote apoptosis by directly interacting with the IAPs and disrupting their ability to inactivate with these caspases (Figure 1.7). In addition to IAPs, the intrinsic pathway can be negatively controlled by the anti-

apoptotic Bcl-2 family members (Section 1.6.3.3) such as Bcl-2 and Bcl-xl, which can directly bind and subsequently inhibit the role of pro-apoptotic proteins Bax and Bad (Figure 1.7) (Rajguru *et al,* 2012).

1.6.1.3 The Execution Phase of Apoptosis

The extrinsic and intrinsic pathways both converge at the execution phase of apoptosis (Figure 1.7) (Jain *et al,* 2014). Here there is the activation of a series of caspases, which are activated at the caspase recognition site by proteolytic cleavage between the large and small domains forming heterodimers. Caspases are sub-divided into initiator caspases (caspase 8, 9 and 10) which have long pro-domains and executioner caspases (caspase 3, 6, and 7) which have short pro-domains (Jain *et al,* 2014) (Figure 1.7). The executioner caspases coordinate the destruction of cell structural proteins and activate enzymes that cause the dramatic morphological and biochemical changes characteristic of apoptosis (Section 1.6.2) (Figure 1.7 and 1.8).

Caspase 3 is considered to be the most important executioner caspase and is activated by the initiator caspases (Figure 1.7) (Belloc *et al,* 2000). Therefore activation of caspase 3 pathway is considered as a hallmark of apoptosis and its measurement is often considered to indicate early apoptosis prior to the appearance of the typical morphological changes of apoptosis (Belloc *et al,* 2000). The precursor form of caspase 3 is predominantly synthesised in the cytoplasm, and activated caspase 3 is translocated from the cytoplasm into the nucleus of the cell by diffusion. This causes disruption of nuclear-cytoplasmic barrier leading to the nuclear morphological changes seen during apoptosis (Belloc et al, 2000).

When caspase 3 is activated, it is also play a crucial role in the destruction of the nucleus of the cell through several substrates: 1) The cleavage the Inhibitor of Caspase activated DNAse (I^{CAD}) of the Caspase-Activated Deoxyribonuclease (CAD) that responsible for chromosomal DNA degradation within the nuclei, resulting in nuclear condensation. 2) Breakdown of structural nuclear proteins (nuclear lamina) further driving chromatin condensation and nuclear fragmentation. 3) Cleavage of cytoskeletal proteins and the cleavage of poly (ADP-ribose) polymerase (PARP); which allows DNA to fragment without repair. Together these actions of caspase 3 contribute to the typical morphological changes seen in apoptosis (Section 1.6.2) (Elmore, 2007).

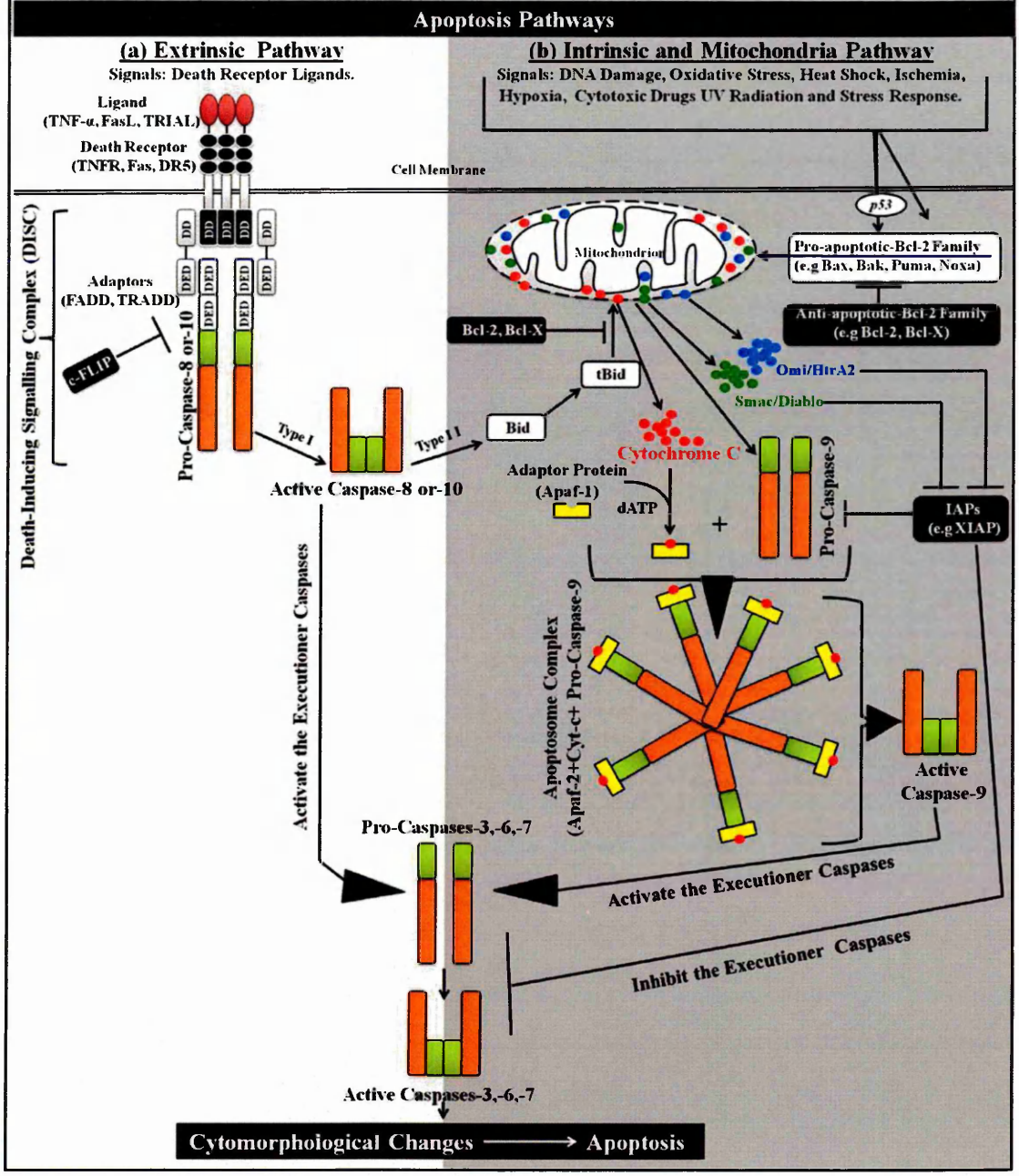


Figure 1.7: Apoptosis pathways. (a) The extrinsic apoptosis pathway - activated by the binding of a ligand to a death receptor. (b) The intrinsic pathway- activated by cellular stress. Each pathway activates its own initiator caspase (8, 9 or 10) which will activate caspase 3. The execution pathway results in characteristic features of apoptosis. This can be negatively regulated by Bcl-2, c-FLIP and IAPs proteins. Modified from Elmore, 2007 and Wong, 2011.

1.6.2 Morphological Features of Apoptosis

During apoptosis the cell shrinks and chromatin condenses; the cell membrane blebs and fragments giving rise to pyknotic apoptotic bodies (Figure 1.8) (Doonan *et al*, 2008). The typical features of apoptosis can be visualised using Hoechst 33342 staining and fluorescence microscopy.

Live Cells	Typical morphological features of Apoptotic cells			
Clear-edged, Round	Shrinkage & Condensation	Formation of Blebbing	Fragmentation	Apoptotic Bodies
				

Figure 1.8: Typical morphological features of live and apoptotic cells.

1.6.3 Regulators of Apoptosis

When considering the physiological importance of apoptotic responses, it is clear that tight regulations of the signalling cascades are required. Three major regulators of apoptosis signalling have been identified including Cellular FLICE-inhibitory protein (c-FLIPs), Bcl-2 family members, Inhibitors of Apoptosis Proteins (IAPs) and p53 (Figure 1.7) (Portt *et al*, 2011).

Generally, the regulation of apoptosis pathways is controlled by a balance between pro-apoptotic and anti-apoptotic proteins (Gewies, 2003). The extrinsic (death receptor) pathway is regulated by the death inhibitory molecule: c-FLIP, which controls the expression of activating ligands and cytoplasmic adapter molecules such as FADD and TRADD which are needed for procaspase 8 or 10 activation (Fridman and Lowe, 2003). The intrinsic pathway is regulated by the pro- and anti- apoptotic members of the Bcl-2 family, within the mitochondria (Gewies, 2003). The IAPs provides a further level of control at the interface between the intrinsic and extrinsic pathways (Fridman and Lowe, 2003). p53 regulates apoptosis via modulation of key control points in both extrinsic and intrinsic pathways (Fridman and Lowe, 2003; Gewies, 2003). The tumour suppressor protein p53 has a critical role in regulation of Bcl-2 family of proteins; however the exact mechanisms have not yet been completely interpreted (Fridman and Lowe, 2003).

1.6.3.1 Cellular FLICE –Inhibitory Protein (c-FLIP)

Cellular FLICE-inhibitory protein (c-FLIP) is a master anti-apoptotic regulator for the extrinsic pathway (Figure 1.7) (Safa *et al*, 2008; Portt *et al*, 2011; Safa, 2012). Functionally, c-FLIP binds to FADD and/or caspase 8 or 10 and TRAIL receptor 5 (DR5) in a ligand-dependent and ligand-independent fashion, which in turn inhibits DISC formation and subsequent activation of the caspase cascade. This protects cells from the death receptor mediated apoptosis (Figure 1.7) (Safa *et al*, 2008; Portt *et al*, 2011; Safa, 2012). In addition, c-FLIP can also suppress induction of apoptosis triggered by chemotherapy agents in cancer (Safa, 2012). c-FLIP can be

expressed as a long (c-FLIP-L) or a short (c-FLIP-S) protein; each being structurally and functionally different (Safa *et al*, 2008; Safa, 2012). c-FLIP-L is similar to caspase 8, it contains two DEDs at its N-terminal, and a large and small caspase-like domain without catalytic activity; however, c-FLIP-S contains only two DEDs and a small C-terminus (Safa *et al*, 2008). Particularly, c-FLIP-S prevents the initial step of pro-caspase 8. Whilst c-FLIP-L permits the first cleavage step, releasing the small subunit of caspase 8, while preventing the second cleavage between the large caspase subunit and the DED domains (Safa *et al*, 2008; Safa, 2012). c-FLIP has been shown to have multifunctional roles in a number of signalling pathways. It is capable of activating and/or up-regulating many cytoprotective and pro-survival signalling proteins, such as Nuclear factor-κB (NF-κB), Protein kinase B (PKB) (also known as Akt) and extracellular-signal-regulated kinase (ERK) (Portt *et al*, 2011; Safa, 2012). Over expression of c-FLIP has been shown to strongly activate the transcriptional factor NF-kβ and might therefore have a role in regulation of NF-kβ dependent gene expression; which could affect cellular proliferation in response to stimulation of death receptors (Safa *et al*, 2008; Portt *et al*, 2011; Safa, 2012). Furthermore, up- regulation of c-FLIP has been reported in numerous tumour types including those of colon carcinoma, melanoma and Hodgkin lymphoma (Safa, 2012). For this reason, c-FLIP has been considered as an important target for cancer therapy (Safa *et al*, 2008; Safa, 2012).

1.6.3.2 Inhibitors of Apoptosis (IAPs)

Both the extrinsic and intrinsic apoptotic pathways converge on a common pathway of caspase activation, which is directly regulated by inhibitors of apoptosis (IAPs) (Figure 1.7) (Nachmias *et al*, 2004; Mannhold *et al,* 2010; Portt *et al*, 2011). IAPs are a group of structurally related proteins which were originally recognised in baculoviruses (Nachmias *et al*, 2004). IAP family members have one or more repeats of a highly conserved 70 amino acids domain called the baculovirus IAP repeat (BIR) sequence, which is located at the amino-terminus (Nachmias *et al*, 2004). To date, eight human IAPs have been identified: XIAP, c-IAP1, c-IAP2, survivin, NAIP, Bruce, ILP-2, and livin (Nachmias *et al*, 2004). The most-well characterised of IAPs is XIAP or X-linked IAP, which is the most potent human inhibitor of caspases (Portt *et al*, 2011). These proteins inhibit apoptosis in two ways: they bind to pro-caspases and prevent their activation, or they bind directly to the caspases and inhibit their activity (Figure 1.7) (Portt *et al*, 2011). In particular, IAPs directly inhibited the effector caspases: 3, 6 and 7. They act through suppression of caspase catalytic activity (Figure 1.7) (Portt *et al*, 2011). Furthermore, IAPs can inhibit the initiator caspases such as caspase 9, which is activated after the release of cytochrome c (Figure 1.7) (Nachmias *et al*, 2004). IAPs can also be upregulated by proteolytic cleavage mediated by effector caspases (Figure 1.7) (Nachmias *et al*, 2004). Alternatively, they can also be down regulated by Smac/Diablo and Omi/HtrA2 (Figure 1.7) (Nachmias *et al*,

2004). Additionally, IAPs expression is regulated by survival signals from growth factor receptors (Mannhold *et al,* 2010). An overexpression of IAPs has been observed in a number of cancers, and believed to be associated with a resistance to apoptosis in cancer cells. Thus, IAPs are considered as one of the potential targets for cancer therapy (Mannhold *et al,* 2010).

1.6.3.3 Bcl-2 Family Members

The Bcl-2 family of proteins include pro-apoptotic and anti-apoptotic proteins, which play an essential role in the regulation of apoptosis, particularly in intrinsic apoptosis; as they reside upstream of irreversible cellular damage and act mainly at the mitochondria level (Czabotar *et al,* 2014). To date, a total of 20 genes have been recognised in the Bcl-2 family (Czabotar *et al,* 2014). All the Bcl-2 members are positioned on the outer mitochondrial membrane and responsible for mitochondrial membrane permeability. The Bcl-2 family of proteins can be recognised by the presence of sequence motifs that are known as Bcl-2 homology domains (BH1 to BH4) (Gewies, 2003). The function of the Bcl-2 family members depends on their Bcl-2 homology (BH) domains. They are classified into three groups: (1) Anti-apoptotic proteins such as Bcl-2, Bcl-x, Bcl-xl, Bcl-x_S, Bcl-w, Mcl-1 and BAG, which contain all four BH domains and protect the cell from apoptotic stimuli. (2) The pro-apoptotic proteins group contain only BH-3 domain these include: Bid, Bim, Noxa, Puma, Bad, Bmf, Hrk, and Bik, these regulate the anti-apoptotic Bcl2 proteins and are the promoter of apoptosis; and final (3) the pro-apoptotic proteins group contain all four BH domains; which include Bax, Bak, and Bok which are the promoter of apoptosis (Czabotar *et al,* 2014). Indeed, the maintenance of mitochondrial membrane potential depends, on the ratio and activity balance between pro-apoptotic and anti-apoptotic signals of the Bcl-2 family members (Ouyang *et al*, 2012; Czabotar *et al,* 2014). These proteins can help determine if the cell is committed to apoptosis or can escape apoptosis (Elmore, 2007). It is believed that the major mechanism of action of the Bcl-2 family of proteins is the regulation of small pro-apoptotic molecules such as cytochrome c, Smac/DIABLO, and Omi/HtrA2 that release from the mitochondria during changes in the mitochondrial membrane permeability (MMP) (Figure 1.7) (Ouyang *et al*, 2012; Czabotar *et al,* 2014). Bax is considered the key pro-apoptotic Bcl-2 family member and can induce cell death via mitochondrial permeabilisation (MMP) (Figure 1.7) (Ouyang *et al*, 2012). The Bcl-2 family is commonly over-expressed in cancers including leukaemia, specifically in chronic lymphocytic leukaemia (CLL) (Ouyang *et al,* 2012). It is important to note that the reduction of Bcl-2 expression may promote apoptotic responses to anti-cancer drugs; however an increase in expression can lead to resistance to chemotherapeutic drugs and even radiation therapy (Ouyang *et al,* 2012).

1.6.3.4 The Role of p53 Tumour Suppressor Gene in Apoptosis

p53 (Section 1.5.2.4) was first tumour suppresser gene linked to apoptosis (Bai and Zhu, 2006). A variety of stimuli activate p53 which then regulates cell-cycle arrest (Section 1.5.2.4), DNA repair, apoptosis and differentiation, through transcriptional activation of specific target genes that carry p53 DNA binding sites (Bai and Zhu, 2006). The different functions of activated p53 are sophisticate and highly dependent on co-expression of the other factors which are affected by cell type and by the severity and persistence of conditions of cell stress and genomic damage. In regard of induction of apoptosis, p53 can directly or indirectly regulate the expression and/or release of pro-apoptotic and anti-apoptotic proteins that control both extrinsic and intrinsic apoptotic pathways (Fridman and Lowe, 2003; Hu and Kavanagh, 2003). Accumulation of p53 within the cell nucleus is often seen after DNA damage. During this DNA damage, p53 protein will arrest the cell cycle allowing time for cells to repair the DNA. If the damage cannot be repaired, then p53 acts as pro-apoptotic signal (Fridman and Lowe, 2003). Activated p53 can positively regulate the pro-apoptotic proteins including Apaf-1, Bax and multiple BH3-proteins such as Noxa, Puma and Bid. These induce cytochrome c release from the mitochondria and induce the intrinsic apoptotic pathway (Figure 1.7) (Fridman and Lowe, 2003; Bai and Zhu, 2006). p53 can also bind to one or more anti- apoptotic mitochondrial proteins such as Bcl-xl, and suppress Bax/ Bak mitochondrial pore formation and the release of cytochrome c (Figure 1.7) (Fridman and Lowe, 2003; Bai and Zhu, 2006). Furthermore, p53 can also trigger expression of death receptors, such as Fas and DR5, which results in activation of extrinsic apoptosis (Figure 1.7) (Fridman and Lowe, 2003; Bai and Zhu). The cellular concentration of p53 is tightly regulated by Mouse double minute 2 (Mdm2) protein, which itself is a transcriptional target of p53 and can trigger the degradation of p53 by the proteasome-ubiquitin system (Bai and Zhu, 2006).

Loss of p53 function confers genomic instability, diminished cell cycle regulation, and impaired apoptosis (Ouyang *et al*, 2012). Alteration of p53 is the most common mutation in human cancer including leukaemia, especially in myeloid leukaemia (Ouyang *et al*, 2012). The loss of p53 in myeloid progenitor's cells is associated with a high risk of developing AML, since p53 has a role in controlling cell proliferation through limiting self-renewal of normal HSCs (Ouyang *et al*, 2012). Tumours that maintain wild type p53 have a better prognosis, as well as a better response to therapy. p53 is an essential pro-apoptotic factor and tumour inhibitor, thus several anti-tumour drugs can exert their roles by targeting p53-related signalling pathways (Ouyang et al, 2012).

1.7 Therapeutic Intervention in Leukaemia: Role in Targeting the Cell Cycle and Apoptosis

Many chemotherapy drugs affect DNA synthesis, the mechanism of action however varies between drugs, although they all interfere with biochemical processes that control cellular replication, which ultimately lead to arrest of cell cycle and induction of apoptosis (Wu, 2006; Sak, 2012; Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Chemotherapeutic agents can be classified according to their chemicals structure and site of action on DNA synthesis (Figure 1.9) (Sak, 2012; Pokhare, 2012; Mohan *et al*, 2013; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). The major classes of chemotherapeutic agents are: topoisomerase inhibitor agents; alkylating agents; anti-metabolite agents; and mitosis inhibitors (Figure 1.9) (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). These chemotherapeutic agents are further sub-divided due to their affect on the cell cycle into cell cycle-specific drugs and non-cell cycle-specific drugs (Figure 1.9) (Lamson and Brignall, 1999; Wu, 2006; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

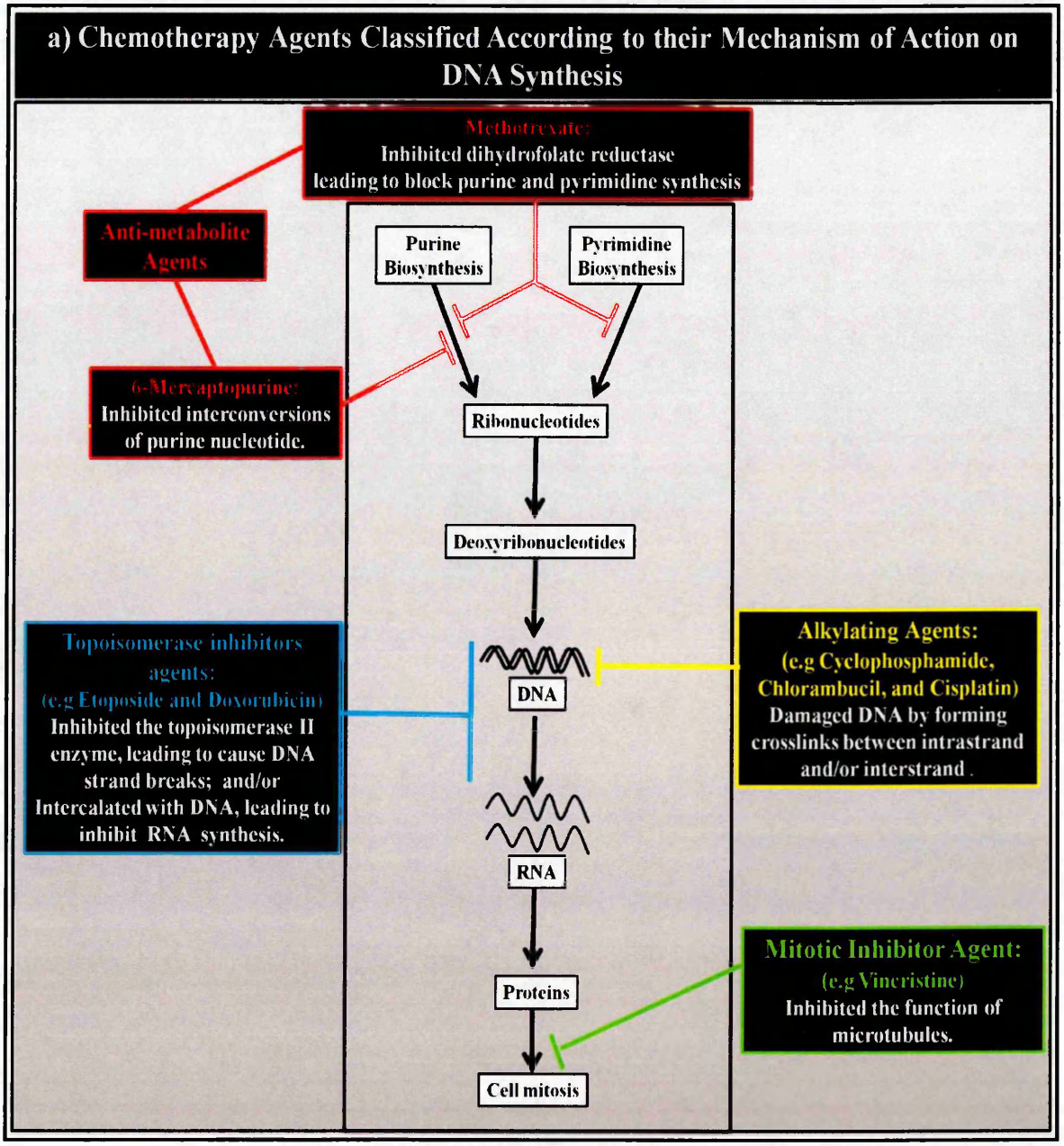
1.7.1 Topoisomerase Inhibitors Agents

Normally, topoisomerases are responsible for controlling the 3D structure of DNA. They regulate the cleavage, unwinding and re-joining of DNA. Furthermore, they are involved in DNA replication, chromatid segregation and transcription. Inhibition of topoisomerase enzymes results in inhibit of DNA replication and results in DNA damage and cell death. Hence topoisomerase inhibitors agents have been applied therapeutically for the treatment of cancer (Hande, 1998; Baldwin *et al,* 2005; Thakur, 2011; Thorn et al, 2011; Tacar *et al,* 2013). There are two classes: Topoisomerase I inhibitors such as topotecan and irinotecan, which binds to the enzyme–DNA complexes, stabilizing it and preventing DNA replication; and Topoisomerase II inhibitors such as etoposide and doxorubicin, which stabilize the complex between topoisomerase II and DNA that causes strand breaks and eventually inhibit DNA replication and led to programmed cell death (apoptosis) (Hande, 1998; Baldwin *et al,* 2005; Thakur, 2011; Thorn et al, 2011; Pokhare, 2012; Tacar *et al,* 2013). The class of topoisomerase II inhibitors are commonly used to treat leukaemias, mainly acute leukaemia types, including ALL and AML.

1.7.1.1 Etoposide

Etoposide is approved for the treatment of acute leukaemia (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). It works by inhibiting the topoisomerase II enzymes. It prevents the re-ligation, synthesis, replication and transcription of DNA, thus generating double-strand DNA breaks which induces apoptosis (Hande, 1998; Baldwin *et al,*

2005; Thakur, 2011; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Etoposide also induces caspase 8 activity, which results in mitochondrial damage and subsequent downstream activation of caspase 9 and 3, and DNA fragmentation and the morphological indicative of apoptosis (Thakur, 2011). Topoisomerase II exists as two highly homology isoforms (alpha- and beta-topoisomerase II) (Thakur, 2011). Etoposide specifically targeted the alpha-topoisomerase II enzyme (Thakur, 2011). This isoform is usually highly formed and produced during G_2/M phase of cell cycle process, for this reason etoposide is considered and sub-classified as cell cycle specific chemotherapy agent as it causes cell cycle arrest at the G_2/M phase (Figure 1.9) (Thakur, 2011).



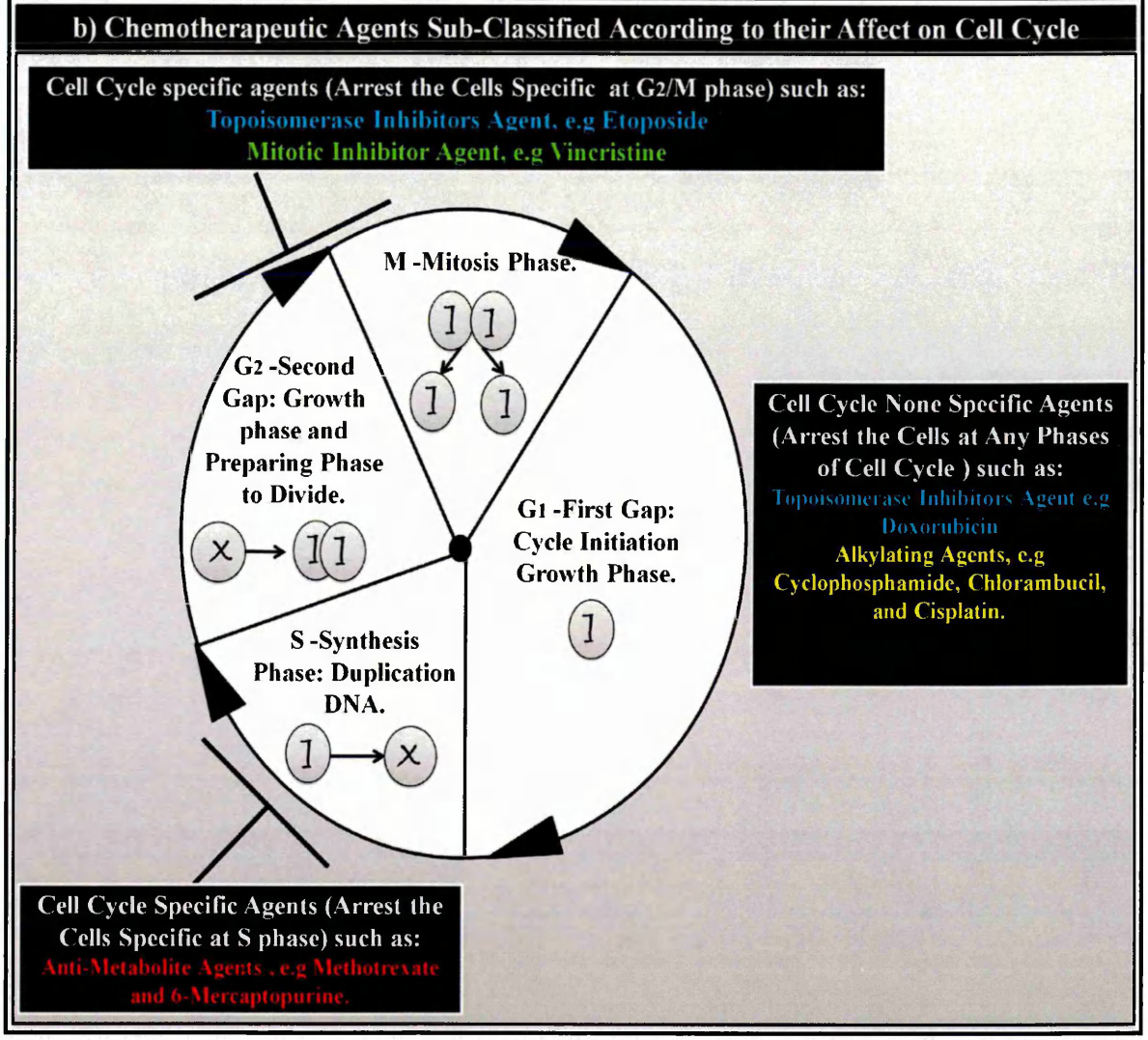


Figure 1.9: Major targets and mechanism of action of chemotherapy agents. (a) Chemotherapy agents are classified according to their mechanism of action on DNA synthesis. (b) Chemotherapy agents are sub-classified also according to their effect on cell cycle.

1.7.1.2 Doxorubicin

Doxorubicin is approved for treatment of acute leukaemia (Thorn et al, 2011; Tacar *et al,* 2013; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). It has three mechanisms of action: 1) It becomes intercalated in the spaces between the nucleotides of DNA or RNA, thus inhibiting DNA and RNA synthesis (Takimoto and Calvo, 2008; Thorn *et al*, 2011; Pokhare, 2012; Tacar *et al,* 2013); 2) it inhibits topoisomerase II, preventing the relaxing of supercoiled DNA and thus blocking DNA transcription and replication, resulting in DNA damage and cell death (apoptosis) (Pommier *et al*, 2010); and 3) it generates free oxygen radicals that damage DNA, proteins and the cell membrane, triggering apoptosis and cell death (Thorn *et al*, 2011). Doxorubicin is also sub-classified as a cell cycle non-specific drug, as it can arrest cells in any phases of cell cycle (Figure 1.9) (Takimoto and Calvo, 2008; Thorn *et al*, 2011; Pokhare, 2012). Doxorubicin is one of the most widely used chemotherapeutic agents

and is generally prescribed in combination with cyclophosphamide and cisplatin (Cancer Research UK, 2014).

1.7.2 Alkylating Agents

Alkylating agents work by modification of DNA bases. They interfere with DNA replication and transcription which leads to DNA mutations (Takimoto and Calvo, 2005; Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Alkylating agents act via two different mechanisms: 1) the alkyl groups (C_nH_{2n+1}) of the drug attaches to the DNA bases at the N7 position of a guanine, resulting in fragmentation of the DNA by repair enzymes as they attempt to replace the alkylated bases. This prevents DNA synthesis and RNA transcription. Alternatively 2) alkylating agents form intra-strand and inter-strand crosslinks between DNA; this prevents DNA synthesis or transcription (Takimoto and Calvo, 2005). Both mechanisms disrupt DNA synthesis and transcription and ultimately resulting in cell death (Takimoto and Calvo, 2005; Pokhare, 2012; Leukaemia & lymphoma Research, 2014). These alkylating chemotherapy agents can be further characterised as being non-cell cycle specific agents, as they can induced arrest of the cell cycle in any phases (Figure 1.9). Cyclophosphamide (Takimoto and Calvo, 2005; Shanafelt *et al,* 2007), chlorambucil (Rai *et al,* 2000) and cisplatin (Florea and Büsselberg, 2011; Praveen *et al*, 2013) are typical alkylating chemotherapy agents approved for the treatment of leukaemia (Figure 1.9).

1.7.2.1 Cyclophosphamide

Cyclophosphamide is approved for all types of leukaemia (Shanafelt *et al,* 2007; Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Cyclophosphamide is a nitrogen mustard alkylating agent that forms DNA crosslinks and inhibits DNA synthesis and induces apoptosis (Hall and Tilby, 1992; Shanafelt *et al,* 2007). For leukaemia treatment, cyclophosphamide is commonly applied alone, or in combination with other chemotherapy agents such as doxorubicin or rituximab and/or with radiotherapy for the treatment of CLL (Leukaemia & lymphoma Research, 2014).

1.7.2.2 Chlorambucil

Chlorambucil is approved to treat most types of leukaemia, but it is mainly applied for the treatment of patients with CLL (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Chlorambucil is a nitrogen mustard alkylating agent that formed DNA crosslinks, resulting in inhibition of DNA synthesis, induction of apoptosis and arresting the cell cycle by up-regulation of p53 (Rai *et al,* 2000).

1.7.2.3 Cisplatin

Cisplatin is approved to treat all types of leukaemia and can be used alone or in combination with other drugs (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). The mechanism of action of cisplatin is mediated by its direct interaction with DNA. It forms intra-strand cross-links between the DNA, resulting in inhibition of DNA synthesis, induction of apoptosis and arrest of the cell cycle (Siddik, 2003; Florea and Büsselberg, 2011). Cisplatin can decrease the activity of ATPase and altered the cellular transports mechanisms, which can cause mitochondrial damage (Siddik, 2003). It also causes direct or indirect damage of the proteins such as p53, Bax, Bcl-2, and caspases; which are involved in the apoptotic; which leads to cell death (Siddik, 2003).

1.7.3 Anti-Metabolite Agents

Anti-metabolite agents are a group of molecules that become incorporated into DNA and RNA synthesis (Kaye, 1998; Sorbello and Brtino, 2001; Sahasranaman *et al,* 2008; Pokhare, 2012; Rossi, 2013). Anti-metabolite agents are structurally related to native nucleosides, amino acids or vitamins (Pokhare, 2012). Most anti-metabolite agents have a similar structure to nucleosides, which are the building blocks of DNA and RNA (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Nucleoside has three functional groups: a nucleobase, a sugar and a phosphate group (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). The nucleobase is normally classified into purines including guanine and adenine; and pyrimidines including cytosine, thymine and uracil (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Anti-metabolite agents are commonly incorporated into these nucleobases or nucleosides of the DNA or RNA, thus inhibiting the DNA polymerase, kinases and enzymes (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). This results in inhibition of the DNA synthesis and an induction of cell death by apoptosis (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Anti-metabolite agents are sub-classified as cell cycle specific agents because their maximal cytotoxic effects are specifically in S phase. Anti-metabolites are considered as one of the first effective chemotherapeutic agents discovered and are classified into three types according to the substances with which they interfere (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). They include: folic acid analogues such as methotrexate, which inhibits dihydrofolate reductase (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Purine analogues like 6-mercaptopurine that inhibits interconversion of purine nucleotide (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Finally, pyrimdine analogues like 5-fluorouracil, which inhibits

thymidylate synthase (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Anti-metabolite agents are commonly applied to the treatment of leukaemia and lymphoma (Leukaemia & lymphoma Research, 2014), but are also used for the treatment of some solid tumours such as breast cancer (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

1.7.3.1 Methotrexate

Methotrexate is approved, alone or with other drugs for the treatment of all type of leukaemia, however in practise they are mainly used for treating ALL that has spread to the central nervous system (CNS) or to prevent this from occuring (Kaye, 1998; Sorbello and Brtino, 2001; Chabner and Roberts, 2005; Rossi, 2013; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Normally, folic acid is an important element for the synthesis of nucleotides (Chabner and Roberts, 2005). It was empirically observed in patients with leukaemia, that diets low in folate produced lower white cell counts than observed in leukaemia patients on normal folate diets (Chabner and Roberts, 2005). Methotrexate is a folic acid antagonist that inhibiting dihydrofolate reductase and prevents the conversion of dihydrofolate to the tetrahydrofolate required for purine and pyrimidine synthesis; which are both essential for DNA or RNA synthesis and cell division (Figure 1.9) (Kaye, 1998; Sorbello and Brtino, 2001; Rossi, 2013). Subsequently, the inhibition of dihydrofolate reductase results in inhibition of cells proliferation, induction of cell death (apoptosis) and arresting the cell cycle at S phase (Figure 1.9) (Kaye, 1998).

1.7.3.2 6-Mercaptopurine

6-Mercaptopurine is approved to treat childhood leukaemias, especially ALL and AML; and sometimes CML (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). 6-Mercaptopurine is a thiopurine analogue of the native purine bases and nucleotides of guanine within DNA (Sahasranaman *et al,* 2008). Mercaptopurine is applied primarily for the maintenance of patients in remission with ALL and is given in combination with MTX for this purpose (Leukaemia & lymphoma Research, 2014). 6-Mercaptopurine is activity metabolised by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) to form 6-thioinosinic acid and 6-methylmercaptopurine ribonucleotide (MMPR), which in turn inhibits a number of the enzymes of purine nucleotides inter-conversion. This results in an alteration of the DNA and RNA synthesis and function; which subsequently leads to DNA damage, apoptosis and cell cycle arrest at S phase (Figure 1.9) (Sahasranaman *et al,* 2008). 6-MP can induce apoptosis through the activation of intrinsic pathway via an up-regulation of p53 expression (Sahasranaman *et al,* 2008).

1.7.4 Mitotic Inhibitor Agents

Cancer cells divide more rapidly than normal cells, for this reason they are more susceptible to inhibition of mitosis (Jiang *et al*, 2006; Chan *et al*, 2012). Mitotic inhibitor such as vincristine is approved for acute leukaemias (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Vincristine is a mitotic spindle poison which acts by binding to tubulin and thus inhibits assembly of the spindle during mitosis and arrests the cells at metaphase (Chan *et al*, 2012). The use of mitotic inhibitor chemotherapy agents are commonly associated with peripheral nerve damage leading to paraesthesia, loss of reflexes and weakness (Leukaemia & lymphoma Research, 2014). It can cause also other side effects including, nausea, vomiting, alopecia and mylosuppression (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

1.7.5 Targeted Therapy

Targeted therapy is the type of treatment that uses drugs or other substances to work by interfering with specific molecules, which are specifically associated with cancer growth. They commonly attack cancer cells more specifically than traditional chemotherapy agents (Downing, 2008). There are some targeted therapies have been approved by the FDA for leukaemia treatment. Examples of these targeted therapies include, immunotherapy, tyrosine kinase inhibitor therapy (e.g. Imatinib) and proteasome inhibitor therapy (e.g. Bortezomib) (Downing, 2008; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014).

17.5.1 Tyrosine Kinase Inhibitor Therapy: Imatinib

Tyrosine kinase inhibitors such as Imatinib are designed to specifically inhibit translocated Bcr-abl, the latter containing a tyrosine kinase domain. Tyrosine kinases are typically responsible for the activation of many proteins that regulate signal transduction, growth, proliferation and survival of hematopoietic and cancer cells (Gilhot, 2004). Imatinib ultimately inhibits cell proliferation and induces apoptosis (Patell *et al,* 2010). Imatinib is sub-classified as a cell cycle non-specific agent; as it can arrest cells at any phase of the cell cycle (Gilhot, 2004). Imatinib is approved to treat newly diagnosed adult and paediatric patients with chronic phase Philadelphia-chromosome positive chronic myeloid leukaemia (Ph+ CML). These are patients with Philadelphia-chromosome-positive chronic myeloid leukaemia (Ph+ CML) in blast crisis, accelerated phase, or chronic phase after failure of interferon-alpha therapy (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). In addition, it is FDA approved to be used in combination with chemotherapy, to treat adult patients with relapsed or refractory Philadelphia-chromosome-positive acute lymphoblastic leukaemia (Ph+ ALL) and

in paediatric patients with newly diagnosed Philadelphia chromosome-positive acute lymphoblastic leukaemia (Ph+ ALL) (Leukaemia & lymphoma Research, 2014).

1.7.5.2 Proteasome Inhibitor Therapy

Proteasomes are multi-enzyme complex found in all cells. Normally, the proteasome plays an important role in control of degrading of more than 80% of ubiquitin-tagged cellular proteins, which control the functions of cellular division, growth, cell cycle and cell death (Niewerth *et al*, 2013; Koyama *et al*, 2014; Liu *et al*, 2014). In particular, proteasome control cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, tumour suppressor genes, oncogenes, and transcriptional activators and inhibitors (Niewerth *et al*, 2013; Koyama *et al*, 2014; Liu *et al*, 2014). The major function of proteasomes is the activation of the transcription factor nuclear factor-kB (NF-kB) (Fuchs *et al*, 2009). Hence, proteasomes are important in maintaining normal cellular function and homeostasis. Clinically, proteasome inhibitor agents such as bortezomib are being used as monotherapy or in combination with standard therapies for the treatment of haematological malignancies, particularly multiple myeloma (Liu *et al*, 2014). Both pre-clinical and clinical studies reported that cancer cells are more sensitive to proteasome inhibitor agents than normal hematopoietic cells (Fuchs *et al*, 2009; Niewerth *et al*, 2013; Koyama *et al*, 2014; Liu *et al*, 2014).

1.7.5.2.1 Bortezomib

Bortezomib (Velcade) is the first proteasome inhibitor approved for the treatment of multiple myeloma and mantle cell lymphoma (Cancer Research UK, 2014). It is considered also as novel treatment strategy in acute leukaemias (ALL and AML) (Niewerth *et al*, 2013; Cancer Research UK, 2014). Functionally, bortezomib inhibits proteasome by binding reversibly to the chymotrypsin-like site in the 26S-core of the proteasome (Niewerth *et al*, 2013). Commonly, bortezomib-mediated proteasome inhibition affects the stability of multiple signalling pathways (Fuchs *et al*, 2009). In particular, bortezomib leads to decreased NF-kB activity, and an increased p53-mediated transcription of genes involved in apoptosis and dysregulation of the cell cycle. This includes increased p21- and p27-mediated induction of cell cycle arrest, and promotion of apoptosis via Bax-mediated inhibition of Bcl-2 (Fuchs *et al*, 2009). This drives the activation of apoptosis and cell death pathways (Fuchs *et al*, 2009). Cancer cells are more susceptible to the effects of bortezomib than normal cells, due to the reversibility of proteasome inhibition in normal cells (Fuchs *et al*, 2009). However, sometimes when bortezomib is insufficient, it may inconsistently activate NF-kB (Fuchs *et al*, 2009). This reversal of bortezomib activity can result in drug resistance mediated through P-glycoprotein (Fuchs *et al*, 2009). In addition, bortezomib is associated with moderate side effects including fatigue, diarrhoea, painful peripheral neuropathy, thrombocytopenia, bleeding, anaemia and

neutropenia (Cancer Research UK, 2014). Additionally, it can increase the infection risk due to a drop in the number of white blood cells (Cancer Research UK, 2014).

1.7.6 Major Problems of Current Leukaemia Treatments

1.7.6.1 Side Effects

The current leukaemia treatments are associated severe side effects (Table 1.9). The specific side effects for each treatment are represented in Table 1.8.

1.7.6.2 Resistance to Anti-Cancer Treatments

Drug resistance is considered as a major problem for leukaemia therapy. Resistance can occur to standard chemotherapy drugs, targeted therapy agents or even radiation treatment; (Luqmani, 2005; Mohan *et al*, 2013). Drug resistance can develop through a number of pathways: (a) An expression of multidrug resistance (MDR1) gene and an up-regulation of p-glycoprotein membrane transporters which remove chemotherapy drug from the cancer cells, resulting in a reduced intracellular drug accumulation and loss of therapeutic efficacy. (b) Drug inactivation due to conjugation with cellular glutathione. (c) Up regulation of survival factors such as NF-kB transcription factor and/or the anti-apoptotic proteins Bcl-2 and Bcl-xl. (d) Mutations in the p53 tumour suppressor gene which leads to an increased cell cycle and resistance to cell death. And finally (e) An increased in DNA repair in cells by nucleotide excision repair (NER) mechanisms (Luqmani, 2005). Each anti-cancer agent has a different resistance mechanisms and this shown in Table 1.10.

1.7.6.3 Disease Recurrence

In the case of chronic cancer such as CML, recurrence may be expected as part of the cycle of the disease. In other cases, recurrence may be unexpected, particularly if the remission has lasted a significant amount of time. For instance, current chemotherapy treatment in patients with AML will result in complete remission in 50% to 90% of patients; but between 10% and 25% of patients will have primary refractory disease and the majority of those who gain remission will relapse within 3 years of diagnosis. The treatment of relapsed leukaemia is complex and well-controlled trails in this group of patients are uncommon. In addition, more than 80% of children with AML experience complete remission (CR), but later more than 30% of these children suffer recurrence, and the long-term survival rate is only about 50%. After relapse, the survival is very poor and ranges from 21% to 33% (Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Together, this indicates that effective and new leukaemia therapies are urgently needed. One such strategy, which could hold promise for the future of leukaemia therapies, is by using none-toxic bioactive compounds such as polyphenols that are isolated from natural sources (fruits and vegetables).

Leukaemia Treatment Classification	Agents Name Or Method of Treatment	Side Effects and Toxicities	References
Alkylating Agents	**Cyclophosphamide**	Major side effects: Alopecia, fatigue, nausea, vomiting, stomach ache, diarrhoea, haemorrhagic cystitis and suppression of haematopoiesis.	Shanafelt *et al,* 2007; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
		Triggers mutagenic and teratogenic effects	Kirshon et al, 1988; Leukaemia & lymphoma Research, 2014
		An increased risk of developing skin cancer, bladder cancer or acute myeloid leukaemia.	Radis et al, 1995; Shanafelt *et al,* 2007; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
	Chlorambuci	Major side effects: Myelosuppresion, anaemia, thrombocytopenia, neutropenia, hyperuricemia, pulmonary fibrosis, alopecia, nausea, vomiting, hepatotoxicity and sometime skin reactions.. An increased risk of development some cancers	Rai *et al,* 2000; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
	Cisplatin	Major side effects: Nausea, vomiting, neurotoxicity, nephrotoxicity, hearing loss, in addition to myelosuppresion and immunesuppression.	Hansen *et al,* 1989; Florea and Büsselberg, 2011.
Anti-Metabolite Agents	**Methotrexate**	Major side effects: Nausea, vomiting, diarrhoea, fatigue, myelosuppression, gastritis, acute pneumonitis and kidney failure.	Kaye, 1998; Sorbello and Brtino, 2001; Rossi, 2013.
		High doses can led to damage to the central nervous system.	Rossi, 2013.
	6-Mercaptopurine	Major side effects: Sore throat and mouth, fatigue, alopecia, highly fever .nausea, vomiting, diarrhoea and skin rash, , myelosuppression, liver toxicity, jaundice and abdominal swelling.	Sahasranaman *et al,* 2008; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Topoisomerase II Inhibitor Agents	**Etoposide**	Major side effects: Fatigue, nausea, vomiting, alopecia, skin rash, fever, myelosuppression, increased heart rate, hematopoietic, lymphoid and liver toxicity. Increases the risk of a second cancer including AML.	Thakur, 2011; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
	Doxorubicin	Major side effects: Cardiotoxicity.	Thorn *et al*, 2011; Lyman *et al,* 2011.
		Other side effects: Fatigue, nausea, vomiting, diarrhoea, alopecia, fever, oral mucositis, esophagitis, skin rashes, liver dysfunction and suppression of haematopoiesis.	Rossi, 2013; Tacar *et al,* 2013.
Mitotic Inhibitor Agent	**Vincristine**	Major side effects: Weakness, nausea, vomiting, alopecia, peripheral nerve damage leading to paraesthesia, loss of reflexes, and myelosuppression.	Jiang *et al*, 2006; Chen *et al*, 2012; Cancer Research UK, 2014; Leukaemia & Lymphima Resarch 2014.
Targeted therapies	**Imatinib**	Major side effects: Nausea, vomiting, diarrhoea, oedema, muscle cramps, and skin rash.	Gilhot, 2004; Dolly and Apostolia, 2009; Patel *et al*, 2010; Leukaemia & Lymphima Resarch 2014.
	Bortezomib (Velcade)	Major side effects: Fatigue, diarrhoea, painful peripheral neuropathy, thrombocytopenia, bleeding, anaemia and neutropenia and increased the infection risk due to a drop in the number of white blood cells.	Fuchs *et al*, 2009; Cancer Research UK, 2014.

Table 1.9: Summary of side effects and toxicities of the current leukaemia treatments.

Anti Cancer Agents	Resistance Mechanisms	References
Alkylating Agents: Cyclophosphamide, Chlorambuci & Cisplatin	***Resistance to alkylating agents can arise through multiple mechanisms:*** ➢ Increased DNA repair ➢ Reduced cellular uptake and accumulation ➢ Decreased cellular permeability to the drug ➢ Increased efflux ➢ Increased drug inactivation ➢ Increased in the synthesis of glutathione (GSH) through conjugation reactions with glutathione S-transferase (GST) which is protective against DNA damage ➢ Inhibition of transmission of DNA damage recognition signals to the apoptotic pathway.	Hall and Tilby, 1992; Siddik, 2003; Florea and Büsselberg, 2011; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Anti-Metabolite Agents: Methotrexate	***Resistance to methotrexate can arise through multiple mechanisms:*** ➢ Impaired transport into cell ➢ Increased efflux due to overexpression of multi-drug resistance protein (MDR) ➢ Increased target expression of thymidylate synthase ➢ Increased in dihydrofolate reductase (DHFR) concentration due to gene amplification and altered affinity of methotrexate to DHFR ➢ Activation of survival pathways such as ERBB signalling pathways ➢ Increased expression of anti-apoptotic proteins e.g. FLIP, and Bcl-2	Kaye, 1998; Sorbello and Brtino, 2001; Chabner and Roberts, 2005; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Anti- Metabolite Agents: 6-Mercaptopurine	***Resistance to 6-mercaptopurine can arise through two mechanisms:*** ➢ Decrease in the activity of hypoxanthine-guanine phosphoribosyl transferase (HGPRT). ➢ Elevated the alkaline phosphatase levels, which dephosphorylate thiopurines nucleotides resulting in cellular loss of ribonuceosides, particularly occurred in acute leukaemia.	Sahasranaman *et al,* 2008; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Topoisomerase II Inhibitor Agents: Etoposide & Doxorubicin	***Resistance to topoisomerase II inhibitor agents can arise through multiple mechanisms:*** ➢ Overexpressed of multi-drug resistance protein (MDR). ➢ Reduced cellular uptake and accumulation, resulting from overexpression of a cell surface permeability glycoprotein. ➢ Altered topoisomerase II activity prevents doxorubicin and etoposide binding the topoisomerase–DNA complex, allowing the broken strands to be repaired. ➢ Decreased apoptosis due to mutation of p53. ➢ Increased in the synthesis of glutathione (GSH) through glutathione S-transferase (GST) which is protective against DNA damage	Thakur, 2011; Thorn *et al*, 2011; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Mitotic Inhibitor Agent: Vincristine	***Resistance to mitotic inhibitor agent such as vincristine can arise through multiple mechanisms:*** ➢ Overexpressed of multi-drug resistance protein (MDR1). ➢ Reduced cellular uptake and accumulation ➢ Mutations in Tubulin ➢ Chromosomal instability	Jiang *et al*, 2006; Chan *et al*, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Targeted therapy: Imatinib	***Resistance to Imatinib can arise through multiple mechanisms:*** ➢ Overexpressed of multi-drug resistance protein (MDR1). ➢ Reduced intracellular uptake of imatinib. ➢ Point mutation in *BCR-ABL*, which interferes with binding. ➢ Bcr-abl gene amplification or overexpression at the mRNA and protein levels. ➢ Chromosomal aberrations.	Dolly and Apostolia, 2009; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014.
Targeted Therapy: Bortezomib (Velcade)	***Resistance to bortezomib can arise through multiple mechanisms:*** ➢ Overexpressed of multi-drug resistance protein (MDR1) mediated through P-glycoprotein. ➢ Reduced intracellular uptake of bortezomib. ➢ Insufficient doses of bortezomib, which may inconsistently activate survival factor such as NF-kB. ➢ Mutation in the binding site for bortezomib. ➢ Enhanced anti-apoptotic mechanisms.	Fuchs *et al*, 2009; Cancer Research UK, 2014.

Table 1.10: Summary of resistance mechanisms to current anti-cancer leukaemia treatments

1.8 Potential of Naturally Sourced Polyphenols

There has been great interest in bioactive agents from natural sources which have anti-cancerous activity (Giovannini *et al,* 2007; Dai *et al,* 2010). The bioactive components that have shown the most potential include polyphenols and polyacetylenes (Huang *et al*, 2007; McDougall *et al*, 2010; Zaini *et al*, 2011; Dahlawi et al, 2012, Spagnuolo *et al*, 2012; Zaini *et al*, 2012; Dahlawi *et al*, 2013). Epidemiological data has shown that diets rich in polyphenols significantly improve the survival and quality of life in patients with cancer (Giovannini *et al,* 2007; Dai *et al,* 2010). Furthermore, these compounds are well tolerated with few side effects (Dai *et al,* 2010).

1.8.1 Polyphenols Overview

Polyphenols are the biggest group of phytochemicals and considered as one of the most numerous and ubiquitous group of plant metabolites (Giovannini *et al,* 2007; Sharif *et al,* 2010; Tsao, 2010). They are an essential part of the human diet (Giovannini *et al,* 2007). Generally, polyphenols can be extracted from fruits (e.g. rhubarb, cherries, berries, apples, citrus); vegetables (e.g. onion, soy beans, celery); herbs, roots, spices (e.g. turmeric, gingko); and green and black tea (Giovannini *et al,* 2007; Huang *et al,* 2007; Yang *et al,* 2008; Jaganathan and Mandal, 2009; Dai *et al,* 2010). Polyphenols have received great attention among nutritionists, food scientists and medical researches due to their roles in human health (Dai *et al,* 2010; Sharif *et al,* 2010; Tsao, 2010). Studies over the years strongly support a role for polyphenols in the prevention of degenerative diseases, particularly cancers, cardiovascular diseases and neurodegenerative diseases (Dai *et al,* 2010; Sharif *et al,* 2010; Tsao, 2010). They have been identified that polyphenols are strong antioxidants, which can defend against oxidative stress caused by excess reactive oxygen species (ROS) (Dai *et al,* 2010; Sharif *et al,* 2010; Tsao, 2010). Subsequently, in the last few years, the recognition and development of such agents for the treatment of cancer has become a major area of research (Patel *et al,* 2007; Dai *et al,* 2010; Sharif *et al,* 2010; Tsao, 2010). Polyphenols have great potential for the treating cancer as they are widely availability in foods; they have low toxicity and biological benefits (Dai *et al,* 2010). Furthermore, in recent *in vivo* and *in vitro* studies polyphenols have been shown to have anti-mutagenic, anti-inflammatory, anti-proliferative, anti-progression properties; and are capable of inducing cell cycle arrest and cell death through multiple signalling pathways (Dai *et al,* 2010).

1.8.2 The Main Classification of Polyphenols

Dietary polyphenols are the most abundant antioxidants in human diets with over 8,000 structural variants and different compounds (Giovannini *et al,* 2007; Han *et al,* 2007; Huang *et al,* 2007; Yang *et al,* 2008; Jaganathan and Mandal, 2009; Dai *et al,* 2010; Tsao, 2010). They are secondary metabolites of plants and are characterised by having at least one aromatic ring(s) bearing one or more hydroxyl moieties and are described as polyhydroxylated phytochemicals or polyphenols (Figure 1.11) (Dai *et al,* 2010). Polyphenols are classified into groups based on their chemical structure, particularly according to the number of phenolic rings and by the structural elements that link the phenolic rings. The major classification include: 1) Flavonoids; 2) Anthaquinones; 3) Stilbenes; 4) Phenolic acids; 5) Tannins and 6) Lignans (Dai *et al,* 2010). The most abundant polyphenols in plants are flavonoids, phenolic acids, stilbenes and anthaquinones, of which flavonoids account for 60% of dietary polyphenols (Han *et al,* 2007; Dai *et al,* 2010). The considerable diversity of their chemical structures influences their bioavailability, biological properties, anti-oxidant activity and specificity to react with enzymes and cell receptors (Giovannini *et al,* 2007). This study focuses on three classes of polyphenols: Flavonoids, Anthaquinones and Stilbenes; and from these classes eight compounds have been selected for investigation based on their previously shown anti-proliferative, pro-apoptotic and/or growth inhibitory affects in solid tumours; and thus may have potential in the treatment of leukaemia. The selected polyphenols include: flavonoids (e.g. quercetin, apigenin and chrysin); anthraquinones (e.g. emodin, aloe-emodin and rhein); and stilbenes (e.g. cis-stilbene and trans-stilbene).

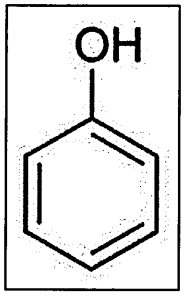


Figure 1.10: Basic structure of polyphenols (Phenol) (Han *et al,* 2007).

1.8.2.1 Flavonoids

Flavonoids are extensively distributed among the plant kingdom (Sandha *et al*, 2011; Kumar and Pandey, 2013). Flavonoids are the most abundant polyphenols in human diets and represent the most important polyphenolic class, with more than 4000 compounds (Huang *et al,* 2007). They are found in fruits (e.g. Apples, blueberries), vegetables (e.g. Onions, broccoli, parsley, and celery), nuts, seeds, flowers and tea (Sandha *et al*, 2011). These polyphenols are an integral part of our daily diet (Sandha *et al*, 2011). The presence of flavonoids in food is commonly responsible for taste, colour, prevention of fat oxidation, as well as enzymes or

vitamins degradation (Sandha *et al*, 2011). Flavonoids are low molecular weight bioactive polyphenols which play a crucial role in photosynthesis (Sandha *et al*, 2011). Chemically, flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B) linked by a heterocyclic pyrane ring (C) (Figure 1.11) (Tsao, 2010; Kumar and Pandey, 2013). Most flavonoids bear this type of 2-phenyl-benzo-γ-pyrane structure (Tsao, 2010; Kumar and Pandey, 2013). Due to the B ring relative to the C ring, as well as the hydroxylation pattern and variations in the C ring (such as functional groups (ketones, hydroxyls) and presence of a double bond or not in the C ring); flavonoids can be further divided into different sub-classes such as flavonols (e.g. quercetin and kaempferol), flavones (e.g. apigenin and chrysin), flavanones (e.g. hesperetin, and naringenin) (Table 1.11) (Tsao, 2010; Kumar and Pandey, 2013). Their general structures are shown in Table 1.11. The most abundant flavonoids in the diet are flavanols (e.g. quercetin) and flavones (e.g. apigenin) (Sandha *et al*, 2011; Kumar and Pandey, 2013). The general dietary intake of flavonoids is estimated to be 1-2 g/day (Kumar and Pandey, 2013). The average intake of flavonols and flavones was reported to be 23 mg/day, among which, flavonol quercetin contributed 16 mg/day (Kumar and Pandey, 2013). The basic structures of flavonoids are aglycones; while in plants, most of these compounds exist as glycosides (Figure 1.11) (Kumar and Pandey, 2013). The biological activities of the flavonoid compounds are usually dependent on both the structural difference and the glycosylation patterns (Kumar and Pandey, 2013). These flavonoids exhibit a wide range of biological activities including: antioxidant, anti-inflammatory, anti-allergic, anti-bacterial, anti-viral, anti-tumour activities, they are cytotoxic and have been used as a treatment of neurodegenerative diseases and used as a vasodilator (Sandha et al, 2011; Kumar and Pandey, 2013).

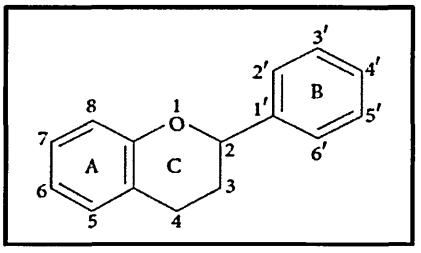


Figure 1.11: Basic Flavonoid Structure (Kumar and Pandey, 2013).

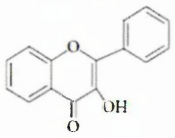
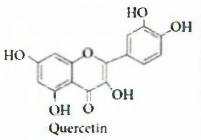
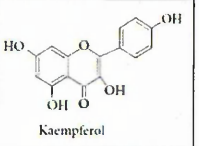
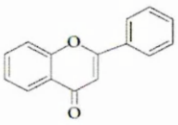
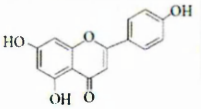
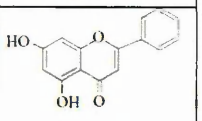
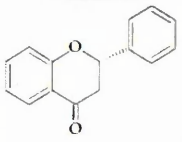
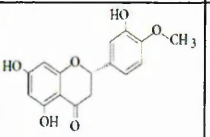
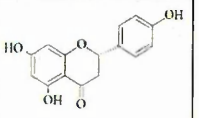
Major Classes of Flavanoid	Structure backbone	Examples for the Major Representative Compounds	
Flavonols		 Quercetin	 Kaempferol
Flavones		 Apigenin	 Chrysin
Flavanones		 Hesperetin	 Naringenin

Table 1.11: Chemical structure of major types of flavonoids and their representative compounds used in this study (Kumar and Pandey, 2013).

1.8.2.2 Anthaquinones

Anthraquinones are a group of functionally diverse aromatic chemicals structurally related to anthracenes. The basic chemical structure of anthraquinone is an anthracene ring (tricyclic aromatic) with two ketone groups in position C-9 and C-10 (Figure 1.12) (Dave and Ledwani, 2012). In plant, anthraquinones are mostly present as sugar derivatives such as glycosides, but they are also present in the free glycones (Huang *et al,* 2007). Anthraquinones are found mainly in medical rhubarb (Huang *et al,* 2007; McDougall *et al,* 2010), or herbs (e.g., senna, purslane and aloe); plus peas, cabbage, lettuce and beans (Dave and Ledwani, 2012). Six key anthraquinones have therapeutic potential in the treatment of cancer included aloe-emodin, emodin, rhein, chrysophanol, physcion and danthron, aloe, senna and purslane (Huang *et al,* 2007; McDougall *et al,* 2010; Zhang *et al*, 2010). The major anthraquinone derivatives including, emodin (1, 3, 8-trihydroxy-6-methylanthraquinone, ~2.6%), aloe-emodin (1, 8-dihydroxy-3-hydroxyl-methyl anthraquinone, ~1.8%), rhein (1, 8-dihydroxy-3-carboxyanthraquinone, ~1.9%) (Figure 1.13) (Huang *et al*, 2007). Anthraquinone compounds are commonly used as laxative, or for the treatment of fungal infections (Dave and Ledwani, 2012). Recent studies have indicated that a number of anti-neoplastic effects could be found among studied anthraquinones, including emodin, aloe-emodin, and rhein (Zhang *et al*, 2010; Dave and Ledwani, 2012).

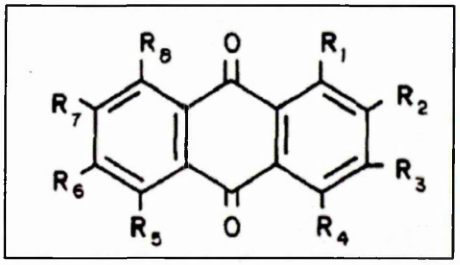


Figure 1.12: Basic chemical structure of anthraquinone (Huang *et al* 2007).

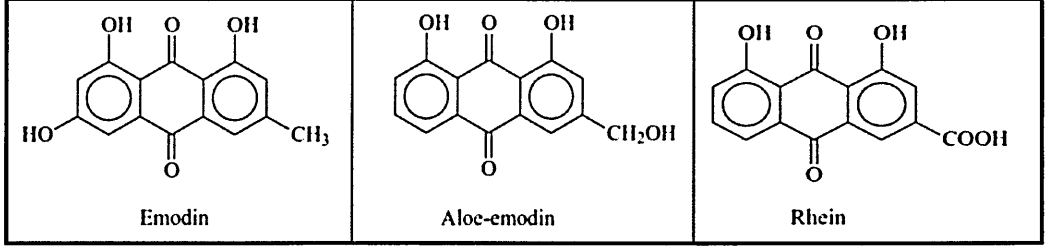


Figure 1.13: Chemical structures of the major anthraquinones: Emodin, Aloe-emodin and Rhein (Huang *et al* 2007).

1.8.2.3 Stilbenes

Stilbenes are an important class of plant polyphenols that have been shown to have anti-fungal and anti-microbal effects and are synthesised in plants in response to injury or infection (Pandey and Rizvi, 2009). Stilbenes are mainly found in grapes and peanuts (Han *et al,* 2007); thus they make up a relatively small part of human diet, (0.3–7mg as aglycones and 15 mg as glycosides in grapes). Stilbenes are found in such low quantities, any protective effect of this molecule is unlikely via normal nutritional intakes (Shen *et al,* 2009). Stilbenes have been found to have diverse bioactivity; they have been shown to have antioxidant, anti-malarial, anti-microbial, anti-inflammatory and cytotoxic properties (Shen *et al,* 2009). They have also been shown to be hepatoprotective, and are currently being evaluated for the treatment of Alzheimer's disease and tumour formation (Shen *et al,* 2009).

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge; the basic backbone for stilbenes is a C6-C2-C6 structure (Shen *et al,* 2009). Structurally, the stilbenes are characterised by the presence of a 1, 2-diphenylethylene nucleus with hydroxyls substituted on the aromatic rings, and can be divided into two isomers (Figure 1.14). The first isomer is called *trans*-stilbene or (*E*)-stilbene (*trans*-1, 2-diphenylethylene) (Figure 1.14); and the second isomer is called *cis*-stilbene or (*Z*)-stilbene (*cis*-1, 2-diphenylethylene) (Figure 1.14) (Shen *et al,* 2009). *Cis*-stilbene and *trans*-stilbene are structurally very similar the only difference is in the potency of their biological effect (Gosslau *et al*, 2008). The small difference in their configuration affects cell uptake and hence their biological activity (Gosslau *et al*, 2008).

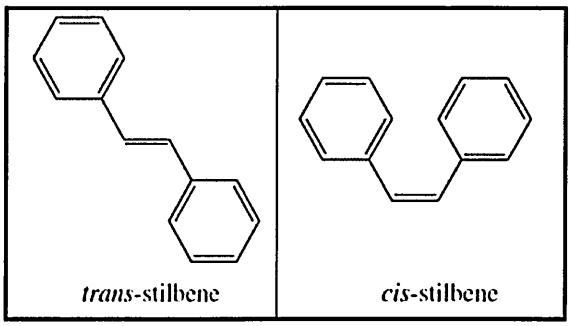


Figure 1.14: Typical structure of stilbene isomers: (A) *Trans*-stilbene and (B) *Cis*-stilbene (Gosslau *et al*, 2008).

1.8.3 Polyphenols - Bioavailability

The most abundant dietary polyphenols in our diet do not necessary have the best bioavailability profile (D'Archivio *et al*, 2010; Manach *et al*, 2004), because they may have a lower intrinsic activity, a poor absorptive rate from the intestine, or are highly metabolised, or rapidly elimination from the body (Manach *et al*, 2004). So, it is not only necessary to know how much of polyphenol is present in specific food, but to also know if its bioavailable (D'Archivio *et al*, 2010).

In general, bioavailability of polyphenols is mostly influenced by their chemical structure and their molecular size; as this will determine the rate and extent of intestinal absorption (Manach *et al,* 2004; Williamson and Manach, 2005; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). In food, some polyphenols are present as aglycones or glucosides (with no sugar group); these can be directly absorbed from the small intestine without requiring hydrolysis (Manach *et al,* 2004; Williamson and Manach, 2005; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). However, most of the polyphenols present as glycosides, polymers or esters forms, and these native forms cannot be absorbed until they hydrolysed by intestinal enzymes or by the colonic microflora (Manach *et al,* 2004; Williamson and Manach, 2005; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). The gut microflora once they have hydrolysed the glycosides into aglycones; these can then be metabolised into aromatic acids (Manach *et al,* 2004; D'Archivio *et al,* 2010). During the course of absorption, polyphenols are conjugated firstly in the small intestine and later in the liver (Manach *et al,* 2004; D'Archivio *et al,* 2010).

Once in the liver the polyphenols are subjected to three main types of conjugation: methylation, sulphation, and glucuronidation (Manach *et al,* 2004; D'Archivio *et al,* 2010). All these modifications deeply affect the biological activity of polyphenols (D'Archivio *et al,* 2010). Subsequently, circulating polyphenol metabolites are not free in blood; they are instead bound to albumin (Manach *et al,* 2004; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). Generally, the physiological concentrations of polyphenols metabolites within blood plasma do not exceed 10μM (Manach *et al,* 2004; D'Archivio *et al,* 2010).

In order to enter the tissues, polyphenols are metabolised. Metabolites of polyphenols are eliminated in bile or urine depending of the size of the conjugated metabolites (Manach *et al,* 2004; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). Particularly, large conjugated metabolites are more likely to be eliminated in the bile, while small conjugates are preferentially excreted in urine (Manach *et al,* 2004; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). However it is important to note that the total amount of polyphenols metabolites excreted in urine is directly correlated to plasma concentrations (Manach *et al,* 2004).

Previous studies in human showed that the most readily absorbed polyphenols were phenolic acid, catechins, flavanones, and quercetin glucoside. However kinetics of this absorptive process was different for each polyphenol (Manach *et al,* 2004; D'Archivio *et al*, 2010). The least well absorbed polyphenols are large molecular weight polyphenols such as the anthocyanins (Han *et al,* 2007; D'Archivio *et al*, 2010). A few researchers have investigated the kinetics and extent of polyphenol absorption by measuring plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenol, provided as pure compound, a plant extract, or whole food/beverage (Williamson and Manach, 2005; Mendoza *et al*, 2011). To date, the data available on bioavailability of polyphenols however is still very limited, but there is evidence that flavonoid compounds such quercetin consumed in a normal diet can result in micromolar concentrations in blood plasma (Hollman *et al*, 1997; Mendoza *et al*, 2011). Furthermore, quercetin has showed to have a relatively long plasma half-life of 11–28 h, and a 50-100 mg dose would lead to concentrations of up to 0.75–1.5 μM in plasma (Manach *et al,* 2004; D'Archivio *et al,* 2010; Mendoza *et al*, 2011). However, further investigations are required to determine whether the effects of the polyphenols *in vitro* are relevant to their clinical use, and to consider their bioavailability and whether these treatment concentrations are achievable in plasma.

18.4 Evidence of the Potential of Polyphenols in the Treatment of Cancers

18.4.1 *The Potential of Flavonoids in the Treatment of Cancer*

(A) Quercetin

Previous studies demonstrated that quercetin has anti-proliferative effects in breast (MDA-MB-231 and MCF-7), cervical (HeLa), liver (HepG2), lung (A-549) and in two leukaemia (HL60 and K562) cell lines (Choi *et al,* 2008; Chien *et al,* 2009; Chou *et al,* 2010; Huang *et al*, 2009; Zhang *et al*, 2009; Priyadarsini *et al,* 2010;,Serrano *et at,* 2006) (Robaszkiewicz *et al,* 2007; and Csokay *et al*, 1997; Kang *et al,* 1997). These studies reported a range of IC_{50} values for quercetin between 20-278 μM (Kang *et al,* 1997; Chien *et al,* 2009) (Table 1.12). In the majority of these studies quercetin induce apoptosis by the activation of the mitochondrial- and caspase 3-dependent apoptotic pathways (Choi *et al,* 2008; Chien *et al,* 2009; Huang *et al*, 2009; Zhang *et al*, 2009; Chou *et al*, 2010; Priyadarsini *et al,* 2010) (Table 1.12). Quercetin was shown to induce apoptosis through inhibition of the major survival signals such as Akt, and extracellular regulated kinase (ERK) and NF-kappa B in hepatoma cell lines (HepG2) (Serrano *et at,* 2006); and through the inhibition of cytosolic protein kinase C (PKC) and phosphoinositol-3-kinase (P13K) in two leukaemia cell lines (HL60 and K562) (Csokay *et al*, 1997; Kang *et al,* 1997) (Table 1.12). In addition, quercetin has been shown to induce S-phase arrest in breast cancer cell lines (MCF-7) by decreasing the level of CDK2, cyclins A and B proteins and increasing the levels of the p53 and p57 protein (Chou *et al,* 2010). It also induced

G_2/M phase arrest in cervical cancer cell lines (HeLa) (Huang *et al*, 2009; Zhang *et al*, 2009; Priyadarsini *et al,* 2010) (Table 1.12).

Quercetin			
Cancer Cell Lines	**IC_{50} Values**	**Targets and Mechanisms of Action**	**References**
Breast Cancer Cell Lines (MDA-MB-435)	100 μM at 24 h	Inhibited cells proliferation. Induced the apoptosis through mitochondrial- and caspase 3-dependent pathways.	(Choi *et al,* 2008)
Breast Cancer Cell Lines (MDA-MB-231)	278 μM at 24 h	Inhibited cells proliferation. Induced the apoptosis through mitochondrial- and caspase 3-dependent pathways.	(Chien *et al,* 2009)
Breast Cancer Cell Lines (MCF-7)	92.4 μM at 48 h	Inhibited cells proliferation. Arrested cell cycle at S phase by decreasing the level of CDK2, cyclins A and B proteins; and increasing the levels of the p53 and p57 protein. Induced the apoptosis through mitochondrial- and caspase 3-dependent pathways.	(Chou *et al,* 2010)
Cervical Cancer Cell Lines (HeLa)	50 μM at 24 h	Inhibited cells proliferation. Arrested cell cycle at G_2/M phase. Induced apoptosis via an up-regulation of pro-apoptotic Bcl-2 family proteins, cytochrome c, Apaf-1 and caspase 3 activity, down-regulation of anti-apoptotic Bcl-2 proteins, and reduction of mitochondrial membrane potential; by increase the levels of the p53 and p57 protein.	(Huang *et al,* 2009)
Cervical Cancer Cell Lines (HeLa)	20 μM at 24h	Inhibited cells proliferation. Arrested cell cycle at G_2/M phase. Induced apoptosis via an up-regulation of pro-apoptotic Bcl-2 family proteins, cytochrome c, Apaf-1 and caspase 3 activity, plus down-regulation of anti-apoptotic Bcl-2 proteins, and reduction of mitochondrial membrane potential.	(Zhang *et al,* 2009)
Cervical Cancer Cell Lines (HeLa)	80 μM at 24 h	Inhibited cells proliferation. Induced apoptosis via an up-regulation of pro-apoptotic Bcl-2 family proteins and caspase 3 activity; plus down-regulation of anti-apoptotic Bcl-2 proteins, and reduction of mitochondrial membrane potential.	(Priyadarsini *et al,* 2010)
Hepatoma Cell Lines (HepG2)	87μM at 18 h	Inhibited cells proliferation. Induced apoptosis associated with inhibition of the major survival signals like Akt, and extracellular regulated kinase (ERK); JNK/AP-1 activation and inhibition of NF-kappa B.	(Serrano *et at,* 2006)
Lung Cancer Cell Lines (A-549)	50 μM at 24 h	Inhibited cells proliferation. Arrested cell cycle at S and G_2/M phases	(Robaszkiewicz *et al,* 2007)
Promyeolytic Leukaemia Cell Lines (HL-60)	20 - 30 μM at 24 h	Inhibited cells proliferation. Induced apoptosis via inhibition of cytosolic protein kinase C (PKC) and membrane TPK, and reduction of phosphoinositol-3-kinase (P13K).	(Kang *et al,* 1997)
Chronic Myeloid Leukaemia Cell Lines (K562)	5.5 μM at 12h	Inhibited cells proliferation Induced apoptosis via inhibition of cytosolic protein kinase C (PKC), and reduction of phosphoinositol-3-kinase (P13K).	(Csokay *et al,* 1997)

Table 1.12: The potential of quercetin in the treatment of cancer based on previous studies.

(B) Apigenin

Apigenin has been targeted as a candidate in the development of non-cytotoxic anti-cancer medicines (Choi and Kim, 2009; Shukla and Gupta, 2010). Previous studies demonstrated that apigenin has anti-proliferative and pro-apoptotic effects on colorectal (SW480, HT-29 and Caco-2) (Wang *et al,* 2000), human cervical (HeLa), neuroblastoma (NUB-7 and LAN-5), hepatoma (HepG2, Hep3B, PLC/PRF/5) (Zheng *et al,* 2005), human breast cancer (MDA-MB-453 and MCF-7) (Way *et al*, 2004; Choi and Kim, 2009) and six leukaemia (THP-1, U937, HL60, JURKAT, K562 and NIH3t3) cell lines (Vrago *et al,* 2006) (Table 1.13). Apigenin was reported as having an IC_{50} values ranging between 40-70μM in colon, cervical and breast cancer cell lines (Wang *et al,* 2000; Way *et al*, 2004; Zheng *et al,* 2005; Choi and Kim, 2009) and between 30-50μM in the leukaemia cell line (Vrago *et al,* 2006) (Table 1.13). Most of the previous studies found that apigenin was a potent apoptotic agent. It was reported that apigenin inhibited cell growth and induced apoptosis via inhibition of the PI3K, and activitation of the Akt kinase and HER2/neu signalling pathways in MDA-MB- 453 and MCF-7 breast cancer cell lines (Way *et al,* 2004) (Table 1.13). In the MDA-MB-453 breast cells apigenin was shown to induce apoptosis via both the intrinsic and extrinsic pathways by the activation of caspases 3, -8 and -9 (Choi and Kim, 2009). Apigenin was also shown to induce apoptosis in six leukaemia cell lines (THP-1, U937, HL60, JURKAT, K562 and NIH3t3) through activation of the intrinsic pathway via an increased caspase 3 and 9 activity. It was also shown to increase reactive oxygen species (ROS) levels in these leukeamia cells and inhibited the activation of cytosolic protein kinase C (PKC) (Vrago *et al,* 2006) (Table 1.13). Apigenin has also been shown to induce G_0/G_1 arrest in human cervical cancer (HeLa) cells through elevating of p53, $p21^{wafl/cipl}$ (Zheng *et al,* 2005) and G_2/M arrest in human colon carcinoma (SW480, HT-29 and Caco-2) through inhibition in p34 (cdc2) kinase, and cyclin B1 proteins levels (Wang *et al,* 2000) (Table 1.13).

Apigenin			
Cancer Cell Lines	**IC_{50} Values**	**Targets and Mechanisms of Action**	**References**
Human Colon Carcinoma Cell lines (SW480, HT-29, and Caco-2).	40 μM at 24 h for SW480 50 μM at 24 h for HT-29 70 μM at 24 h for Caco-2	Inhibited cells proliferation. Arrested cell cycle at G_2/M through inhibition in p34 (cdc2) kinase, and cyclin B1 proteins levels.	(Wang *et al,* 2000)
Breast Cancer Cell Lines (MDA-MB- 453 and MCF-7)	40 μM at 24 h	Inhibited cells proliferation. Inhibition the HER2/neu-overexpressing accompanied by the induction of apoptosis processes	(Way *et al,* 2004)
Breast Cancer Cell Lines (MDA-MB-453)	59.44 μM at 24 h	Inhibited cells proliferation. Induced apoptosis via both the intrinsic and extrinsic pathways by the activation of caspases-3,-8 and -9.	(Choi and Kim, 2009)
Cervical Cancer Cell Lines (HeLa), **Neuroblastoma Cell Lines (NUB-7 and LAN-5),** **Hepatoma Cell Lines (HepG2, Hep3B, PLC/PRF/5)**	35.89 μM at 24 h	Inhibited cells proliferation. Arrested cell cycle at G_0/G_1 phase in human cervical cancer (HeLa) cells through elevating of p53, $p21^{wafl/cip1}$. Induced apoptosis.	(Zheng *et al,* 2005)
Leukaemia Cell Lines (THP-1, U937, HL60, JURKAT, K562 and NIH3t3)	30 and 50 µM at 24h	Inhibited cells proliferation Induced apoptosis by intrinsic pathway via increased caspase 3 and -9 activity plus an increase in reactive oxygen species (ROS) level, phosphorylation of the MAPKs and p38 kinase expression and activation of cytosolic Protein Kinase C (PKC).	(Vrago *et al,* 2006)

Table 1.13: The potential of apigenin in the treatment of cancer based on previous studies.

(C) Chrysin

Previous studies demonstrated that chrysin has anti-proliferative and pro-apoptotic effects in gastric (SGC-7901), colorectal (HT-29 and HCT-116), cervical (HeLa), hepatic (HepG2), nasopharyngal (CNE1) (Li *et al,* 2011), breast (MDA-MB-231), prostate (PC3) (Parajuli *et al,* 2009), glioma (U87-MG and U-251, C6) (Weng *et al,* 2005; Parajuli *et al,* 2009), and three leukaemia (U937, THP-1 and HL60) cell lines (Monasterio *et al,* 2004; Rmos and Aller, 2008) (Table 1.14). The reported IC_{50} values for chrysin in the solid tumours were between 16 and 100 µM (Weng *et al,* 2005; Parajuli *et al,* 2009; Li *et al,* 2011); and in the leukaemia cell lines were between 16 and 35 µM (Monasterio *et al,* 2004; Rmos and Aller, 2008) (Table 1.14). These studies reported that chrysin was able to induce apoptosis through caspase activation involving caspase 8 and 3 (Monasterio *et al,* 2004; Li, *et al*, 2011); and through inactivation of the Akt signalling (Monasterio *et al,* 2004; Ramos and Aller, 2008) (Table 1.14). Chrysin was also shown to significantly inhibite cell proliferation, induced apoptosis through inhibition of

proteasome activity and caused arrested the cell cycle at G_0/G_1 phase in breast (MDA-MB-231), glioma (U87-MG U-251 and C6) and prostate (PC3) cell lines (Weng *et al,* 2005; Parajuli *et al,* 2009) (Table 1.14). This was accompanied by a reduction in the levels of phosphorylation of pRb; inhibition in the activity of cyclin dependent kinase-2 (CDK2) and -4 (CDK4); and elevation in the levels of cyclin dependent kinase inhibitor ($p21^{waf/, cip1}$); without any change in p53 protein levels (Weng *et al,* 2005; Parajuli *et al,* 2009) (Table 1.14).

Chrysin			
Cancer Cell Lines	**IC_{50} Values**	**Targets and Mechanisms of Action**	**References**
Human Gastric Adenocarcinoma (SGC-7901), Colorectal Adenocarcinoma (HT-29 and HCT-116), Cervical Cancer Cell Lines (HeLa), Hepatoma Cells Lines (HepG2), Human Nasopharyngal Carcinoma Cell Lines (CNE1).	40 μM at 2 h	Inhibited cells proliferation.	(Li *et al,* 2011)
		Induced apoptosis through the caspase cascade activation by increasing the activity levels of caspase 8 and caspase 3.	
Glioma Cell lines (C6).	50 μM at 24, 48 and 72 h	Inhibited cells proliferation.	(Weng *et al,* 2005)
		Arrested cell cycle at G_0/G_1 phase by a reduction in the levels of phosphorylation of Retinoblastoma (Rb) proteins, inhibition the activity of cyclin dependent kinase 2 (CDK2) and 4 (CDK4).	
		Induced apoptosis.	
Breast Cancer Cell Lines (MDA-MB-231), Malignant Glioma (U87-MG and U-251), Prostate Cancer Cell Lines (PC3).	100 µM at 24 h	Inhibited cells proliferation.	(Parajuli *et al,* 2009)
		Arrested cell cycle at G_0/G_1 phase by a reduction in the levels of phosphorylation of Retinoblastoma (Rb) proteins, inhibition the activity of cyclin dependent kinase 2 (CDK2) and 4 (CDK4); and elevation in the levels of cyclin dependent kinase inhibitor ($p21^{waf1/cip1}$) without any change in p53 protein level.	
		Induced apoptosis through inhibition the activity of proteasome.	
Histiocytic Lymphoma Leukaemia Cell Lines (U937)	16 µM at 24 h 32 µM at 48 h	Inhibited cells proliferation.	(Monasterio *et al,* 2004)
		Accumulated cells in sub-G_1 phase of cell cycle.	
		Induced apoptosis by increasing the activity levels of caspase 8 and caspase 3 via the over expression of BCL-2 levels; and through reduction in the level of the member inhibitor of apoptosis proteins XIAP and inactivation of Akt phosphorylation.	
Histiocytic Lymphoma Leukaemia Cell Lines (U937) Promyeolytic Leukaemia Cell Lines (HL-60) Acute Monocytic Leukaemia Cell Lines (THP-1)	25 µM at 8 h 16 µM at 24 h	Inhibited cells proliferation	(Rmos and Aller, 2008)
		Induced apoptosis via a decrease the Akt phosphorylation and mitochondrial dysfunction.	

Table 1.14: The potential of chrysin in the treatment of cancer based on previous studies.

1.8.4.2 *The Potential of Anthraquinones Compounds: Aloe-emodin, Emodin and Rhein in the Treatment of Cancer*

The anti-cancer activity of emodin has been the most highly investigated anthraquinone (Table 1.14). Emodin has been shown to inhibit cells proliferation, arrested the cell cycle at G_0/G_1 phase and induced apoptosis in HL-60 promyeolytic leukaemia cell lines through with activation of caspase 3, up-regulation of p53 and p21 and down regulation the activation of the tyrosine kinase, phosphoinositol-3-kinase (P13K), protein kinase C (PKC), NF-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling cascade (Chen *et al*, 2002) (Table 1.15). Additional studies reported that emodin was able to induce the apoptosis in JURKAT and WEHI-3 leukaemia cell lines through the activation of caspase cascade involving caspase 9 and caspase 3, up-regulation of PARP expressions and elevation the reactive oxygen species (ROS) (Wei *et al,* 2009; Chang *et al,* 2011) (Table 1.15). Similarly emodin along with aloe-emodin and rhein has been shown to have anti-cancer activity in human tongue cancer (SCC-4) (Chen *et al,* 2010). These anthraquinones have been shown to inhibit cell proliferation, arrest the cell cycle and induce the apoptosis, through the production of reactive oxygen species (ROS) and mitochondria-dependent pathways (Table 1.15) (Chen *et al,* 2010). Based on this simgle study, emodin has shown to be one of the most potent ployphenols, with an IC_{50} of 30 μM. This is considerabily lower than the IC_{50} for aloe-emodin and rhein which were 100 μM, at 24 and 48 h (Chen *et al,* 2010) (Table 1.15).

Anthraquinones			
Cancer Cell Lines	**IC_{50} Values**	**Targets and Mechanisms of Action**	**References**
Emodin, Aloe-emodin and Rhein			
Human Tongue Cancer Cell Lines (SCC-4).	30 μM at 24 h and 48 h for emodin. 100 at 24 h and 48 h for aloe-emodin and rhein.	Inhibited cell proliferation. Arrested the cell cycle at G_0/G_1 Induced apoptosis, through the production of reactive oxygen species (ROS) and mitochondria-dependent pathways.	(Chen *et al,* 2010)
Emodin			
HL-60 - Promyeolytic Leukaemia Cell lines	40 μM at 24 h	Inhibited cell proliferation. Arrested the cell cycle at G_0/G_1 Induced apoptosis through with activation of caspase 3, up-regulation of p53 and p21 and down regulation the activation of the tyrosine kinase, phosphoinositol-3-kinase (P13K), protein kinase C (PKC), NF-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling cascade.	(Chen *et al,* 2002)
T-lymphocytic leukaemia Cell Lines (JURKAT)	20 μM at 24 h	Inhibited cell proliferation. Induced apoptosis through the activation of caspase 9 and caspase 3, up-regulation of PARP expressions; elevated ROS.	(Wei *et al,* 2009)
Murine Leukaemia Cell Lines (WEHI-3)	100 μM at 24h and 48 h	Inhibited cell proliferation. Induced apoptosis through the activation of caspase 9 and caspase 3, up-regulation of PARP expressions; elevated ROS.	(Chang *et al,* 2011)

Table 1.15: The potential of anthraquinones (emodin, aloe-emodin and rhein) in the treatment of cancer based on previous studies.

1.8.4.3 *The Potential of Stilbenoids in the Treatment of Cancer*

The stilbenoids have been shown to inhibit cell proliferation and induce apoptosis in lung (A549) (Lee *et al*, 2004; Weng *et al*, 2009), prostate (DU145 and PC3) (Shankar *et al*, 2007), breast (BT-549), colon (HT29) (Yang *et al,* 2002) and one leukaemia (HL60) cell lines (Saiko *et al,* 2006; Simoni *et al,* 2006). Cis-stilbene and trans-stilbene have also been shown to be effective at inhibiting growth of human lung cancer cells. The reported IC_{50} of trans-stilbene and cis-stilbene in human lung cancer (A549) cells, were 6.36 and 0.03 μM, respectively, at 24 h (Lee *et al*, 2004). Cis-stilbene was found to be more effective than trans-stilbene and shown to inhibit cell growth, induce cell cycle arrest at G2/M, through the down-regulation of checkpoint protein cyclin B1 and induce apoptosis through an elevation of the pro-apoptotic protein p53, the cyclin-dependent kinase inhibitor p21, and the release of cytochrome c in the cytosol in lung cancer (A549) cells [(Lee *et al*, 2004), and S phase in leukaemia (HL60) cells (Simoni *et al*, 2006; Saiko *et al,* 2006). Similarly in lung (A549 and CH27) (Weng *et al*, 2009) and prostate cancer (DU145 and PC3) cell lines (Shankar *et al*, 2007). *Trans*-stilbene was shown to induce apoptosis through reduction of mitochondrial membrane potential, elevation the Bax/Bcl-2 ratio, activation of caspase 9 and 3, and subsequent cleavage of PARP (Shankar *et al*, 2007; Weng *et al*, 2009); causing the generation of reactive oxygen species (ROS) (Shankar *et al*, 2007). An evaluation of a series *trans*-stilbene benzenesulfonamide derivatives on 60 human cancer cell lines showed that *trans*-stilbene compounds were more potent on breast cancer (BT-549) and colon cancer (HT29) cell lines with IC_{50} values as low as 0.20 and 0.55 μM at 24 h, respectively (Yang *et al,* 2002) (Table 1.16).

Stilbenoids			
Cancer Cell Lines	**IC_{50} Values**	**Targets and Mechanisms of Action**	**References**
***Trans*-stilbene**			
Lung Cancer Cell Lines (A549) & Lung Squamous Carcinoma Cell Lines (CH27)	92μM at 24 h for A549 cells & 39 μM at 24 h for CH27 cells.	Induced the apoptosis through reduction of mitochondrial membrane potential, elevation the Bax/Bcl-2 ratio, activation of caspase 9 and 3, and cleavage of PARP	(Weng *et al*, 2009)
Prostate Cancer Cell Lines (DU145 and PC3)	25 μM at 48 h.	Inhibited cell growth. Induced apoptosis through reduction of mitochondrial membrane potential via elevation the Bax/Bcl-2 ratio, activation of caspase 9 and 3, and cleavage of PARP generating reactive oxygen species.	(Shankar *et al*, 2007)
Breast Cancer Cell Lines (BT-549) & Colon Cancer Cell Lines (HT29)	0.20 μM at 24 h for BT-549 cells.& 0.55 μM at 24 h for HT29 cells.	Inhibited cell growth. Induced apoptosis.	(Yang *et al,* 2002)
***Cis*-stilbene**			
Promyelocytic Leukaemia Cell Lines (HL-60)	0.15 μM at 24 h.	Inhibited cell growth. Arrested cell cycle at S phase & apoptosis.	(Simoni *et al,* 2006)
Promyelocytic Leukaemia Cell Lines (HL-60)	12 μM at 24 h.	Inhibited cell growth. Arrested cell cycle at S phase & apoptosis.	(Saiko *et al,* 2006)

Table 1.16: The potential of Stilbenoids in the treatment of cancer based on previous studies.

1.8.5 The Potential of Polyphenols in the Treatment of Leukaemia

Polyphenols can affect the overall process of carcinogenesis in many ways. In particular, polyphenols contribute to counteracting oxidative stress and the prevention of the onset of cancer (Lambert *et al,* 2005). Polyphenols have been shown to suppress cell proliferation and hence growth of many cancer cell lines; induce apoptosis or/and arrest the cell cycle. From the previous *in vitro* studies, it can be seen that polyphenols can activate the signalling pathways and regulate cell cycle proteins (Cyclin D1 as an example), growth factors (e.g. EGF, HER-2), transcription factors (e.g. NF-κB, PPAR, p53), protein kinases (e.g. PKC, PI3K, IKK, Akt, MAPK), pro-apoptotic proteins (caspases, PARP, Bax and Bak) and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xl) that are important in the control of cancer (Han *et al,* 2007; Dai *et al,* 2010). Previous studies clearly show the potential of these molecules in the treatment of cancer (Table 1.12, 1.13, 1.14, 1.15 and 1.16). Much of the work undertaken has demonstrated the pro-apoptotic and anti-cancerous activity of these polyphenols has focused on the treatment of solid tumours cancers, of the breast, colon, and liver; however this evidence led us to believe that these molecules may have potential in the treatment of leukaemia. A few studies using one or two leukaemia cell lines have shown that this may be possible, although more detailed work is required. It is essential to study the effects on polyphenols in a number of leukaemia cells lines, including cell lines that are representative of both acute and chronic myeloid and lymphoid leukaemia. Further it is important to determine the IC_{50} values of each studied polyphenol in each cell lines as this will enable a direct comparison of the effectiveness of each in the treatment of leukaemia; and the identification of the most potent polyphenols for treatment of each leukaemia type. From this is may be possible to produce a short-list of polyphenols that could be use alongside standard leukaemia chemotherapy and determine if they could be used as an adjunctive treatment. In this fashion it can be determined whether any synergistic, additive, or antagonistic action of this polyphenols on the action of standard chemotherapy treatments.

The evidence so far suggests that it may be possible for the polyphenols to enhance action of the standard chemotherapy treatments; and thus it may be possible to help in overcoming resistance to standard chemotherapy treatments, improve their performance and help in reducing treatment dosages and toxic side effects, all of which are critical in improving patient prognosis. So, based on evidence from solid tumours and some leukaemia cell lines, it is possible that polyphenols and related bioactive molecules may have potential in the treatment of leukaemia; furthermore they could be used individually or in combination with existing therapies. However, it is necessary to expand this knowledge, to look at all types of leukaemia including: acute and chronic, lymphoid and myeloid leukaemia.

1.9 General Hypothesis

1.9.1 Polyphenols Modulate Apoptotic and Proliferative Responses to Chemotherapy Agents in Leukaemia Cells

The general hypotheses of this research are: Polyphenols inhibit cellular proliferation and have a pro-apoptotic effect on leukaemia cell lines vs. non-tumour cells, and they work synergistically with standard chemotherapeutic agents; and this action is reliant on a modulation in glutathione (GSH) levels within the leukaemia cells. Using a hypothesis-driven approach we aim to evaluate the potential for polyphenols in the treatment of leukaemia.

1.9.2 General Aims

The specific aims for this study were to:

(1) Investigate the effect of 8 polyphenols: quercetin, chrysin, apigenin, emodin, aloe-emodin, rhein, cis-stilbene and trans-stilbene on cellular proliferation, cell cycle and apoptosis (Chapter 2). These polyphenols have been specifically selected as they have been shown in previous studies to have the most potential in the treatment of solid cancer tumours, and are representative of the major polyphenol sub-classes. These polyphenols were tested on a panel of leukaemia cells lines, including human myeloid (KG1a, HL60, THP-1 and K562) and lymphoid (JURKAT, CCRF-CEM, MOLT-3 and U937) leukaemia cell lines, which are representative of the different leukaemia types. Unlike other studies, two control hematopoietic stem cell sources were also tested ($CD34^+$ HSC and $CD133^+$ HSC progenitor cells from cord blood), to investigate the effects of our treatments on normal health blood cells, which is fundamental when developing new chemotherapy treatments. Determine which polyphenols were the most effective at reducing ATP levels as an indicator for cell viability and inducing apoptosis in each of the eight leukemic cell lines, whilst having a limited effect on the non-tumour cells. A direct comparison was made of effectiveness of each polyphenol, an IC_{50} and AP_{50} values were determined for each polyphenol in each cell line.

(2) Investigate the effect of the combination treatment of the most effective polyphenols with the standard chemotherapeutic agents on ATP levels, apoptosis and cell cycle in the two most sensitive leukaemia cell lines (JURKAT and CCRF-CEM) and in the two most resistance leukaemia cell lines (THP-1 and KG-1a) (Chapter 3).

(3) Identify the potential mechanistic pathways for the synergetic and antagonistic combination treatments of polyphenols and chemotherapeutic agents through investigating the extrinsic and intrinsic apoptotic pathways, glutathione levels and DNA damage; in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1 and KG-1a) (Chapter 4).

Chapter 2

Effects of Polyphenols on Proliferation and Apoptosis in Human Myeloid and Lymphoid Leukaemia Cell Lines.

2.1 Introduction

The problems with current treatments have led to the search for new compounds for the treatment of leukaemia. One area that has received great interest is the use of bio-active agents from natural sources (McDougall *et al,* 2010; Zaini *et al,* 2011; Dahlawi *et al,* 2012; Dahlawi *et al,* 2013). Two groups of bioactive components that have shown potential are the polyphenols (Dahlawi *et al,* 2012; Dahlawi *et al,* 2013) and polyacetylenes (Zaini *et al,* 2011). Epidemiological data has shown that diets rich in polyphenols significantly improve the quality of life (Han *et al*, 2007) and survival rates (Dai *et al*, 2013) of patients with a range of chronic diseases, including cancer. Furthermore, these polyphenols are found naturally in a variety of foods and are well tolerated, with few side effects (Dai *et al*, 2013). The selected polyphenols used in this study are representative of 3 different classes of polyphenols, which have been previously shown to have anti-proliferative, pro-apoptotic and/or prevent the progression of solid tumours (Huang *et al,* 2007; Han *et al*, 2007; Patel *et al,* 2007; Jaganathan *et al*, 2009; Sharif *et al*, 2010; Shukla *et al*, 2010; Dai *et al*, 2013) and a handful of leukaemia cell lines, with the most commonly studied being the human promyleocytic: HL-60 cells (Kang *et al*, 1997; Saiko *et al*, 2006; Simoni *et al*, 2006; Vargo *et al*, 2006; Ramos *et al*, 2008). The polyphenols investigated include the flavonol (quercetin), flavones (apigenin and chrysin), anthraquinones (emodin, aloe-emodin and rhein); and two stilbene isomers (*cis*-stilbene and *trans*-stilbene) (Section 1.8.2).

Previous work has demonstrated the pro-apoptotic and anti-cancerous activity of polyphenols in a number of solid tumours (Han *et al*, 2007; Patel *et al,* 2007; Jaganathan *et al*, 2009; Sharif *et al*, 2010; Shukla *et al*, 2010; Dai *et al*, 2013) and a selection of leukaemia cell lines (Kang *et al*, 1997; Saiko *et al*, 2006; Simoni *et al*, 2006; Vargo *et al*, 2006; Ramos *et al*, 2008). However, there has not been a comprehensive comparison of the action of polyphenols within a wide range of leukaemia cell lines. From previous studies, it is difficult to determine which polyphenols have the greatest potential for the treatment of leukaemia. There are no direct comparisons of the IC_{50} values (the concentration which inhibits 50% of cell proliferation) or AP_{50} values (the concentration at which 50% of cells undergo apoptosis) for each polyphenol. Furthermore, it is unclear whether a single polyphenol is affective in all leukaemia types; or whether specific polyphenols are only useful in a single type or subset of leukaemia. For this reason we compared the anti-proliferative and pro-apoptotic effects of the 8 polyphenols that have previously shown potential in solid cancer cell lines, on a panel of leukaemic cell lines that represent the major leukaemia types. These included four myeloid (KG-1a, HL-60, THP-1 and K562), three lymphoid (JURKAT, CCRF-CEM and MOLT-3) human leukaemic cell lines and one histocytic lymphoma cell line (U937). In addition, for the first time we evaluated the

action of these polyphenols on two non-tumour hematopoietic stem progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC) from cord blood.

The purpose of this study was to determine which polyphenols were the most effective at inhibiting cell proliferation and inducing apoptosis in each of the eight leukaemic cell lines, whilst having a limited effect on the non-tumour cells. A direct comparison was made of the IC_{50} and AP_{50} values of each polyphenol in each cell line. Furthermore, we determined the action of each polyphenol on cell-cycle progression.

2.1.1 Hypothesis

This study tested the hypothesis that: Polyphenols reduce ATP levels, induce apoptosis and/or arrest cell cycle progression in leukaemia cell lines.

2.1.2 Aims

The major aims of this study were to investigate the effects of eight polyphenols (quercetin, apigenin, chrysin, emodin, aloe-emodin, rhein, *cis*-stilbene and *trans*-stilbene) on: (1) ATP levels using CellTiter-Glo® luminescent cell viability assay; (2) apoptosis using the NucView caspase 3 activity assay and flow cytometry; and morphological assessment with Hoechst 33342 stained cells and (3) progression of the cell cycle using PI staining and flow cytometry in four human lymphoid leukaemia cell lines (JURKAT, CCRF-CEM, MOLT-3 and U937), four human myeloid leukaemia cell lines (HL60, THP-1, K562 and KG1a) and two non-tumour hematopoietic stem progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC).

2.2 Material and Methods

2.2.1 Preparation of Polyphenols

Polyphenols: quercetin (Enzo), chrysin, apigenin, emodin, aloe-emodin, rhein, cis-stilbene and trans-stilbene (Sigma) were dissolved in ethanol (Sigma). A stock solution of 100 mMol was prepared in 10% (v/v) ethanol (Sigma) in serum free media (Invitrogen) to generate concentrations 0.4, 2, 10, 50, 250, 500 μMol.

2.2.2 Cell Lines and Culture Conditions

Four human myeloid leukaemia cell lines (HL-60 (promyelocytic leukaemia) (ATCC: CCL-240), THP-1 (acute monocytic leukaemia) (ATCC: TIB-202), K562 (chronic myeloid leukaemia) (ATCC: CCL-243) and KG-1a (acute mylogenous leukaemia) (ATCC:CCL-243)]; and three human lymphoid cell lines [JURKAT (peripheral blood T cell leukaemia) (ATCC: TIB-152), MOLT-3 (acute lymphoblastic leukaemia from a patient relapsed following chemotherapy) (ATCC: CRL- 1552), and CCRF-CEM (acute lymphoblastic leukaemia)

(ATCC: CCL-119)]) and one histocytic lymphoma cell line (U937) (ATCC: CRL-1593.2) (Section 1.3.2); together with two non-tumour hematopoietic stem progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC) from cord blood (Stem cell Technologies) were used in this study. All leukaemia cell lines except MOLT-3 were p53-deficient, being either null, or containing mutant p53 (An *et al*, 2000; Durland and Reisman, 2002; Geley *et al*, 1997). MOLT-3 cells express wild type p53 (Cai *et al*, 2001), but are mutant for PTEN (Medyouf *et al*, 2010). All cell lines were tested regularly for mycoplasma contamination using the MycoAlert TM mycoplasma detection kit (Lonza) and were all tested negative throughout the study. Cells were seeded in T75cm² flasks (Invitrogen) in 90% of Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS), 1.5mM L-Glutamine and 100 µg/ml penicillin/streptomycin. The cultures were maintained at 37°C with 5% CO_2 in air.

2.2.2.1 Mycoplasma Analysis Using MycoAlert™ Mycoplasma Detection (Lonza)

One of the most common contaminants present in cell cultures are mycoplasma infections, which can lead to many serious alterations in cell viability, morphology, metabolic function, and gene expression (Uphoff and Drexler, 2005; Young *et al,* 2010; Volokhov *et al,* 2011). The first sign that a cell culture is contaminated with mycoplasma is a reduction in cell proliferation, cell aggregation and a change in cell morphology (Volokhov *et al,* 2011). Thus, regular testing of cell cultures for mycoplasma is essential (Uphoff and Drexler, 2005; Young *et al,* 2010; Volokhov *et al,* 2011). The MycoAlert™ mycoplasma detection assay (Lonza) was used check for mycoplasma contamination monthly (Uphoff and Drexler, 2005; Young *et al,* 2010; Volokhov *et al,* 2011).

The MycoAlert™ assay is a selective biochemical test that uses the activity of certain mycoplasmal enzymes. Any viable mycoplasma is lysed and the mycoplasma enzymes react with the MycoAlert™ substrate, catalyzing the conversion of ADP to ATP. The resultant ATP causes the mono-oxygenation of luciferin in the presence of luciferase, oxygen (O_2) and magnesium (Mg^{2+}) to oxyluciferin, AMP, carbon dioxide and energy in the form of a luminescent signal. This can be measured by a luminometer, and is an indication of the amount of ATP and hence the amount of mycoplasma infection. Briefly, a 2 ml sample of cell culture suspension was removed from each flask of cells into a 20 ml centrifuge tube, and centrifuged at 200 g for 5 minutes. Then, 100µl of culture supernatant was transferred into triplicate wells of a white 96 well plate (Fisher Scientific). One hundred microliters of MycoAlert™ reagent (reconstituted in MycoAlert Buffer from Lonza) was added to each well and incubated at room temperature (RT) for 5 minutes. Luminescence was measured using a Wallac Victor 2 14020 (Reading A). Finally 100 µL of MycoAlert™ substrate (reconstituted

in MycoAlert Buffer, Lonza) was added to each well and the plate incubated at RT for 10 minutes prior to luminescence detection on the Wallac Victor 2 14020 (Reading B). The ATP level in a test sample should be measured before (Reading A) and after (Reading B) addition of the MycoAlert™ substrate, this produces a ratio of ATP levels, indicating the presence or absence of mycoplasma. The MycoAlert ratio was calculated for each well: the equation: Calculate ratio = Reading B/Reading A. A ratio of less than 0.9 was considered to have no mycoplasma contamination.

2.2.3 Analysis of Adenosine-5'-triphosphate (ATP) Levels as Indicator of Cell Viability Using CellTiter-Glo® Luminescent Cell Viability Assay

ATP is essential for the control of intracellular energy and is fundamental for all metabolic process of the cell, and indicative of normal cell function (Gilbert *et al*, 2011). ATP provides energy to sustain protein synthesis, biomass accumulation, growth, and cellular proliferation (Pelicano *et al*, 2003; Gilbert *et al*, 2011). In addition, ATP can mediate a variety of biological functions, including the induction of programmed cell death (apoptosis) (Gilbert *et al*, 2011). Cellular proliferation and apoptosis are energy dependent processes use ATP, during proliferation ATP levels increase in proportion with cell number (Gilbert *et al*, 2011). While, although apoptosis requires ATP, ATP levels will decrease during the loss of metabolic functions until cell death occurs (Gilbert *et al*, 2011). Measuring the ATP levels has been used as an indicator for number of viable cells (Gilbert et al, 2011; Lee *et al,* 2012). There are a number of methods for measuring ATP, however the most reliable and sensitive assay is a bioluminescent method based on the luciferin-luciferase reaction (Gilbert et al, 2011; Lee *et al,* 2012). One such assay is the CellTiter-Glo® Luminescent Cell Viability Assay which unlike traditional colourmetric viability assays such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), this assay measures ATP levels as a luminescent signal rather than a colour change, which can be particularly useful if agents under investigation are coloured.

2.2.3.2 CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay utilises the mono-oxygenation of luciferin in the presence of luciferase, ATP, oxygen (O_2) and magnesium (Mg^{2+}) to oxyluciferin, AMP, carbon dioxide and energy in the form of light (Figure 2.1). The luminescent signal can then be measured by a luminometer detector (Figure 2.1), where the luminescent signal is proportional to the amount of ATP (Lee *et al,* 2012; Promega, 2013).

For ATP analysis, cells were seeded into white 96-well plates (Fisher Scientific) at 2.5 x 10^3 cells per well and treated with each polyphenol (quercetin, apigenin, chrysin, emodin, aloe-emodin, rhein, *cis*-stilbene and *trans* stilbene) (Sigma) at concentrations of 0.4, 2, 10, 50, 250,

500 µMol, together with ethanol vehicle controls at 0.1 % (v/v). Treated cells were incubated at 37^{0}C with 5% CO_2, for 24, 48 and 72 h. Following treatments, 25µl of CellTiter-Glo® Reagent from CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) was added to each well and mixed for 2 min on an orbital shaker and incubated at room temperature (RT) for 10 min to stabilise luminescent signal. The luminescence was measured using Wallac Victor 2 1420 luminescence detector (PerkinElmer, Waltham, USA). The luminescent signal was proportional to the amount of ATP present in the sample. The average from three luminescence readings was calculated and all treated samples were normalised to the controls. All treatments were performed in triplicate, in three independent experiments. The IC_{50} was determined for each polyphenol in each cell line. This was defined as the treatment concentration at which 50% reduction in ATP levels was observed. This was calculated from a linear regression equation of each standard curve for each polyphenol with each cell line. The IC_{25} was also determined in order to provide treatment ranges for apoptosis detection, and cell cycle treatments, but were not used to determine the effectiveness of treatments.

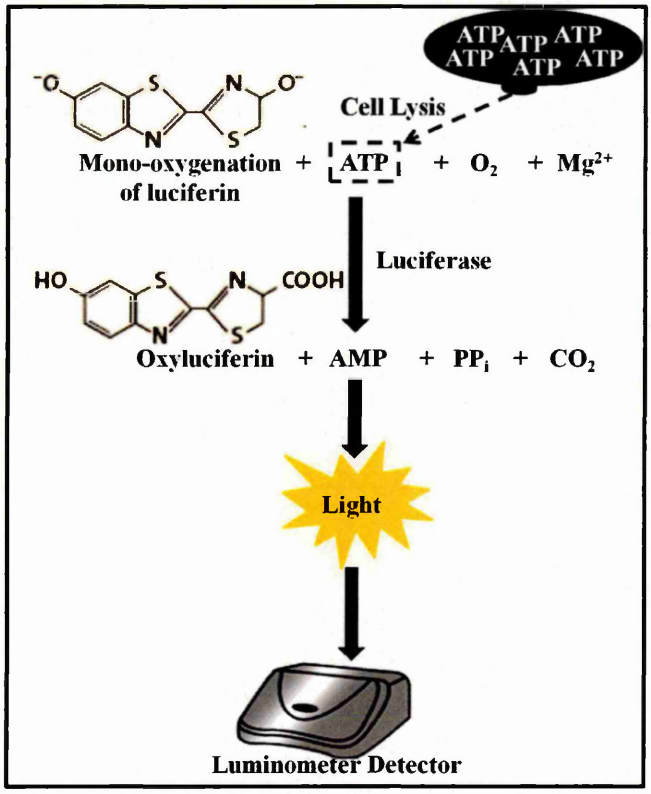


Figure 2.1: Schematic of the CellTiter-Glo® Luminescent Cell Viability Assay. CellTiter-Glo® Reagent was added to cells cultured in serum-supplemented medium. This caused cell lysis and the release of ATP. Luciferin in the presence of luciferase, ATP, oxygen (O_2) and magnesium (Mg^{2+}) is converted to oxyluciferin, AMP, carbon dioxide (CO_2) and energy in the form of a luminescence which can be measured by a luminometer. Modified from Lee *et al,* 2012 and Promega, 2013.

2.2.4 Apoptosis Analysis

2.2.4.1 NucView Caspase 3 Activity Assay Determination by Flow Cytometry

Activation of the caspase 3 is a hallmark of apoptosis, thus determining levels of active caspase 3 is widely applied in cellular assays to identify induction of apoptosis (Lavrik *et al,* 2005). NucView™ 488 caspase 3 substrate is a novel cell membrane-permeable fluorogenic caspase substrate designed by Biotium (Cambridge Biosciences, UK) for detecting active

caspase 3. NucView™ 488 caspase 3 substrate consists of a DNA-fluorogenic dye and a DEVD peptide moiety (with an amino acid sequence of Asp-Glu-Val-Asp). This amino acid sequence corresponds to a sequence within PARP; which is cleaved specifically by caspase 3 (Figure 2.2) (Cen et al, 2008; Skommer *et al*, 2010; Smith *et al*, 2012). The NucView™ 488 substrate is initially is non-functional, until the DEVD peptide (which is highly negatively charged) attaches to a DNA-fluorogenic dye (which is positively charged). Once this occurs the substrate rapidly crosses the cell membrane to enter the cell cytoplasm, where it is cleaved by intracellular caspase 3 activation to release the high-affinity DNA dye; which migrates to the cell nucleus and stains it fluorescent green. This can be measured at 488 nm and analysed by a flow cytometer using FITC settings (Figure 2.2) (Cen et al, 2008; Skommer *et al*, 2010; Smith *et al*, 2012). Using this method, it was possible to determine the AP_{50} concentrations for each polyphenol in each leukaemia cell line. This was defined as the treatment concentration at which 50% of treated cells had undergone apoptosis.

For analysis of caspase 3 activity, cells were treated with each polyphenol at the range of IC_{25} and IC_{50} concentrations as shown determined previously by the CellTiter-Glo® assay. The treatment concentrations for emodin, quercetin and *cis*-stilbene were 0, 0.4, 2, 10, 50 µM; and for apigenin, chrysin, aloe-emodin, rhein and *trans*-stilbene they were 0, 10, 50, 250 µM. Following treatments, 200 µl of each cell suspension was transferred to a flow cytometry tube and 5 µl of caspase 3 activity assay (0.2 mM) (Promega) was added. This was incubated for 10 min in the dark, and each sample was analysed by flow cytometer using a BD FACS Calibur instrument (BD). Ten thousand events were acquired per sample and the data was analysed with Flow Jo software (Tree Star).

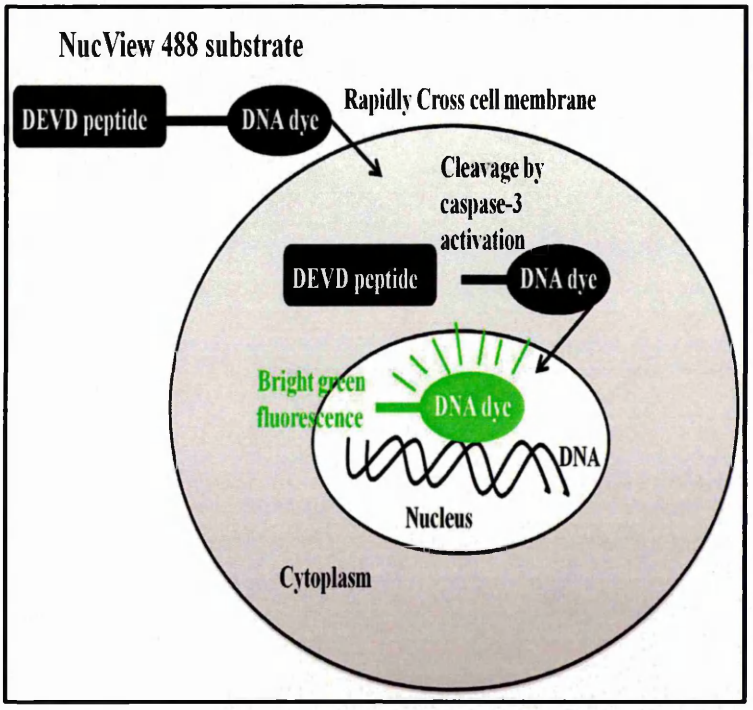


Figure 2.2: The principle of NucView™488 Caspase 3 Substrate staining for intracellular caspase 3 activity detection. NucView™ 488 caspase 3 substrate consists of a DNA-fluorogenic dye and a DEVD peptide moiety specific for caspase 3. This substrate is originally not fluorescent and non-functional until the DEVD peptide (which is highly negatively charged) attached to a DNA-fluorogenic dye (which is positively charged). Now the DNA-fluorogenic dye rapidly crossed the cell membrane to enter the cell cytoplasm, where it is cleaved by intracellular caspase 3 and migrates into the cell nucleus which is now stained a bright fluorescent green.

2.2.4.2 Hoechst 33342 Nuclear Morphological Analysis by Fluorescence Microscopy

Apoptotic cells and nuclear morphology was assessed by fluorescence microscopy following Hoechst 33342 nucleic acid staining. During apoptosis, the cell shrink and the chromatin content condenses. The cell membrane blebs and fragments giving raise to pyknotic apoptotic bodies (Doonan *et al*, 2007; Elmore, 2007). The typical features of apoptosis can be visualised following Hoechst 33342 staining using a fluorescence microscopy. Hoechst 33342 is a permeable blue-fluorescent stain that binds strongly to Adenine-Thymine rich regions in the minor groove of double-stranded DNA (Breusegem *et al,* 2002; Martin *et al,* 2005; Gilbert *et al*, 2011). The fluorescence of Hoechst 33342 stain is very sensitive to DNA conformation and chromatin state in cells. The fluorescence of this stain is enhanced based upon binding to dsDNA AT stretches of at least three AT base pairs (Martin *et al,* 2005). Once the Hoechst 33342 stain is bound to DNA, it generates a 30 fold increase in fluorescence which can be observed by fluorescent microscope (Martin *et al,* 2005; Gilbert *et al*, 2011). Hoechst can enter live and apoptotic cells easily, and the level of fluorescence is proportional to the DNA content. Hoechst 33342 dye provides a brighter staining of the condensed chromatin of apoptotic cells; whilst normal live cells appear round and diffusely stained (Martin *et al,* 2005; Gilbert *et al*, 2011). Using this stain it is possible to identify and quantify the number of apoptotic cells.

For analysis of morphological changes of apoptosis, cells were seeded in 12 well plates 0.5 x 10^6 cells per well and treated for 24 h with each of the polyphenols at wide concentrations of 0.4, 2, 10, 50, 250, 500 µMol, together with ethanol vehicle controls at 0.1 % (v/v). Following polyphenol treatments, cells from each culture well was transferred to eppendorf tubes and centrifuged for 5 min at 400 g at 4ºC. The supernatant was removed, and cells washed in 100 µl PBS. The cells were fixed in 4% (w/v) paraformaldhyde/PBS and cytospins formed (Shandon Cytospin 3 Centrifuge, Thermo). Samples were air dried and stained in 50 µl of 10 µg/ml Hoescht 33342 stain (Sigma) for 10 min in the dark. Slides were mounted in immersion oil and examined using a fluorescence microscope (Olympus). Two hundred cells (live and apoptotic) were counted and the percentage of apoptotic nuclei determined for each sample. Images were captured using LabWorks 4.0 (UVP BioImaging Systems).

2.2.5 Cell Cycle Analysis using Propidium Iodide (PI) and Flow Cytometry

The effect of polyphenols on the progression of cell cycle was studied using a fluorescent DNA-intercalating dye propidium iodide (PI) and analysed by flow cytometery. PI is a red-fluorescent stain that binds to all double stranded nucleic acids, including that of the major groove of double stranded DNA and RNA (Darzynkiewicz, 2010; Darzynkiewicz, 2011). It is

able to quantify the proportion of cells in each phase of cell cycle (G_0/G_1, S and G_2/M); and determine whether cells are accumulated in a specific phase (Figure 2.3) (Thomas, 1993; Tao *et al*, 2004; Leo *et al*, 2011). When analysing cell cycle progression, cell are initially permeabilized and then stained with PI stain together with RNase. The RNase removes any RNA so that the PI can only bind to DNA (Darzynkiewicz, 2010; Darzynkiewicz, 2011). The PI stain once bound to DNA, fluoresces with a broad emission between 600 and 637 nm that can be excited at 488nm and detected using a flow cytometer (Darzynkiewicz, 2010; Darzynkiewicz, 2011). As DNA content increases as cell cycle progresses, cell cycle stage can be easily determined from the concentration of DNA within each cell (Figure 2.3).

For cell cycle analysis, cells were seeded in 12 well plates at 0.5 x 10^6 cells per well and treated for 24 h with dose ranges between IC_{25} and IC_{50} for each polyphenol as shown in the CellTiter-Glo® assay. The treatment doses were 2, 10, 50 µM for quercetin, emodin and *cis*-stilbene (Sigma); 10, 50 250 µM for apigenin (Sigma), and 50, 250, 500 µM for chrysin, aloe-emodin, rhein and *trans*-stilbene (Sigma). Following treatment cells were harvested and centrifuged at 400 g for 5 min. The supernatant was removed, and cells were washed twice in 100 µl cold PBS. Cells were fixed in 100 µl of 80% ethanol/H_2O (v/v) and stored overnight at -20°C. Then, cells were washed twice with cold PBS prior to addition of 300 µl of 50 µg/mL PI (Sigma) and 50 µl of 0.1 unit/mL RNase (Sigma). Samples were PI stained overnight at 4°C and analysed on the flow cytometer with BD FACS Calibur instrument. Ten thousand events were acquired per sample and the DNA histogram of cell cycle phase was analyzed with FlowJo software using the Waston (pragmatic) equation (Tree Star).

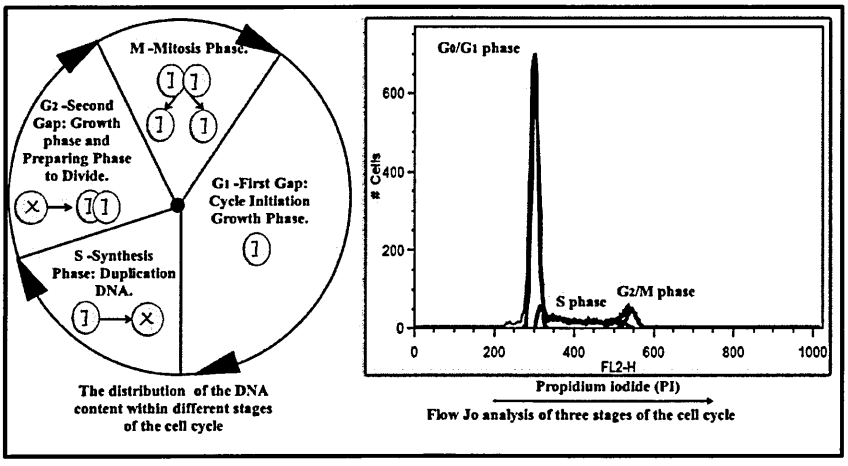


Figure 2.3: The relationship between the cell cycle and DNA histogram. Left picture showed the distribution of the DNA content within different stages of the cell cycle. Right picture showed Flow Jo analysis of three stages of cell cycle using the Waston (pragmatic) equation (Tree Star).

2.2.6 Statistical Analysis

The means and standard deviations (STD) were calculated for CellTiter-Glo®, caspase 3 activity and Hoechst 33342 assays. The median with ranges were calculated for Cell Cycle analysis. Stats Direct software (Stats Direct Ltd, England) was used to test for normality using a Shapiro-Wilks test. The data was non-parametric; a Kruskal-Wallis and Conover-Inman post

hoc test was used to determine statistical significance of the data. Results were considered statistically significant when $p \leq 0.05$.

2.3 Results

2.3.1 Effects of Polyphenol Treatments on ATP Levels in Leukaemia Cell Lines at 24, 48, 72 h

Treatment with polyphenols for 24, 48, 72 h resulted in reduced cell ATP levels in all 8 leukaemia cell lines to a greater extent than in non-tumour cell lines (Figure 2.4, 2.5, 2.6). The lowest dose of polyphenols at which there was a significant inhibition on cellular ATP levels (Table 2.1, 2.3, 2.5), and the IC_{50} values was determined for each polyphenol in each cell line at 24, 48, 72 h (Table 2.2, 2.4, 2.6). Using this data, it was possible to rank the polyphenols in order of effectiveness. Over the different time points, the most effective polyphenols at significantly reducing ATP levels compared to vehicle controls were emodin, quercetin, and *cis*-stilbene ($p<0.05$) (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). A more moderate affect was shown by apigenin and rhein; and the least effective polyphenols were aloe-emodin, *trans*-stilbene and chrysin. Both lymphoid and myeloid leukaemia cell lines were sensitive to emodin, quercetin, and *cis*-stilbene treatment (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). However, it is important to note that each leukaemia cell line demonstrated differing sensitivity with the remaining polyphenols. Generally, the lymphoid cell lines were more sensitive to polyphenol treatment than myeloid cell lines (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6).

Emodin consistently gave the lowest IC_{50} values (5-22 μM at 24 h) (Table 2.2), (3-20 μM at 48 h) (Table 2.4), (2-10 μM at 72 h) (Table 2.6); showing a significant reduction on ATP levels of all leukaemia cell lines ($p<0.05$), with a slightly greater effect on lymphoid than myeloid cells. Emodin also significantly reduced ATP levels in the non-tumour cell lines ($CD34^+$ HSC and $CD133^+$ HSC) ($p<0.05$). However, the IC_{50} in the $CD34^+$ HSC and $CD133^+$ HSC cells (~150 and 250 μM at 24 h) (Table 2.2), (~125 and 160 μM at 48 h) (Table 2.4), (~85 and 95 μM at 72 h) (Table 2.6) were much greater than that seen for all the leukaemia cells, demonstrating selectivity towards malignant verses non-transformed cell lines (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). Similarly, quercetin had a more potent effect on lymphoid cell line (IC_{50} values of 8-20 μM at 24 h, 4.5-10 μM at 48 h and 4-10 μM at 72 h) than myeloid cell lines (IC_{50} values of 33-155 μM at 24 h, 25-100 μM at 48 h and 20-50 μM at 72 h) (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). The least sensitive leukaemia cell line to quercetin treatment with an IC_{50} of 155 μM at 24 h, 100 μM at 48 h, and 50 μM at 72 h was the acute myelogenous leukaemia KG-1a cell line (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). However, the human promyelocytic leukaemia (HL-60) cell line had a much lower IC_{50} values (8 μM at 24 h, 4 μM at 48 h and 3 μM at 72 h), which were similar to those values seen in lymphoid cells (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). In addition, quercetin did not affect the ATP levels of the

non-tumour cell lines, until the treatment dose reached 500 μM at 24 and 48 h, and 250 μM at 72 h (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). *Cis*-stilbene had IC_{50} values of 25-85 μM at 24 h, 25-80 μM at 48 h and 15-75 μM at 72 h, and affected both lymphoid and myeloid cells equally (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). *Cis*-stilbene also did not affect the ATP levels of the non-tumour cell lines, until the treatment dose exceeds 350 μM over the three time points (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6).

Apigenin and rhein had a moderate effect at reducing ATP levels. Apigenin demonstrated a greater effect on the lymphoid cells (IC_{50} values of 140-195 μM at 24 h, 120-160 μM at 48 h and 60-145 μM at 72 h) compared to the myeloid cells (IC_{50} values of 100-500 μM at 24 h, 100-480 μM at 48 h, 60-400 μM at 72 h) (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). In non-tumour cell lines ($CD34^+$ HSC and $CD133^+$ HSC), they did not reach a 50% inhibition of ATP levels until apigenin treatment exceeded 500 μM over the three time points (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6).

Rhein demonstrated a significant decrease on ATP levels of all leukaemia cell lines with IC_{50} values of 135-380 μM at 24 h, 120-260 μM at 48 h and 90-250 μM at 72 h with a similar effect seen in both lymphoid and myeloid cell lines. In the non-tumour cell lines the IC_{50} values were 350-380 μM at 24 h, 260 μM at 48 h and 250 μM at 72 h ($p<0.05$), (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6).

Aloe-emodin, chrysin and *trans*-stilbene were the least effective polyphenols at reducing ATP levels. Aloe-emodin had IC_{50} values between 185-450 μM at 24 h, 180-450 μM at 48 h and 170-380 μM at 72 h (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). These were more than ten times higher than emodin. Aloe-emodin, like emodin, showed a greater effect on lymphoid cell lines than myeloid cell lines (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). Similarly, chrysin demonstrated comparatively high IC_{50} values, and again was more effective on lymphoid cells (IC_{50} values of 128-217 μM at 24 h, 100-200 μM at 48 h and 75-150 μM at 72 h) compared to the myeloid cells (IC_{50} values of 328-500 μM at 24 h, 300-500 μM at 48 h and 270-400 μM at 72 h) (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6).

Trans-stilbene had some of the highest IC_{50} values ranging between 109-500 μM at 24 h, 100-500 μM at 48 h, 95-500 μM at 72 h (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). These were much higher than those values found with its isomer, *cis*-stilbene (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6). Despite the differing effects on the leukaemia cells, aloe-emodin, chrysin and *trans*-stilbene did not cause any significant reduction in ATP levels of the non-tumour cell lines, until the treatment dose reached 500 μM (Figure 2.4, 2.5, 2.6 and Table 2.2, 2.4, 2.6).

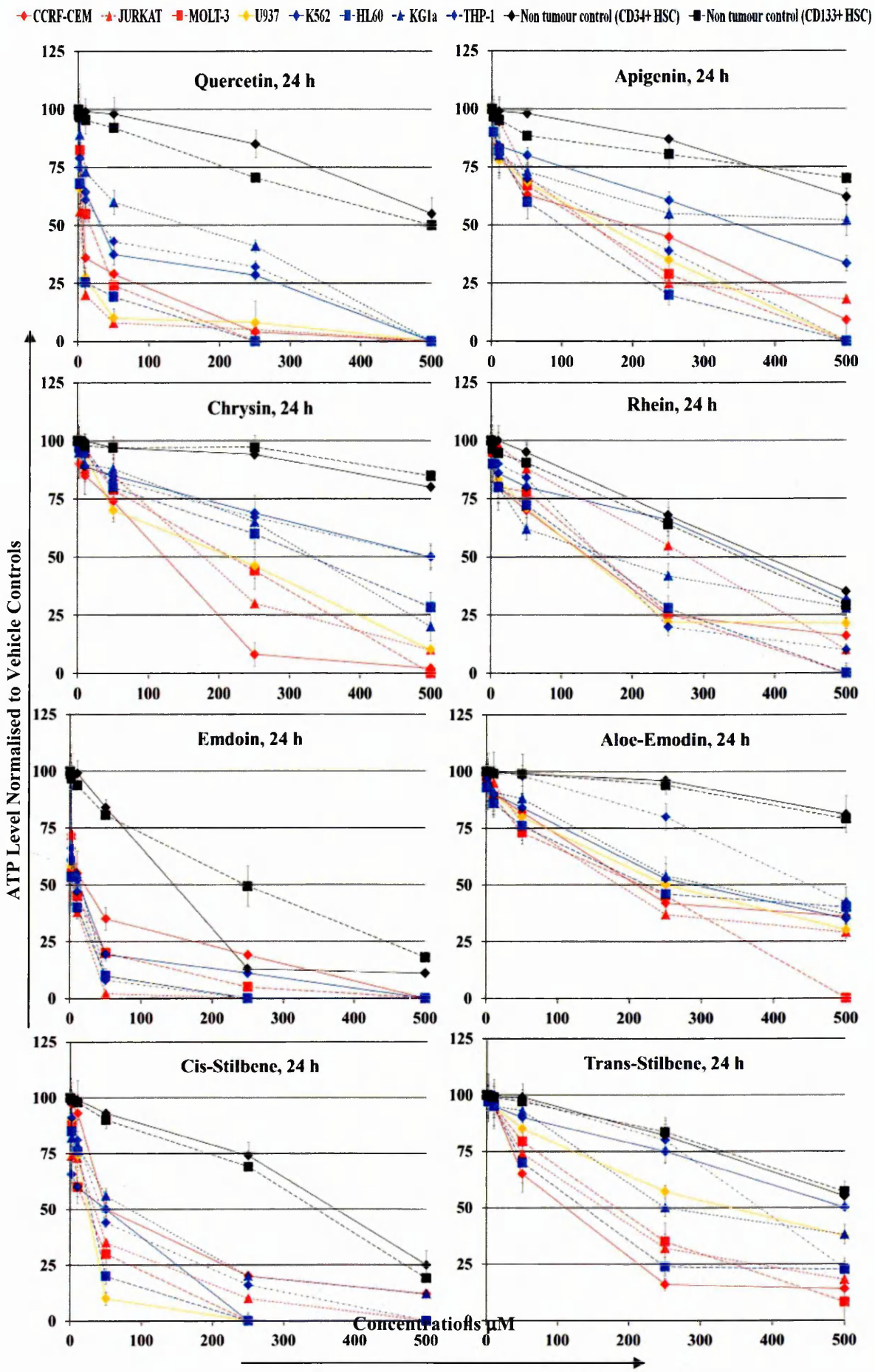

CCRF-CEM
JURKAT
MOLT-3
U937
K562
HL60
KG1a
THP-1
Non tumour control (CD34+ HSC)
Non tumour control (CD133+ HSC)
Quercetin, 24 h
Apigenin, 24 h
Chrysin, 24 h
Rhein, 24 h
Emdoin, 24 h
Aloe-Emodin, 24 h
Cis-Stilbene, 24 h
Trans-Stilbene, 24 h
ATP Level Normalised to Vehicle Controls
Concentrations µM
0
25
50
75
100
125
0
100
200
300
400
500

Figure 2.4: Effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*-stilbene and *trans*-stilbene) on ATP levels of three lymphoid leukaemia (CCRF-CEM, MOLT-3, JURKAT; red lines), one histocytic lymphoma (U937; orange lines), four human myeloid leukaemia cell lines (K562, HL-60, KG-1a , THP-1; blue lines), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC; black lines) at 0, 0.4, 2, 10, 50, 250, 500 µM for 24 h. This was evaluated by CellTiter-Glo® assay. Data was normalised to the vehicle control which was assigned 100% cell viability. The data is expressed as mean ± STD (three independent experiments, each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. Statistical results are summarised in Table 2.1 which shows the lowest dose that induced significant inhibition compared to vehicle control at 24 h. All concentrations above these points were also statistically significant. The IC_{50} for each polyphenol in each cell line at 24 h were determined and shown in Table 2.2.

Cell Types			The Lowest Dose of Polyphenols (µM) At Which There Was a Significant Inhibition of ATP Levels Compared to The Vehicle Control At 24 h.							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	2	50	50	50	2	50	2	50
		CCRF-CEM	2	10	50	50	2	50	10	50
		MOLT-3	2	50	50	50	2	50	2	50
	Myeloid leukaemia	HL60	2	10	50	50	2	50	2	50
		THP-1	2	10	250	50	2	250	2	250
		K562	2	10	250	50	2	50	2	50
		KG1a	10	10	250	50	2	50	2	50
	Histocytic Lymphoma	U937	2	10	50	50	2	50	2	50
Cord blood cells	Non-Tumour Control Cells	$CD34^+$ HSC	250	500	500	250	50	500	250	250
		$CD133^+$ HSC	250	50	500	250	50	500	250	250

Table 2.1: The lowest dose of polyphenols that induced a significant decrease in ATP levels compared to the vehicle controls, $p<0.05$. Polyphenol treatments were: 0, 0.4, 2, 10, 50, 250, 500 µM for 24 h. The polyphenols were ranked in order of activity with respect to significant reduction of cellular proliferation in lymphoid cells (emodin = quercetin > *cis*-stilbene > apigenin > rhein = *trans*-stilbene = aloe-emodin = chrysin); and in myeloid cells (emodin = *cis*-stilbene ≥ quercetin > apigenin > rhein > aloe-emodin = *trans*-stilbene > chrysin). Note that the treatment doses that caused significant inhibition of cellular proliferation in all leukaemia cell lines were much lower than in the non-tumour cells ($CD34^+$ HSC and $CCD133^+$ HSC) at 24 h. Due to the wide range of concentrations used and the number of cell lines investigated, it was not possible to indicate significance levels on Figure 2.4 and thus, Table 2.1 indicates the lowest dose of polyphenol at which significance was obtained for each cell line, providing the statistical analysis for Figure 2.4.

Cell Types			Polyphenols IC_{50} in μM at 24 h							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell Lines	Lymphoid leukaemia	JURKAT	10	143	180	277	9	185	38	163
		CCRF-CEM	10	195	128	140	22	211	53	109
		MOLT-3	20	140	217	158	8	220	25	180
	Myeloid leukaemia	HL60	8	100	328	150	5	225	32	135
		THP-1	37	180	500	158	10	450	45	380
		K562	33	350	500	380	13	309	53	500
		KG1a	155	500	335	169	15	310	85	250
	Histocytic Lymphoma	U937	8	160	217	135	7	250	30	340
Cord blood cells	Non-Tumour Control Cells	$CD34^+$ HSC	≥ 500	> 500	> 500	380	150	> 500	390	500
		$CD133^+$ HSC	500	> 500	> 500	350	250	> 500	350	500

Table 2.2: The IC_{50} values responsible for 50% inhibition of ATP levels in each leukaemia and non-tumour control cell line following 24 h treatment with each polyphenols. This was determined by CellTiter-Glo® Luminescent assay. The polyphenols were ranked in order of activity with respect to inhibition of 50% proliferation in lymphoid cells (emodin = quercetin > *cis*-stilbene > apigenin > *trans*-stilbene ≥ chrysin = rhein > aloe-emodin); and in myeloid cells (emodin = *cis*-stilbene ≥ quercetin > apigenin = rhein > aloe-emodin = *trans*-stilbene = chrysin). Non-tumour cells ($CD34^+$ HSC and $CD133^+$ HSC) did not reach 50% inhibition until the polyphenol treatments excessed 500 μM; the only exceptions were emodin, rhein and *cis*-stilbene. Note that the highest doses of aloe-emodin, chrysin, rhein and *trans*-stilbene would be clinically impractical, while IC_{50} for quercetin emodin, *cis*-stilbene had much lower doses and thus are potentially more clinically useful.

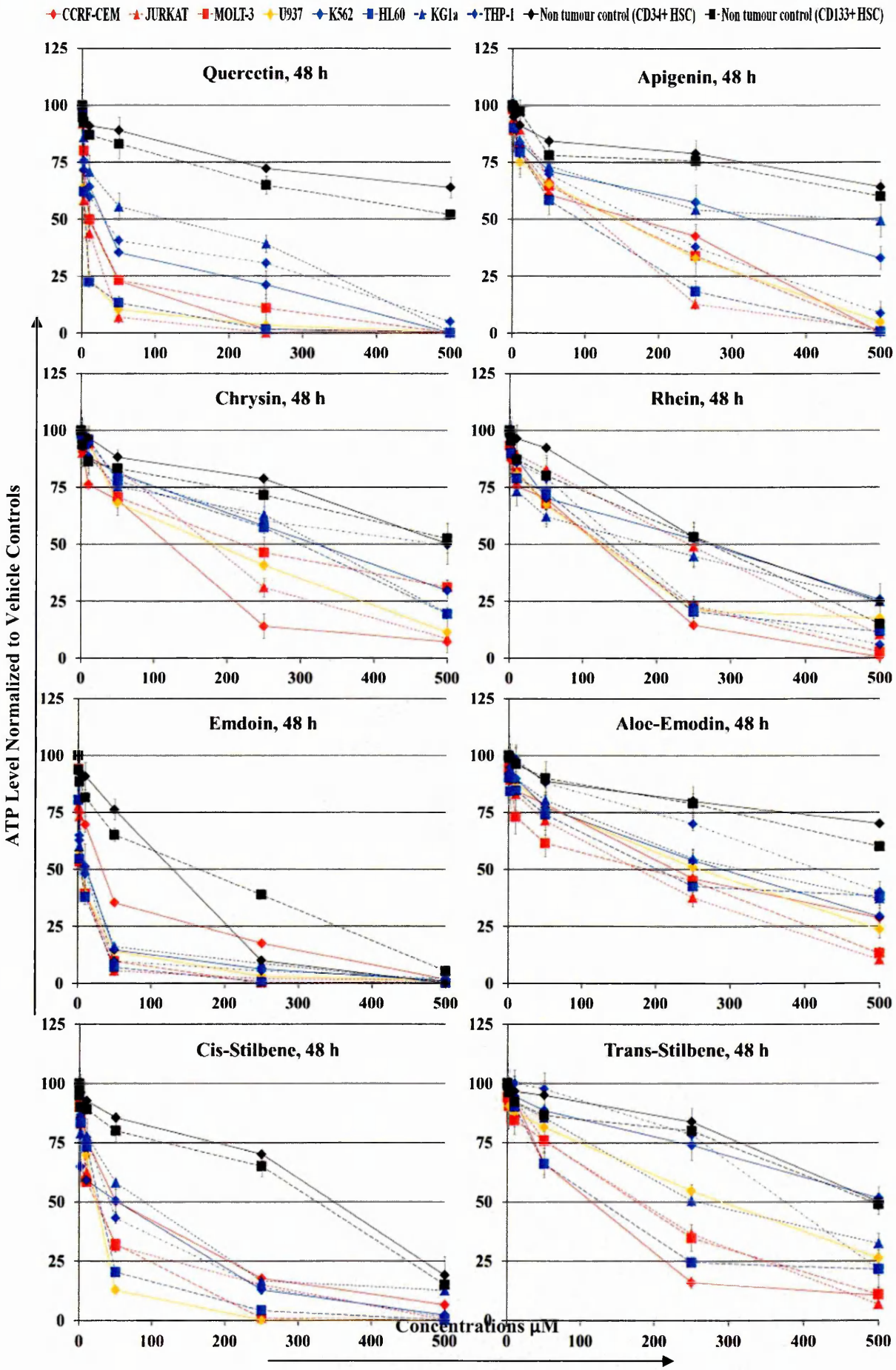

CCRF-CEM
JURKAT
MOLT-3
U937
K562
HL60
KG1a
THP-1
Non tumour control (CD34+ HSC)
Non tumour control (CD133+ HSC)
Quercetin, 48 h
Apigenin, 48 h
Chrysin, 48 h
Rhein, 48 h
Emdoin, 48 h
Aloc-Emodin, 48 h
Cis-Stilbene, 48 h
Trans-Stilbene, 48 h
ATP Level Normalized to Vehicle Controls
Concentrations µM

Figure 2.5: Effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*-stilbene and *trans*-stilbene) on ATP levels of three lymphoid leukaemia (CCRF-CEM, MOLT-3, JURKAT; red lines), one histocytic lymphoma (U937; orange lines), four human myeloid leukaemia cell lines (K562, HL-60, KG-1a , THP-1; blue lines), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC; black lines) at 0, 0.4, 2, 10, 50, 250, 500 μM for 48 h. This was evaluated by CellTiter-Glo® assay. Data was normalised to the vehicle control which was assigned 100% cell viability. The data is expressed as mean ± STD (three independent experiments, each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. Statistical results are summarised in Table 2.3 which shows the lowest dose that induced significant inhibition compared to vehicle control at 48 h. All concentrations above these points were also statistically significant. The IC_{50} for each polyphenol in each cell line at 48 h were determined and shown in Table 2.4.

Cell Types			The Lowest Dose of Polyphenols (μM) At Which There Was a Significant Inhibition of ATP Levels Compared to The Vehicle Control At 48 h.							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	2	10	10	10	0.4	10	2	50
		CCRF-CEM	2	10	10	2	2	10	10	50
		MOLT-3	2	2	50	10	2	10	2	50
	Myeloid leukaemia	HL60	2	10	50	10	0.4	10	2	50
		THP-1	2	10	50	10	2	50	2	250
		K562	2	10	10	10	2	50	2	50
		KG1a	2	10	10	10	2	10	2	50
	Histocytic Lymphoma	U937	2	2	50	10	2	10	10	50
Cord blood cells	Non-tumour control cells	$CD34^+$ HSC	50	50	50	250	10	50	10	250
		$CD133^+$ HSC	50	50	50	10	10	50	10	250

Table 2.3: The lowest dose of polyphenols that induced a significant decrease in ATP levels compared to the vehicle controls, $p<0.05$. Polyphenol treatments were: 0, 0.4, 2, 10, 50, 250, 500 μM for 48 h. The polyphenols were ranked in order of activity with respect to significant reduction of cellular proliferation in lymphoid cells (emodin = quercetin > *cis*-stilbene > apigenin > rhein = *trans*-stilbene = aloe-emodin = chrysin); and in myeloid cells (emodin = *cis*-stilbene ≥ quercetin > apigenin > rhein > aloe-emodin = *trans*-stilbene > chrysin). Note that the treatment doses that caused significant inhibition of cellular proliferation in all leukaemia cell lines were much lower than in the non-tumour cells ($CD34^+$ HSC and $CCD133^+$ HSC) at 48 h. Due to the wide range of concentrations used and the number of cell lines investigated, it was not possible to indicate significance levels on Figure 2.5 and thus, Table 2.3 indicates the lowest dose of polyphenol at which significance was obtained for each cell line, providing the statistical analysis for Figure 2.5.

Cell Types			Polyphenols IC_{50} in μM at 48 h							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	7.5	120	160	250	8	180	30	160
		CCRF-CEM	10	160	100	120	20	210	50	100
		MOLT-3	10	140	200	120	3	190	25	175
	Myeloid leukaemia	HL60	4	100	300	135	4	200	31	160
		THP-1	25	170	500	150	9	450	40	400
		K562	25	300	320	260	11	300	50	500
		KG1a	100	480	320	200	9	310	80	250
	Histocytic Lymphoma	U937	4.5	150	160	120	4.5	250	28	280
Cord blood cells	Non-Tumour Control Cells	$CD34^+$ HSC	≥ 500	≥ 500	500	260	125	≥ 500	400	500
		$CD133^+$ HSC	500	≥ 500	500	260	160	≥ 500	350	500

Table 2.4: The IC_{50} values responsible for 50% inhibition of ATP levels in each leukaemia and non-tumour control cell line following 48 h treatment with each polyphenols. This was determined by CellTiter-Glo® Luminescent assay. The polyphenols were ranked in order of activity with respect to inhibition of 50% proliferation in lymphoid cells (emodin = quercetin > *cis*-stilbene > apigenin > *trans*-stilbene ≥ chrysin = rhein > aloe-emodin); and in myeloid cells (emodin = *cis*-stilbene ≥ quercetin > apigenin = rhein > aloe-emodin = *trans*-stilbene = chrysin). Non-tumour cells ($CD34^+$ HSC and $CD133^+$ HSC) did not reach 50% inhibition until the polyphenol treatments excessed 500 μM; the only exceptions were emodin, rhein and *cis*-stilbene. Note that the highest doses of aloe-emodin, chrysin, rhein and *trans*-stilbene would be clinically impractical, while IC_{50} for quercetin emodin, *cis*-stilbene had much lower doses and thus are potentially more clinically useful.

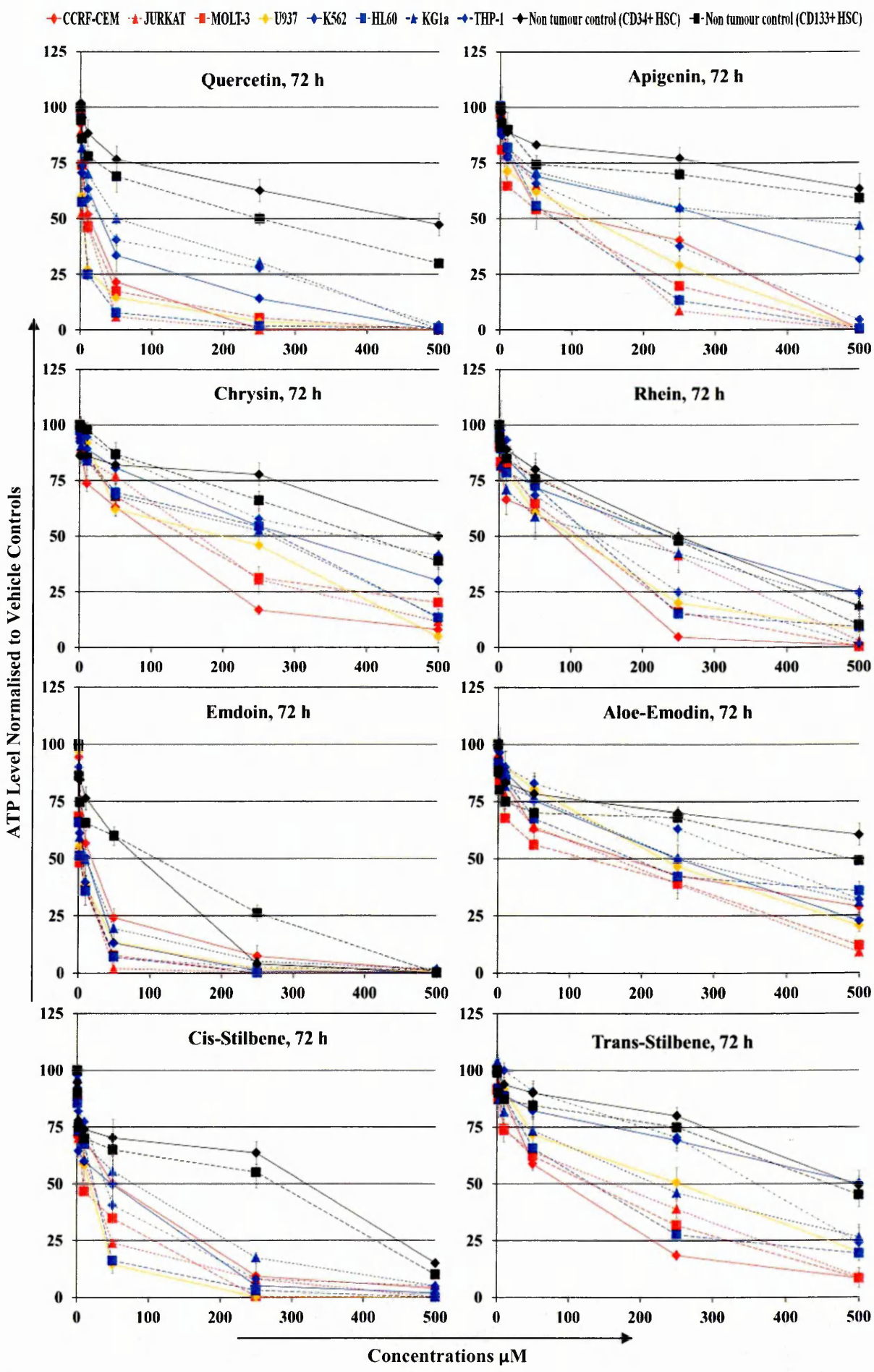

CCRF-CEM
JURKAT
MOLT-3
U937
K562
HL60
KG1a
THP-1
Non tumour control (CD34+ HSC)
Non tumour control (CD133+ HSC)
Quercetin, 72 h
Apigenin, 72 h
Chrysin, 72 h
Rhein, 72 h
Emdoin, 72 h
Aloe-Emodin, 72 h
Cis-Stilbene, 72 h
Trans-Stilbene, 72 h
ATP Level Normalised to Vehicle Controls
Concentrations µM

Figure 2.6: Effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*-stilbene and *trans*-stilbene) on ATP levels of three lymphoid leukaemia (CCRF-CEM, MOLT-3, JURKAT; red lines), one histocytic lymphoma (U937; orange lines), four human myeloid leukaemia cell lines (K562, HL-60, KG-1a , THP-1; blue lines), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC; black lines) at 0, 0.4, 2, 10, 50, 250, 500 µM for 72 h. This was evaluated by CellTiter-Glo® assay. Data was normalised to the vehicle control which was assigned 100% cell viability. The data is expressed as mean ± STD (three independent experiments, each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. Statistical results are summarised in Table 2.5 which shows the lowest dose that induced significant inhibition compared to vehicle control at 72 h. All concentrations above these points were also statistically significant. The IC_{50} for each polyphenol in each cell line at 72 h were determined and shown in Table 2.6.

Cell Types			The Lowest Dose of Polyphenols (µM) At Which There Was a Significant Inhibition of ATP Levels Compared to The Vehicle Control At 72 h.							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	0.4	10	10	10	0.4	10	0.4	10
		CCRF-CEM	2	10	10	2	2	10	2	10
		MOLT-3	2	2	10	10	0.4	10	2	10
	Myeloid leukaemia	HL60	2	10	10	10	0.4	10	2	50
		THP-1	2	10	50	50	2	50	2	250
		K562	2	10	10	10	2	50	2	50
		KG1a	2	10	10	10	2	10	2	50
	Histocytic Lymphoma	U937	2	2	50	10	2	10	10	50
Cord blood cells	Non-Tumour Control Cells	$CD34^+$ HSC	50	50	10	50	10	50	10	250
		$CD133^+$ HSC	50	50	50	10	10	10	10	50

Table 2.5: The lowest dose of polyphenols that induced a significant decrease in ATP levels compared to the vehicle controls, $p<0.05$. Polyphenol treatments were: 0, 0.4, 2, 10, 50, 250, 500 µM for 72 h. The polyphenols were ranked in order of activity with respect to significant reduction of cellular proliferation in lymphoid cells (emodin = quercetin > *cis*-stilbene > apigenin > rhein = *trans*-stilbene = aloe-emodin = chrysin); and in myeloid cells (emodin = *cis*-stilbene ≥ quercetin > apigenin > rhein > aloe-emodin = *trans*-stilbene > chrysin). Note that the treatment doses that caused significant inhibition of cellular proliferation in all leukaemia cell lines were much lower than in the non-tumour cells ($CD34^+$ HSC and $CCD133^+$ HSC) at 72 h. Due to the wide range of concentrations used and the number of cell lines investigated, it was not possible to indicate significance levels on Figure 2.6 and thus, Table 2.5 indicates the lowest dose of polyphenol at which significance was obtained for each cell line, providing the statistical analysis for Figure 2.6.

Cell Types			Polyphenols IC_{50} in μM at 72 h							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	4.9	100	150	200	4.5	170	19	160
		CCRF-CEM	10	145	75	90	18	195	50	95
		MOLT-3	9	60	150	100	2	100	15	100
	Myeloid leukaemia	HL60	3	60	270	125	3	200	20	100
		THP-1	20	160	400	130	7	380	40	390
		K562	20	280	280	250	10	250	50	500
		KG1a	50	400	260	160	9	250	75	200
	Histocytic Lymphoma	U937	4	145	95	100	3.5	235	15	200
Cord blood cells	Non-Tumour Control Cells	$CD34^+$ HSC	450	≥500	500	250	85	≥500	355	500
		$CD133^+$ HSC	250	≥500	450	250	95	500	300	490

Table 2.6: The IC_{50} values responsible for 50% inhibition of ATP levels in each leukaemia and non-tumour control cell line following 72 h treatment with each polyphenols. This was determined by CellTiter-Glo® Luminescent assay. The polyphenols were ranked in order of activity with respect to inhibition of 50% proliferation in lymphoid cells (emodin = quercetin > *cis*-stilbene > apigenin > *trans*-stilbene ≥ chrysin = rhein > aloe-emodin); and in myeloid cells (emodin = *cis*-stilbene ≥ quercetin > apigenin = rhein > aloe-emodin = *trans*-stilbene = chrysin). Non-tumour cells ($CD34^+$ HSC and $CD133^+$ HSC) did not reach 50% inhibition until the polyphenol treatments excessed 500 μM; the only exceptions were emodin, rhein and *cis*-stilbene. Note that the highest doses of aloe-emodin, chrysin, rhein and *trans*-stilbene would be clinically impractical, while IC_{50} for quercetin emodin, *cis*-stilbene had much lower doses and thus are potentially more clinically useful.

2.3.2 Induction of Apoptosis Following Polyphenol Treatments in Leukaemia Cells at 24 h

The effectiveness of polyphenols in inducing apoptosis was assessed by caspase 3 activity assay using flow cytometry and Hoechst 33342 nuclear staining using a fluorescence microscope; these two methods were indicative of early and late phases of apoptosis. For the measurement of caspase 3 activity, the leukaemia cell lines were treated with polyphenols at the IC_{25} and IC_{50} treatment ranges (0.4, 2, 10, 50 μM of quercetin, emodin and *cis*-stilbene, 10, 50, 250 μM of apigenin, 50, 250 μM of chrysin and aloe-emodin, 50, 250, 500 μM of *trans*-stilbene) as determined by CellTiter-Glo® assay for 24 h. For Hoechst 33342 nuclear staining, the leukaemic cell lines were treated with all polyphenols at a wide range of concentrations (0, 0.4, 2, 10, 50, 250, 500 μM) as previously used in CellTiter-Glo® assay for 24 h; This enabled an examination of the differential effects of each polyphenols on the morphological changes of apoptosis plus help to confirm the CellTiter-Glo® results. Using the results from both assays, it was possible to determine the lowest dose of polyphenol (μM) which significant induction of apoptosis (Table 2.7, 2.8); plus the AP_{50} values (Table 2.9) the dose at which there was 50% of apoptosis. By using the AP_{50} values of caspase 3 activity and Hoechst 33342 assays, it was possible to rank the polyphenols in order of effectiveness for apoptosis induction (Table 2.9). The morphological assessment of apoptosis by Hoechst 33342 staining confirmed the induction of apoptosis shown by the caspase 3 activity assays. However it is important to note that the AP_{50} values determined using the caspase 3 activity were lower than those determined by the Hoechst staining, as the later is indiciative of late stages of apoptosis (Figure 2.7, 2.8 and Table 2.9).

All eight polyphenols induced significantly higher levels of apoptosis determined by caspase 3 activity (Figure 2.7) and Hoechst 33342 staining (Figure 2.9) in all leukaemia cell lines compared to the non-tumour cells ($p<0.05$) (Table 2.9). The leukaemia cell lines demonstrated differing sensitivity to the polyphenols. In particular, quercetin (Figure 2.10), chrysin (Figure 2.11), apigenin (Figure 2.12), emodin (Figure 2.13), aloe-emodin (Figure 2.14), *cis*-stilbene (Figure 2.15) had a greater toxicity towards lymphoid leukaemia cell lines than myeloid leukaemia cell lines with AP_{50} values of caspase 3 activity and Hoechst 33342 assays ranging between 19-50 μM for quercetin, 35-130 μM for chrysin, 30-250 μM for apigenin, 2-27 μM for emodin, 50-195 μM for aloe-emodin, 8-50μM for *cis*-stillbene (Figure 2.7, 2.8 and Table 2.9). Emodin, quercetin, *cis*-stilbene and apigenin were the most effective polyphenols at inducing apoptosis according to their AP_{50} values in lymphoid cell lines (Figure 2.7, 2.8 and Table 2.9). The HL-60 cells were the only myeloid cells which were sensitive responded to quercetin, apigenin, chrysin, emodin, aloe-emodin, *cis*-stilbene treatments with AP_{50} values of caspase 3 activity and Hoechst 33342 assays 50 μM, 75-175 μM, 84-129 μM, 6-8.5 μM, 185-190 μM, 31-49 μM respectively (Figure 2.7, 2.8 and Table 2.9). In addition, the THP-1 myeloid cell line

was only sensitive to apigenin and emodin, with AP_{50} values of 110-225 μM (Figure 2.7, 2.8 and Table 2.9). K562 and KG1a were resistant to the most of polyphenol treatments (quercetin, chrysin, emodin, aloe-emodin, *cis*-stilbene treatments), while they were sensitive to apigenin treatment with AP_{50} values between 150-190 μM in K562 cells and 89-235 μM in KG1a cells (Figure 2.7, 2.8 and Table 2.9). Consequently, apigenin was the only effective polyphenol at inducing apoptosis in all myeloid cell lines with AP_{50} values ranging between 84-235 μM. Rhein (Figure 2.7, 2.8, 2.12) and *trans*-stilbene (Figure 2.7, 2.8, 2.16), demonstrated similar sensitivity to both myeloid and lymphoid cell lines with AP_{50} values of caspase 3 activity and Hoechst 33342 assays ranging between 60-265 μM and 40-400 μM respectively (Table 2.9).

In accordance with their AP_{50} values emodin, quercetin, *cis*-stilbene and apigenin were the most effective polyphenols at inducing apoptosis in most of leukaemic cell lines (Figure 2.7, 2.8 and Table 2.9). Emodin induced 50% apoptosis in 5 of the 8 leukaemia cell lines (JURKAT, MOLT-3, HL-60, THP-1 and U937) (Figure 2.7, 2.8, 2.13 and Table 2.9). Quercetin was also a potent polyphenol inducing apoptosis with AP_{50} value ranging between 19-50 μM in lymphoid cell lines and 50-205 μM in myeloid cell lines. Quercetin differential induction of apoptosis in each leukaemia cell line although the AP_{50} values were consistently low (Figure 2.7, 2.8, 2.9 and Table 2.9).

C*is*-stilbene induced caspase 3 activity of early apoptosis and morphological changes characteristic of late apoptosis in the majority of leukaemia cell lines. In particular *Cis*-stilbene showed AP_{50} values ranging between 8-50 μM in three lymphoid cell lines (JURKAT, MOLT-3, and U937) and between 31-49 μM in one myeloid cell line (HL-60); the remaining cell lines were more resistant, and did not reach 50% apoptosis even when treated with a maximal treatment dose (500 μM) (Figure 2.7, 2.8, 2.15 and Table 2.9).

Apigenin was shown to induce apoptosis in all leukaemia cell lines, with AP_{50} values ranging between 35-130 μM in lymphoid cell lines and 84-235 μM in the myeloid cell lines. In contrast to quercetin, apigenin was capable of inducing both an increase in caspase 3 indicating early apoptosis, plus morphological evidence of late apoptosis, in all leukaemia cells lines; including the KG-1a and K562 cells which were resistant to emodin, quercetin and *cis*-stilbene treatment. The other polyphenols investigated; chrysin, rhein, aloe-emodin, *trans*-stilbene induced apoptosis in some leukaemia cell lines, however the AP_{50} values were much higher compared to other polyphenols (Figure 2.7, 2.8 and Table 2.9).

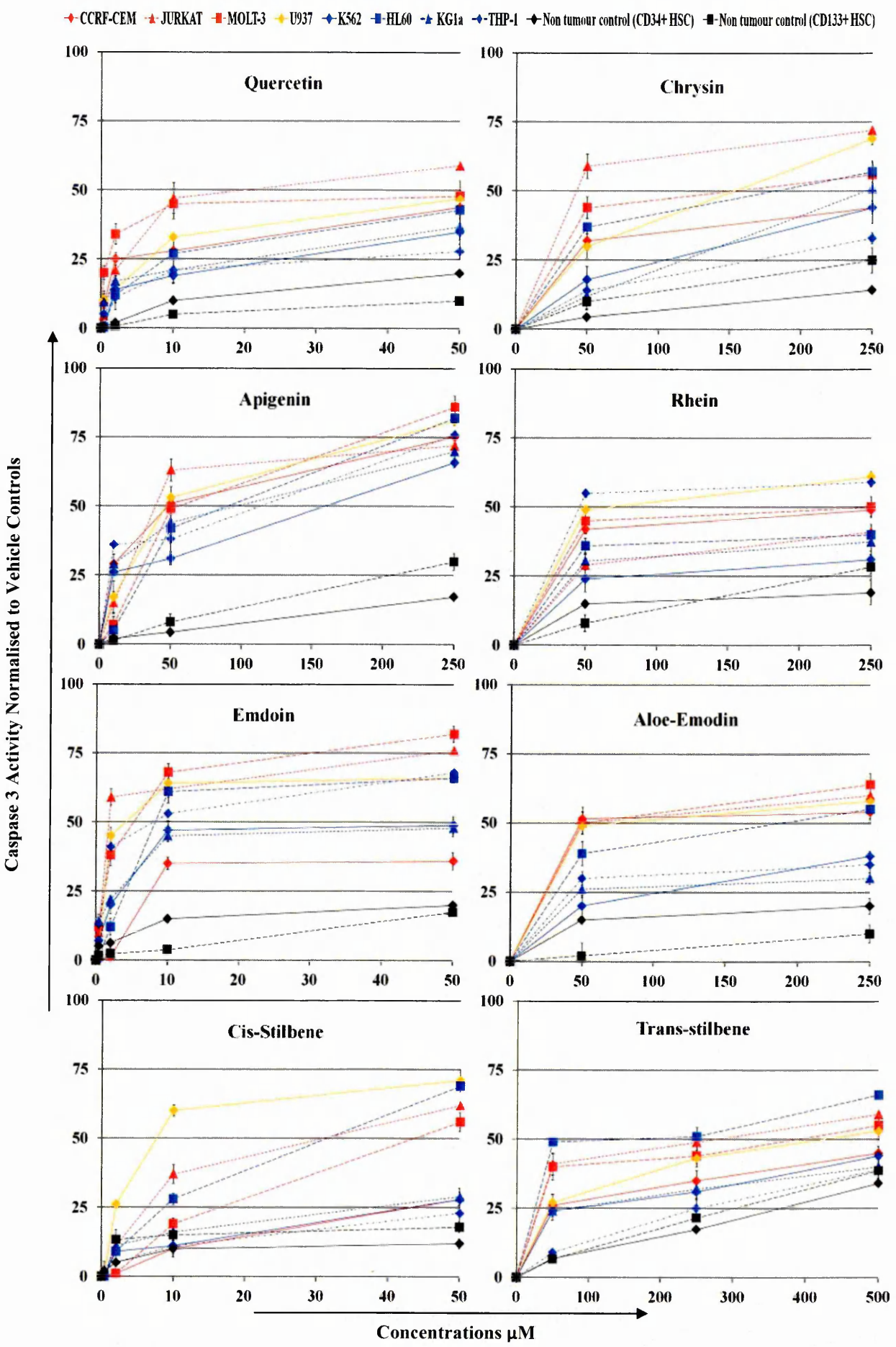

CCRF-CEM
JURKAT
MOLT-3
U937
K562
HL60
KG1a
THP-1
Non tumour control (CD34+ HSC)
Non tumour control (CD133+ HSC)
Quercetin
Chrysin
Apigenin
Rhein
Emdoin
Aloe-Emodin
Cis-Stilbene
Trans-stilbene
Caspase 3 Activity Normalised to Vehicle Controls
Concentrations µM

Figure 2.7: Effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe emodin, *cis*-stilbene and *trans*-stilbene) on apoptosis of three lymphoid leukaemia (CCRF-CEM, MOLT-3, JURKAT; red lines), one histocytic lymphoma (U937; orange lines), four human myeloid leukaemia cell lines (K562, HL-60, KG-1a , THP-1; blue lines), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC; black lines). Apoptosis was assessed using a caspase 3 activity assay and analysed by flow cytometry. Cells were treated with each polyphenol at ranges of IC_{25} and IC_{50} doses as determined by CellTiter-Glo® assay for 24 h. The treatment concentrations for emodin, quercetin and *cis*-stilbene were 0, 0.4, 2, 10, 50 μM; and for apigenin, chrysin, aloe-emodin, rhein and *trans*-stilbene were 0, 10, 50, 250 μM. All data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data is expressed as mean ± STD (three independent experiments, each in triplicate). The statistical significance was determined by comparison with the vehicle control, statistical significant was set at $p \leq 0.05$. Statistical results are summarised in Table 2.7 which shows the lowest dose that induced a significant increase in caspase 3 activity compared to vehicle control. All concentrations above these points were also significant. The AP_{50} for each polyphenol in each cell line were determined and shown in Table 2.9.

Cell Types			The lowest dose of polyphenols (μM) at which there was a significant induction of apoptosis compared to the vehicle control by caspase 3 activity assay at 24 h.							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	2	50	50	50	2	50	10	50
		CCRF-CEM	2	10	50	50	10	50	50	50
		MOLT-3	10	50	50	50	2	50	10	50
	Myeloid leukaemia	HL60	10	50	50	50	10	50	10	50
		THP-1	10	10	250	50	2	50	50	250
		K562	10	10	250	50	2	50	50	50
		KG1a	10	10	250	50	2	50	10	50
	Histocytic Lymphoma	U937	2	50	50	50	2	50	2	50
Cord blood cells	Non-tumour control cells	$CD34^+$ HSC	50	250	250	50	10	250	250	250
		$CD133^+$ HSC	50	250	50	250	50	50	10	250

Table 2.7: The lowest dose of polyphenols which induced significant induction of caspase 3 activity, compared to the control *($p \leq 0.05$).* Apoptosis was assessed by caspase 3 activity assay. The polyphenols were ranked in order of activity with respect to significant induction of apoptosis in lymphoid cells (emodin = quercetin ≥ *cis*-stilbene > apigenin > rhein = *trans*-stilbene = aloe-emodin = chrysin); and in myeloid cells (emodin > quercetin > *cis*-stilbene = apigenin > rhein = aloe-emodin ≥ *trans*-stilbene > chrysin). Note that the treatment doses which caused significant induction of apoptosis in all leukaemia cell lines were much lower than of the non-tumour cells (CD34+). Due to the wide range of concentrations used and the cell lines investigated, it was not possible to indicate significance levels on (Figure 2.7) and thus Table 2.7 provides the lowest doses of polyphenol at which significance was obtained.

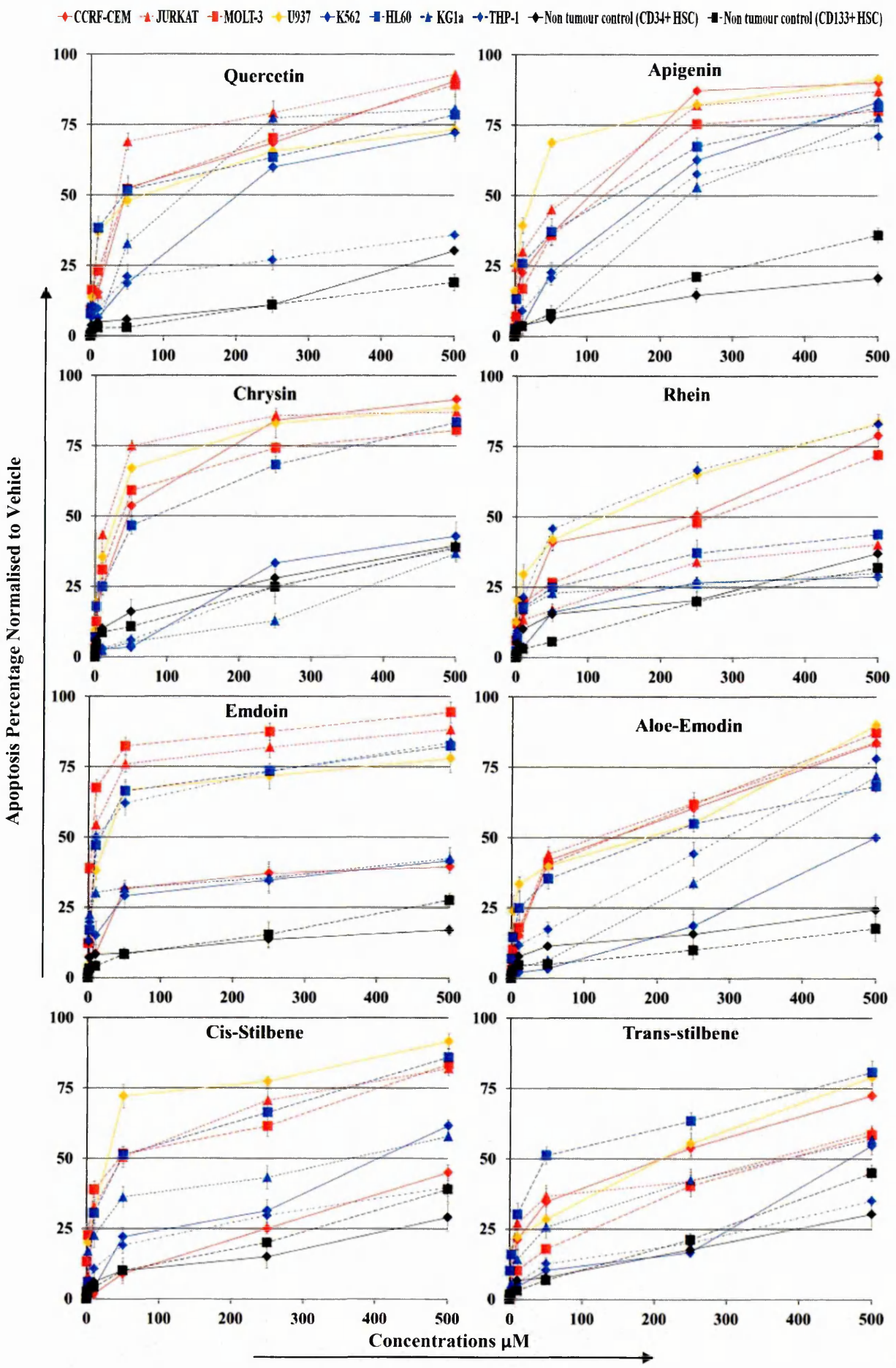

CCRF-CEM
JURKAT
MOLT-3
U937
K562
HL60
KG1a
THP-1
Non tumour control (CD34+ HSC)
Non tumour control (CD133+HSC)
Quercetin
Apigenin
Chrysin
Rhein
Emdoin
Aloe-Emodin
Cis-Stilbene
Trans-stilbene
Apoptosis Percentage Normalised to Vehicle
Concentrations µM
0
25
50
75
100
0
100
200
300
400
500

Figure 2.8: Effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*-stilbene and *trans*-stilbene) on apoptosis of three lymphoid leukaemia (CCRF-CEM, MOLT-3, JURKAT; red lines), one histocytic lymphoma (U937; orange lines), four human myeloid leukaemia cell lines (K562, HL-60, KG-1a , THP-1; blue lines), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC; black lines). Apoptosis was assessed using a Hoechst 33342 staining and examined by fluorescence microscopy. Cells were treated with each polyphenol at 0, 0.4, 2, 10, 50, 250, 500 μM for 24 h. All data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data is expressed as mean ± STD (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significant was set at $p \leq 0.05$. Statistical results are summarised in Table 2.8 which shows the lowest dose that caused significant induction of apoptosis compared to vehicle control. All concentrations above these points were also significant. The AP_{50} for each polyphenol in each cell line were determined and shown in Table 2.9. An example of the morphological assessment of apoptosis for polyphenols showed in following figures; quercetin (Figure 2.9), chrysin (Figure 2.10), apigenin (Figure 2.11), rhein (Figure 2.12), emodin (Figure 2.13), aloe-emodin (Figure 2.14), two *cis*-stilbene (Figure 2.15),and *trans*-stilbene (Figure 2.16).

Cell Types			The Lowest Dose of Polyphenols (μM) at Which There Was a Significant Induction of Apoptosis Compared to The Vehicle Control Using Hoechst 33342 Staining							
			Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Cell lines	Lymphoid leukaemia	JURKAT	2	2	10	10	2	10	10	10
		CCRF-CEM	2	2	2	10	10	10	50	2
		MOLT-3	10	10	2	10	2	2	2	10
	Myeloid leukaemia	HL60	10	2	2	10	2	2	10	2
		THP-1	10	50	250	10	2	10	10	50
		K562	50	50	250	50	10	250	50	50
		KG1a	50	250	250	10	2	250	10	10
	Histocytic Lymphoma	U937	2	2	2	2	2	2	2	2
Cord blood cells	Non-tumour Control cells	$CD34^+$ HSC	250	250	50	50	250	250	50	250
		$CD133^+$ HSC	250	250	50	250	250	250	50	250

Table 2.8: The lowest dose of polyphenols which caused significant induction of apoptosis morphological changes, compared to the control *($p \leq 0.05$).* Apoptosis was assessed by Hoechst 33342 staining and fluorescence microscopy. The polyphenols were ranked in order of activity with respect to significant induction of apoptosis in lymphoid cells (emodin = quercetin ≥ *cis*-stilbene > apigenin > rhein = *trans*-stilbene = aloe-emodin = chrysin); and in myeloid cells (emodin > quercetin > *cis*-stilbene = apigenin > rhein = aloe-emodin ≥ *trans*-stilbene > chrysin). Note that the treatment doses which caused significant induction of apoptosis in all leukaemia cell lines were much lower than of the non-tumour cells (CD34+ HSC). Due to the wide range of concentrations used and the cell lines investigated, it was not possible to indicate significance levels on (Figure 2.8), and thus Table 2.8 provides the lowest doses of polyphenol at which significance was obtained.

Cell Types			Polyphenols AP_{50} in µM															
			Quercetin		Apigenin		Chrysin		Rhein		Emodin		Aloe - Emodin		Cis-Stilbene		Trans-Stilbene	
			C3	Hoe	C3	Hoe	C3	Hoe	C3	Hoe	C3	Hoe	C3	Hoe	C3	Hoe	C3	Hoe
Cell lines	Lymphoid leukaemia	JURKAT	19	25	35	90	40	30	>250	>500	2	9	50	130	31	50	250	310
		CCRF-CEM	50	50	50	100	250	40	200	250	>10	>500	50	150	>50	>500	>500	200
		MOLT-3	50	50	50	125	140	38	200	265	5	9	50	150	44	50	350	400
	Myeloid Leukaemia	HL60	50	50	84	129	175	75	>250	>500	8.5	6	185	190	31	49	40	50
		THP-1	>50	>500	110	220	>250	>500	50	60	7.8	10	>250	283	>50	>500	>500	>500
		K562	>50	205	150	190	>250	>500	>250	>500	>10	>500	>250	500	>50	410	>500	460
		KG1a	>50	125	89	235	>250	>500	>250	>500	>10	>500	>250	360	>50	350	>500	360
	Histocytic lymhpoma	U937	50	50	45	130	150	32	60	140	4	27	50	195	8	20	200	225
Cord blood cells	Non-tumour control cells	$CD34^+$ HSC	>50	>500	>250	>500	>250	>500	>250	>500	>50	>500	>250	>500	>50	>500	>500	>500
		$CD133^+$ HSC	>50	>500	>250	>500	>250	>500	>250	>500	>50	>500	>250	>500	>50	>500	>500	≥500

Table 2.9: The AP_{50} values responsible for 50% induction of apoptosis, determined by: Caspase 3 activity assay (C3) (Figure 2.7) and Hoechst 33342 staining (Hoe) (Figure 2.8). The polyphenol were ranked in order of induction of 50% apoptosis in lymphoid cells (quercetin ≥ emodin = *cis*-stilbene > apigenin > aloe-emodin > chrysin ≥ rhein > *trans*-stilbene). The HL-60 human promyelocytic leukaemia cell line was the only myeloid cell to reach an AP_{50}. The non-tumour cells ($CD34^+$ HSC and $CD133^+$ HSC) did not reach 50% apoptosis with any of the treatment doses investigated. THP-1, K562 and KG-1a myeloid cell lines were the most resistant cell lines, although did they reached an AP_{50} with apigenin treatment.

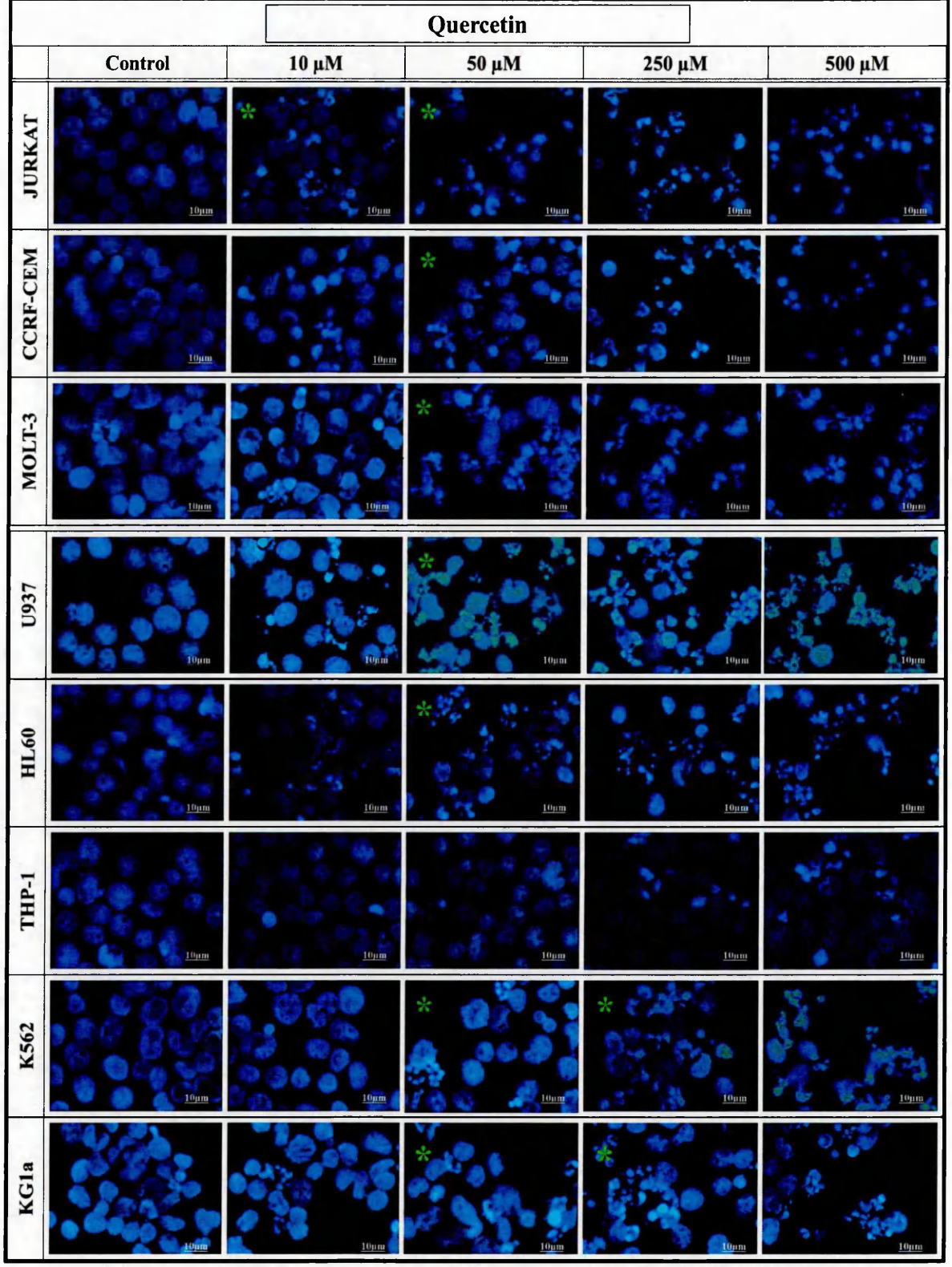


Figure 2.9: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with quercetin at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

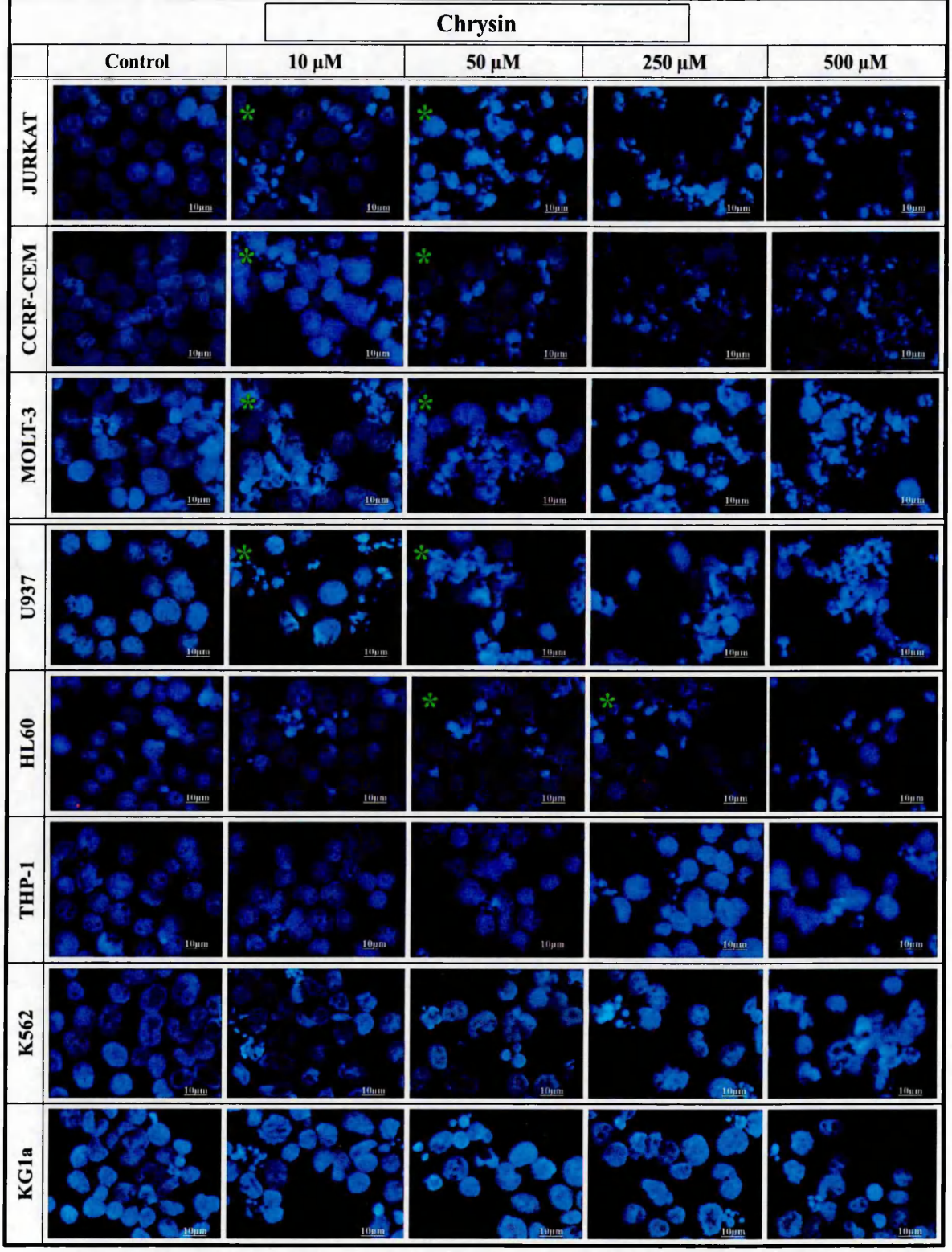


Figure 2.10: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with chrysin at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

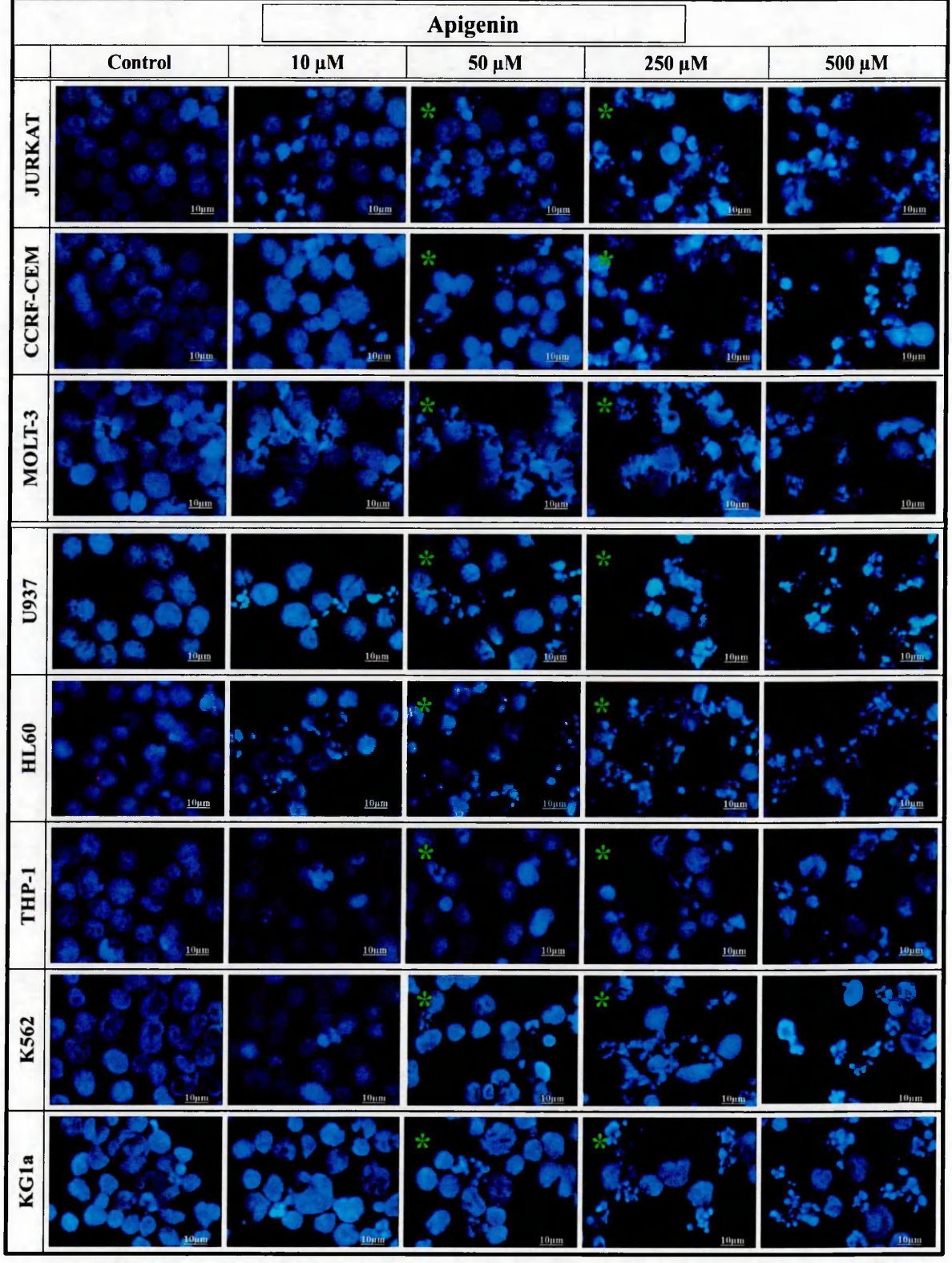


Figure 2.11: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with apigenin at 10, 50, 250, 500 µM for 24 h **on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a).** Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

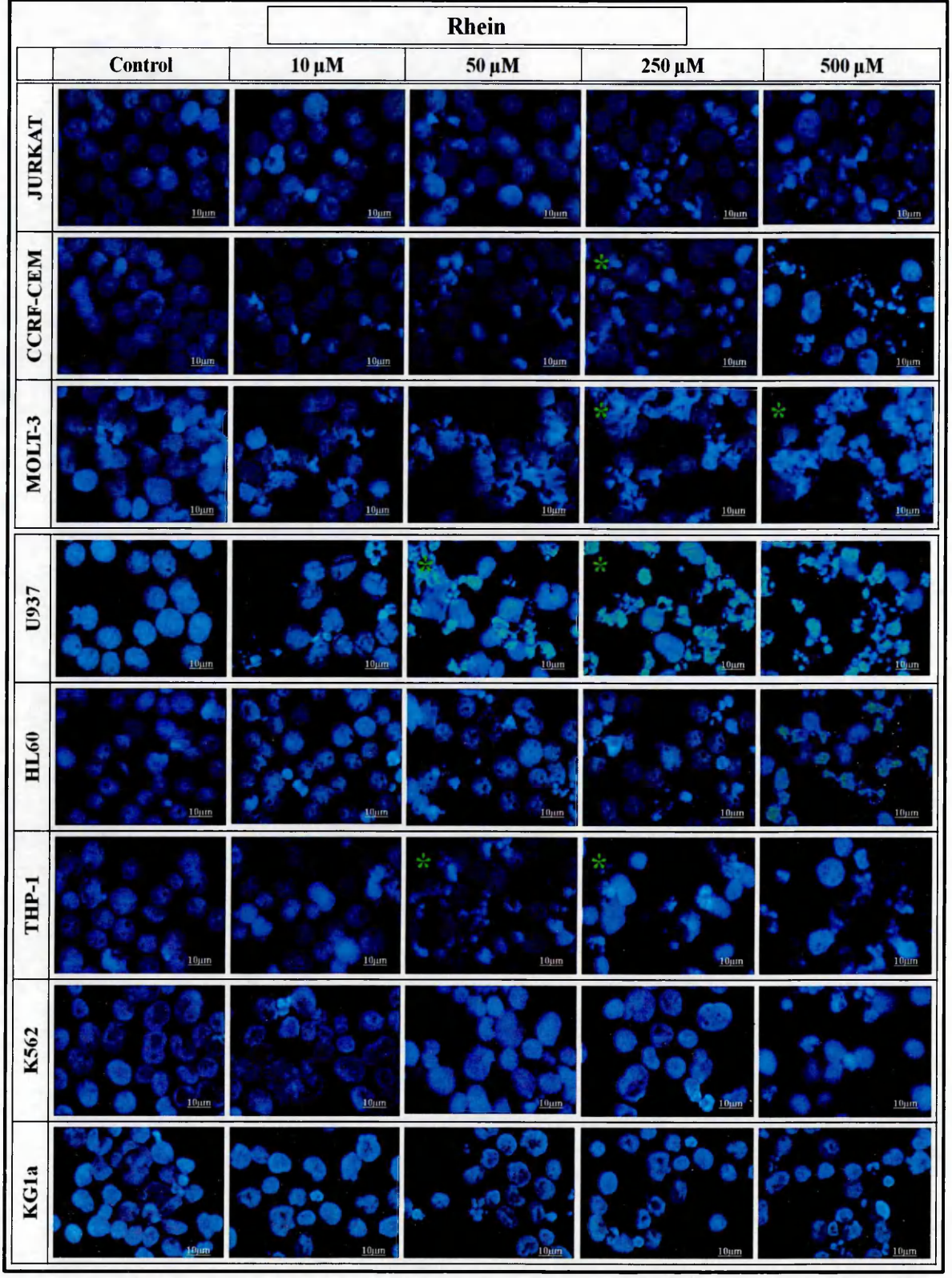


Figure 2.12: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with rhein at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

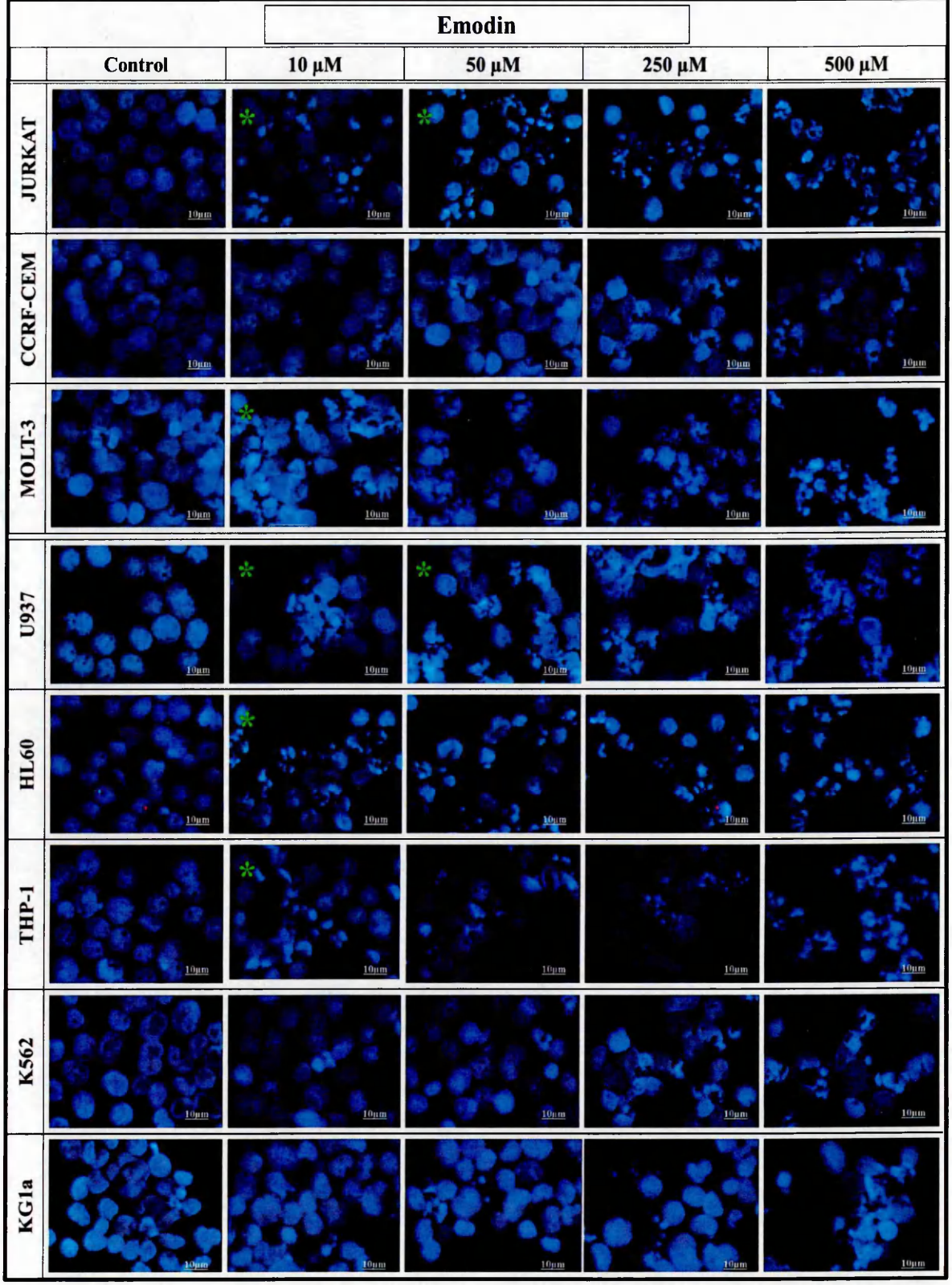


Figure 2.13: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with emodin at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

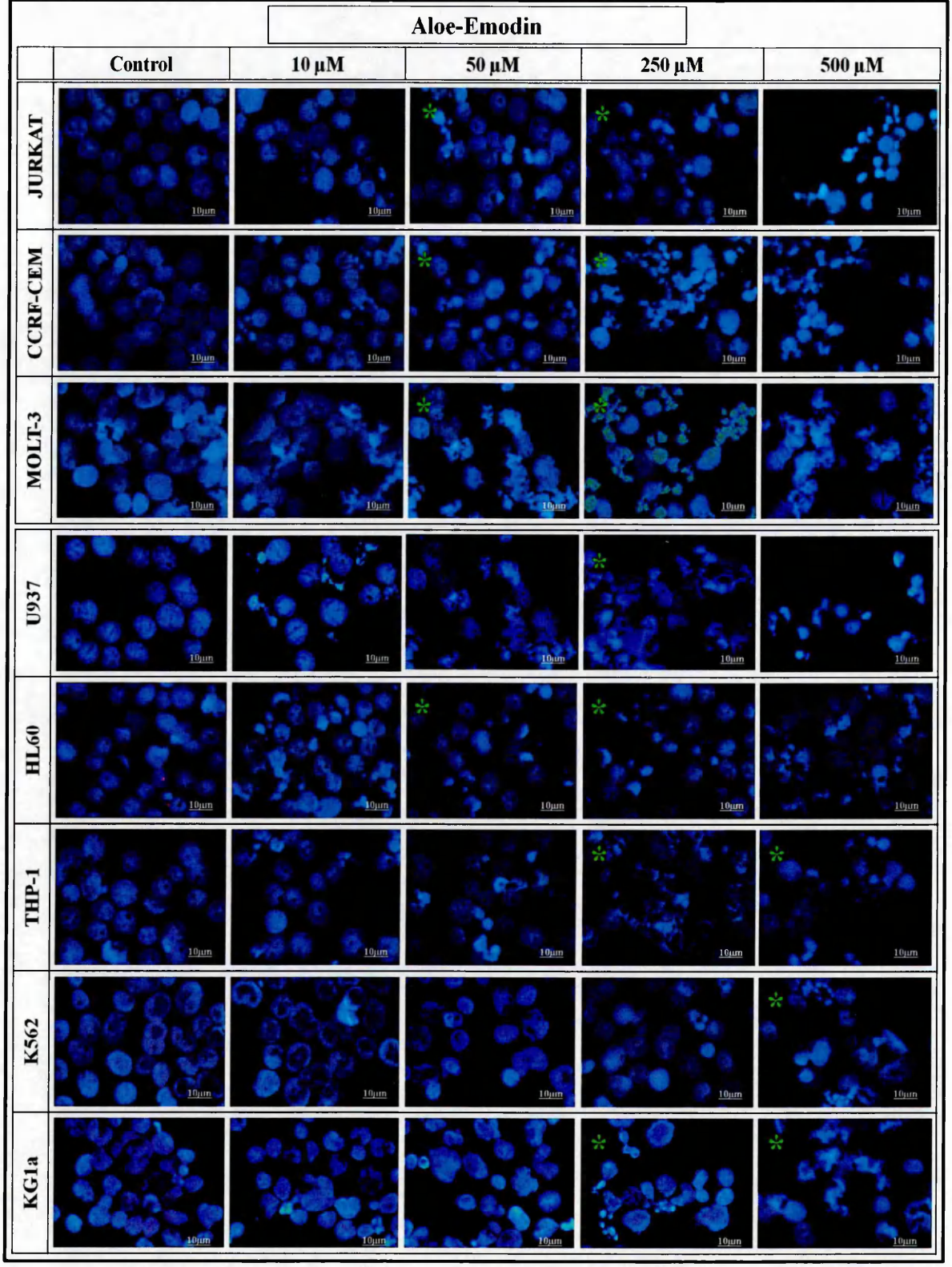


Figure 2.14: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with aloe-emodin at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

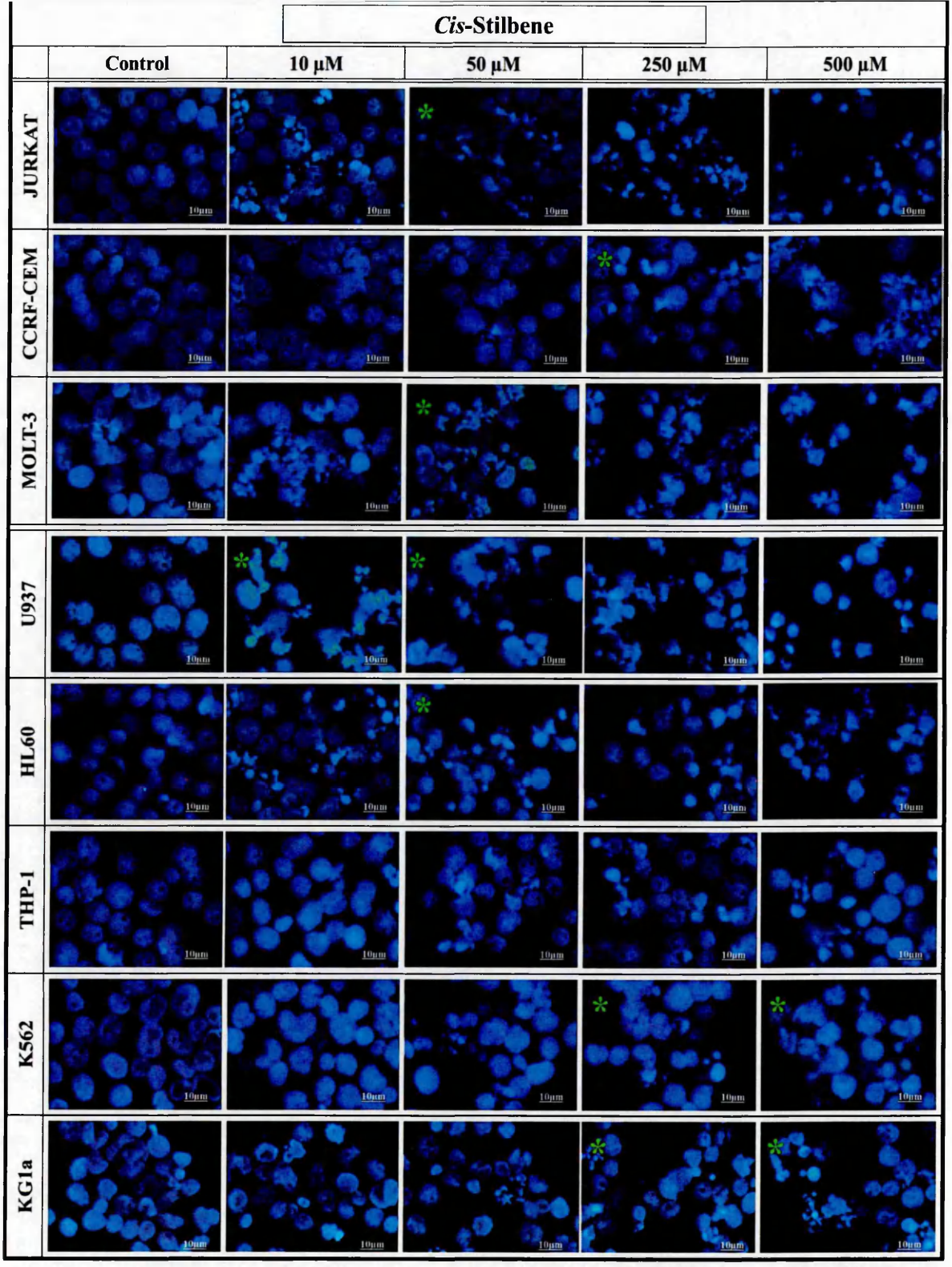


Figure 2.15: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with *cis*-stilbene at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

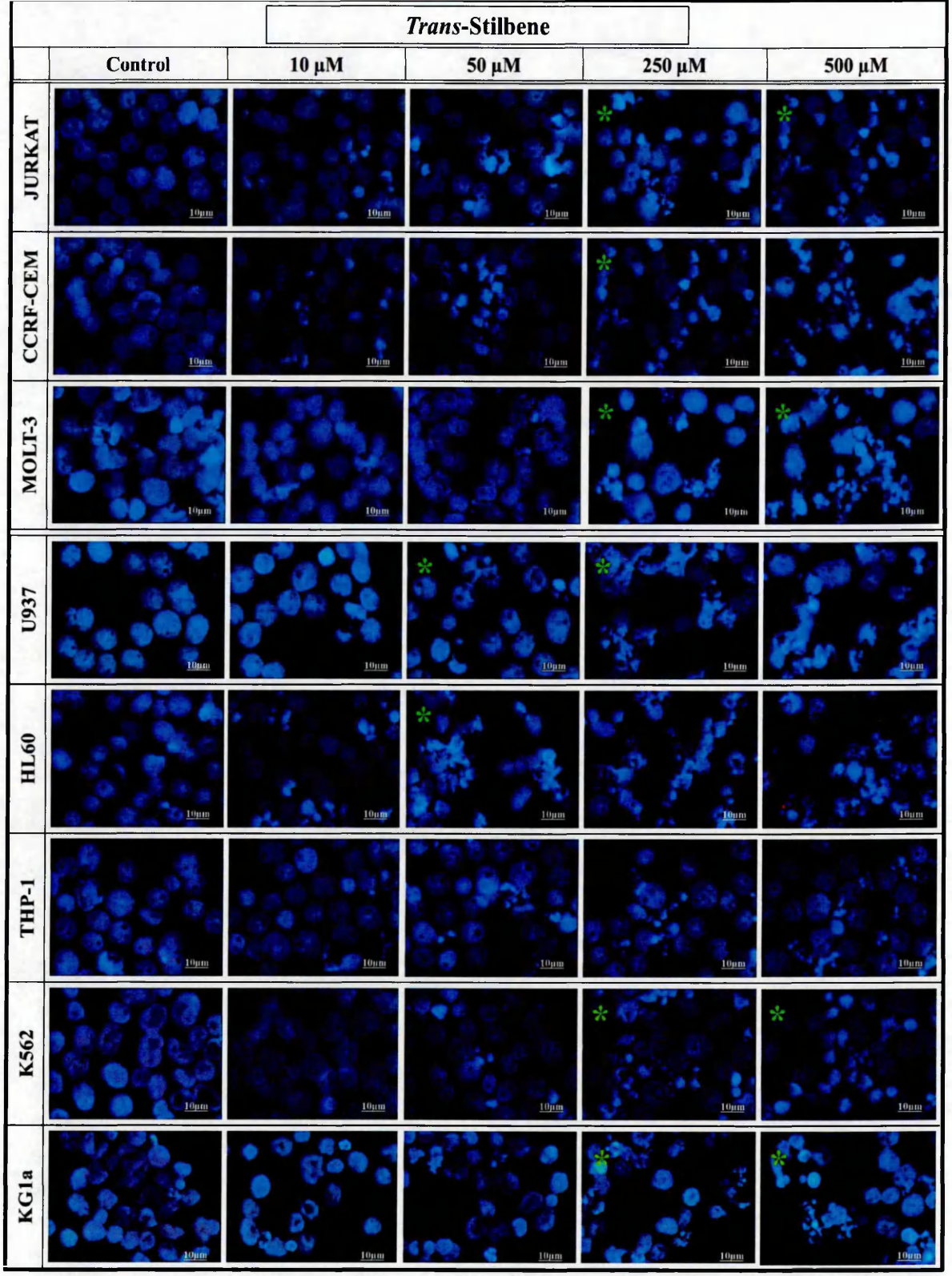


Figure 2.16: An example of the morphological assessment of apoptosis using Hoechst 33342 nuclear staining using a fluorescence microscope, after treatment with trans-stilbene at 10, 50, 250, 500 µM for 24 h on three lymphoid leukaemia cell lines (JURKAT, CCRF-CEM and MOLT-3), one histocytic lymphoma (U937) and four human myeloid leukaemia cell lines (HL-60, THP-1, K562 and KG1a). Scale bar = 10 µm. Apoptotic cells were identified by their irregular shape, intensely stained nuclei, chromatin condensation and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Green star (*) indicates to the treatment doses that induce 50% of apoptosis.

2.3.3 Cell Cycle Accumulation Following Polyphenol Treatments in Leukaemia Cells at 24 h

When assessing the effect of polyphenols of the progression of the cell cycle the IC_{25} and IC_{50} of polyphenols treatment doses as determined by CellTiter-Glo® assay for 24 h were used: 2, 10, 50 μM for quercetin, emodin and *cis*-stilbene, 10, 50, 250 μM for apigenin, and 50, 250, 500 for chrysin, aloe-emodin and *trans*-stilbene. These treatment doses significantly induced cell cycle arrest in all leukaemia cell lines ($p<0.05$) (Table 2.10). There was however no significant arrest in cell cycle with most polyphenols in the non-tumour progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC) (Table 2.10). The only exceptions was observed with rhein, which caused significant arrest at G_2/M phase in $CD34^+$ and $CD133^+$ HSC normal cells when used at the highest treatment dose of 500 μM ($p\leq0.05$) (Figure 2.23, 2.24 and Table 2.10), and with aloe-emodin and *trans*-stilbene, which caused significant arrest at S phase in $CD133^+$ HSC cells when treatment doses of 250 and 500 μM were used ($p\leq0.05$) (Figure 2.27, 2.28, 2.31, 2.32 and Table 2.10). It is important to note that these doses at which there was significant arrest for normal cells would be clinically impractical. The phase of cell cycle accumulation varied according to polyphenol treatment and cell line (Table 2.10). While in general, polyphenols appeared to cause G_0/G_1 phase accumulation in most of leukaemia cell lines (Table 2.10).

Emodin at 2, 10, 50 μM (Figure 2.29, 2.30) caused a more consistent effect significantly increasing the accumulation of cells in G_0/G_1 phase in all leukaemia cell lines ($p\leq0.05$) (Figure 2.25, 2.26 and Table 2.10). Similarly *cis*-stilbene at 2, 10, 50 μM (Figure 2.29, 2.30) and chrysin at 250 and 500 μM (Figure 2.19, 2.20) also significantly induced cells accumulation at G_0/G_1 phase in 7 out of the 8 leukaemia cell lines ($p\leq0.05$) (Table 2.10). Similarly, by rhein at 50, 250, 500 μM also significantly induced cells accumulation in G_0/G_1 phase of cell cycle in 6 out of the 8 leukaemia cell lines ($p\leq0.05$) (Figure 2.23, 2.24 and Table 2.10).

Quercetin, apigenin, aloe-emodin and *trans*-stilbene treatments caused differential effects on arresting the cell cycle phases within the IC_{25} and IC_{50} treatment ranges (Table 2.10). Quercetin (Figure 2.17, 2.18) produced a consistent effect on all myeloid cell lines significantly accumulating cells at G_0/G_1 phase with treatment doses of 2, 10, 50 μM ($p\leq0.05$); while it acted differently on lymphoid cell lines in which significantly accumulated the cells at S phase in JURKAT and CCRF-CEM cells, and G_2/M phase in MOLT-3 and U937 cells with 10, 50 μM treatmetn doses ($p\leq0.05$) (Figure 2.17, 2.18 and Table 2.10).

Apigenin (Figure 2.21, 2.22) induced varied effects on cell cycle within the leukaemia cell lines. In particular, apigenin significantly accumulated in the cells in G_0/G_1 phase for CCRF-CEM, U937, HL60 cells at treatment dose of 50 and 250 μM; in S phase for JURKAT and MOLT-3 cells at treatment dose of 50 and 250 μM, K562 cells at treatment dose of 250 and

500 μM, and KG1a cells at treatment dose of 50, 250 500 μM; and finally in G_2/M phase for THP-1 cells at treatment dose of 50 and 250 μM ($p \leq 0.05$) (Figure 2.21, 2.22 and Table 2.10).

Aloe-emodin (Figure 2.27, 2.28) and *trans*-stilbene (Figure 2.31, 2.32) had similar effects on cell cycle within the leukaemic cell lines. Both treatments caused significant accumulation in cells in G_0/G_1 phase for CCRF-CEM, MOLT-3, THP-1 and U937 cell lines; plus S phase accumulation for JURKAT, HL60 and THP-1 cells; and finally G_2/M phase accumulation for K562 cells mostly at treatment doses of 250 and 500 μM ($p \leq 0.05$) (Table 2.10). The only difference seen between these two polyphenol treatment was shown in KG1a cells, which were significantly arrested at G_0/G_1 phase with aloe-emodin and at G_2/M phase with *trans*-stilbene at 250 and 500 μM treatment doses ($p \leq 0.05$) (Table 2.10).

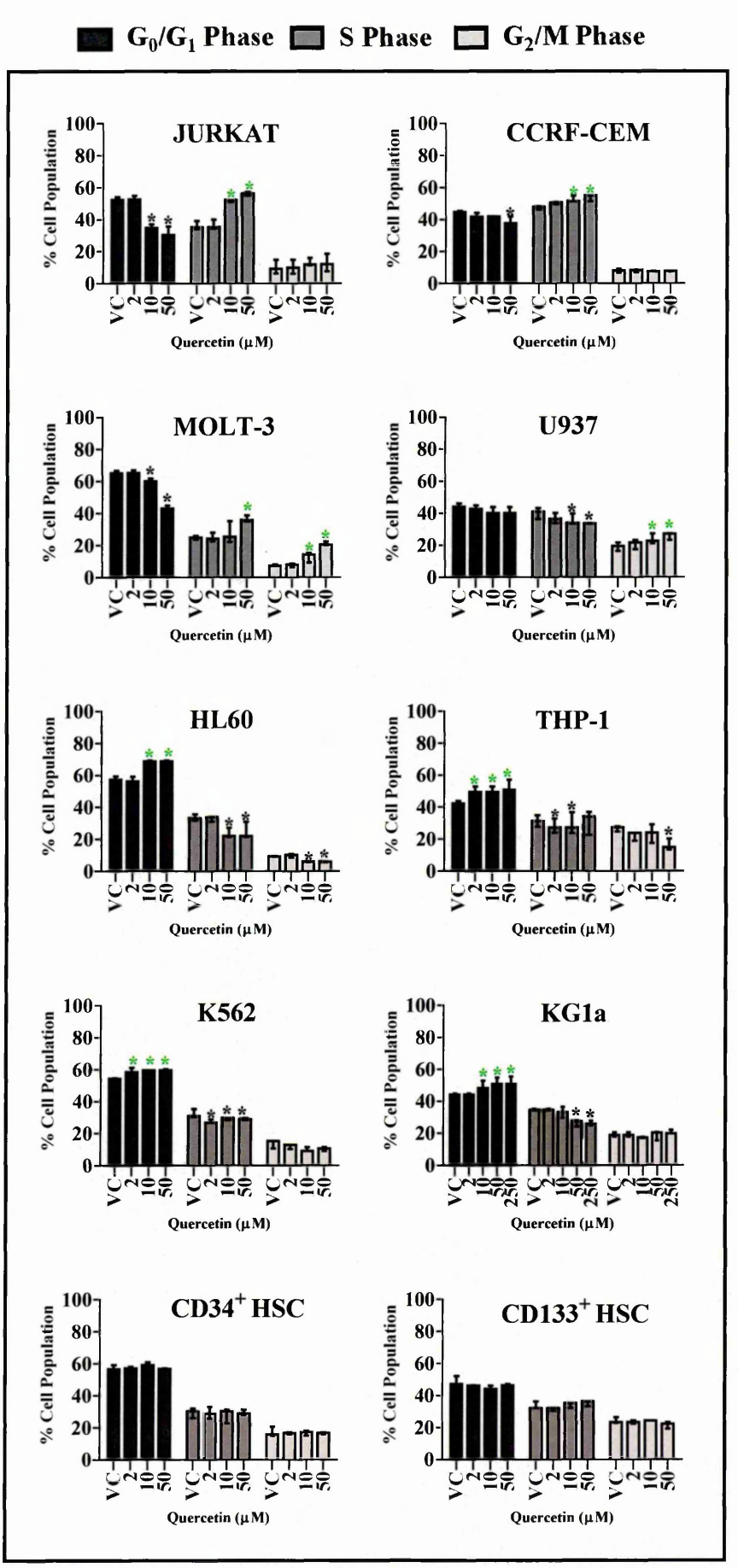

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Quercetin (μM)
VC
2
10
50
250

Figure 2.17: Effect of quercetin on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with quercetin at the range of IC_{25} and IC_{50} doses (at 2, 10, 50 250 µM for KG1a cell lines and at 2, 10, 50 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

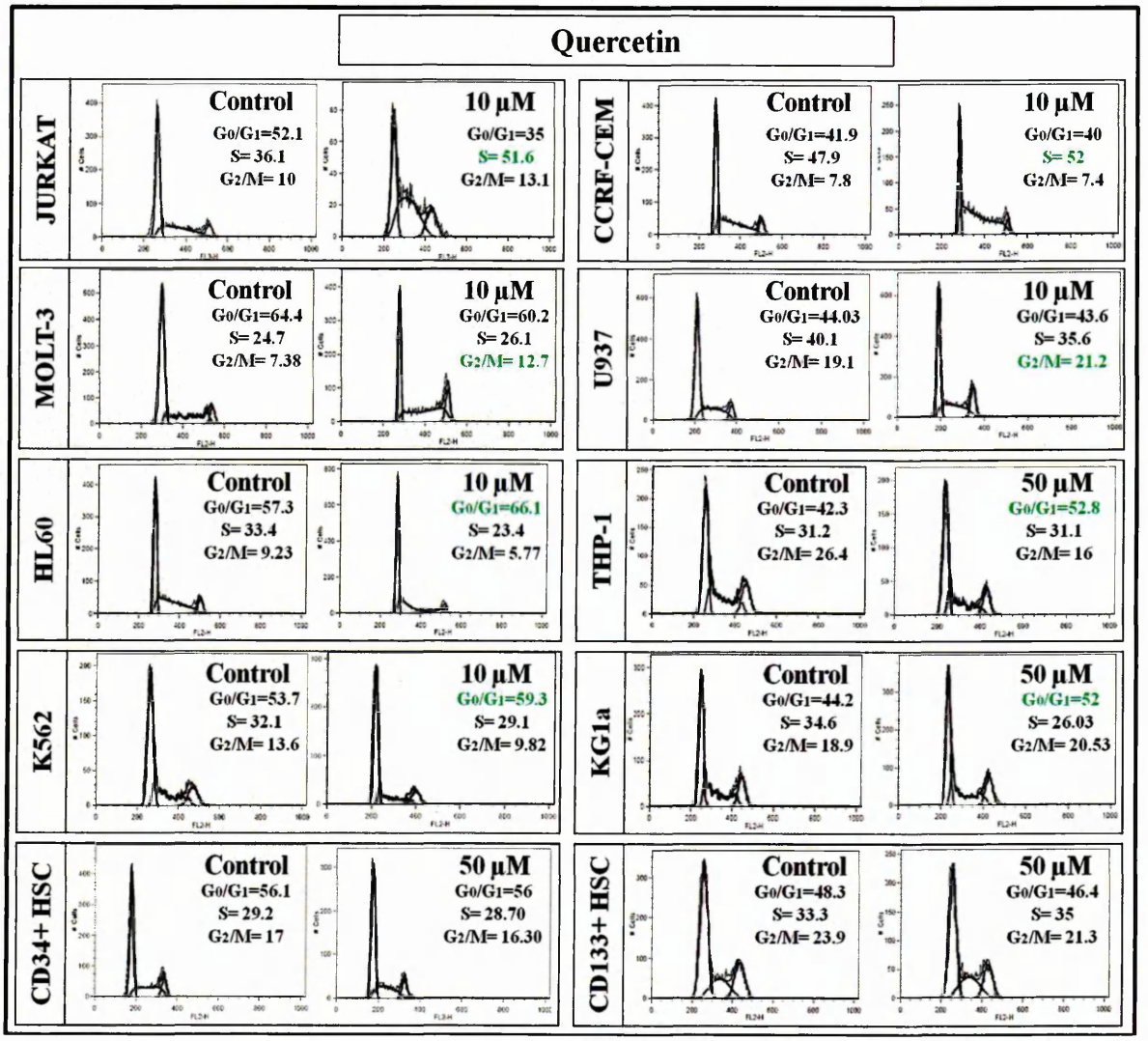


Figure 2.18: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for quercetin treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

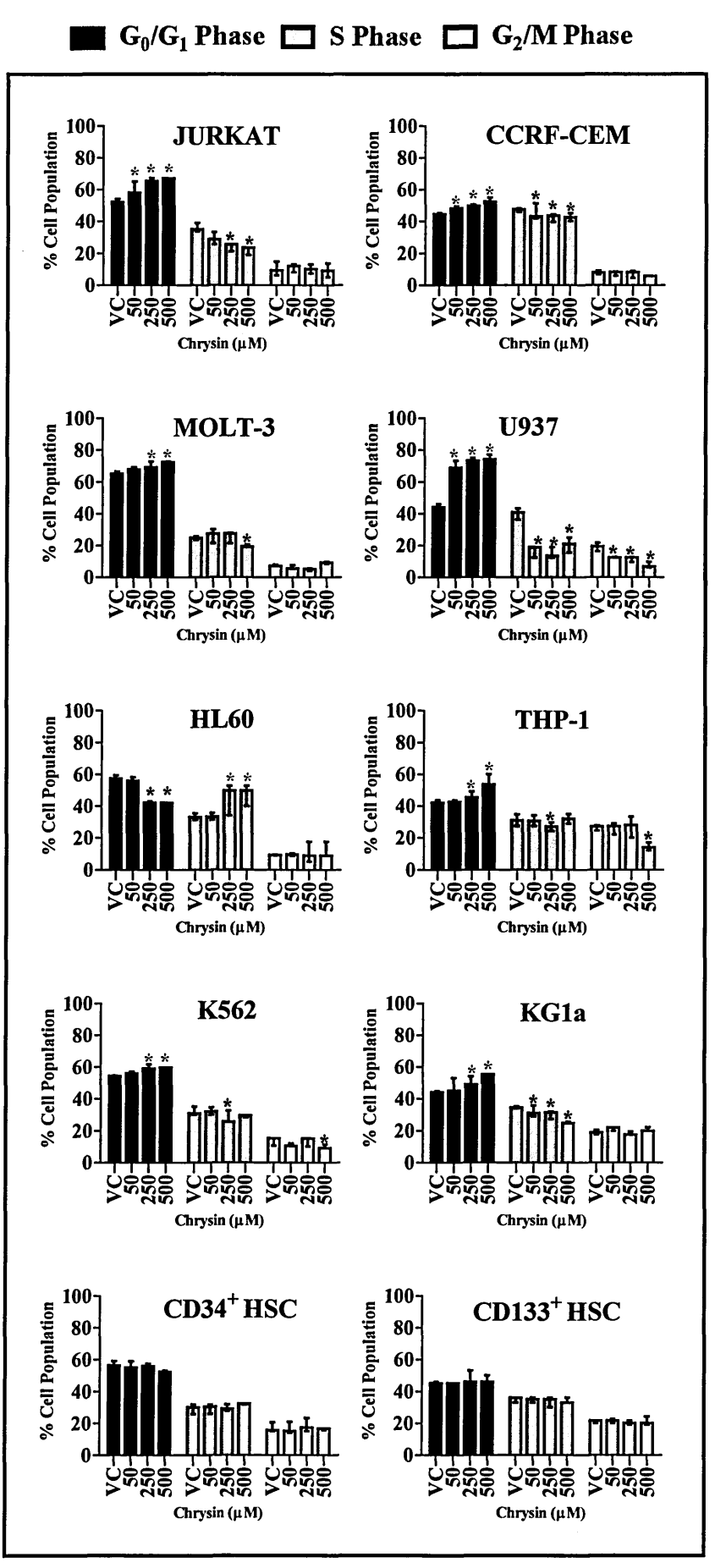

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Chrysin (μM)
VC
50
250
500

Figure 2.19: Effect of chrysin on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with chrysin at the range of IC_{25} and IC_{50} doses (at 50, 250, 500 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

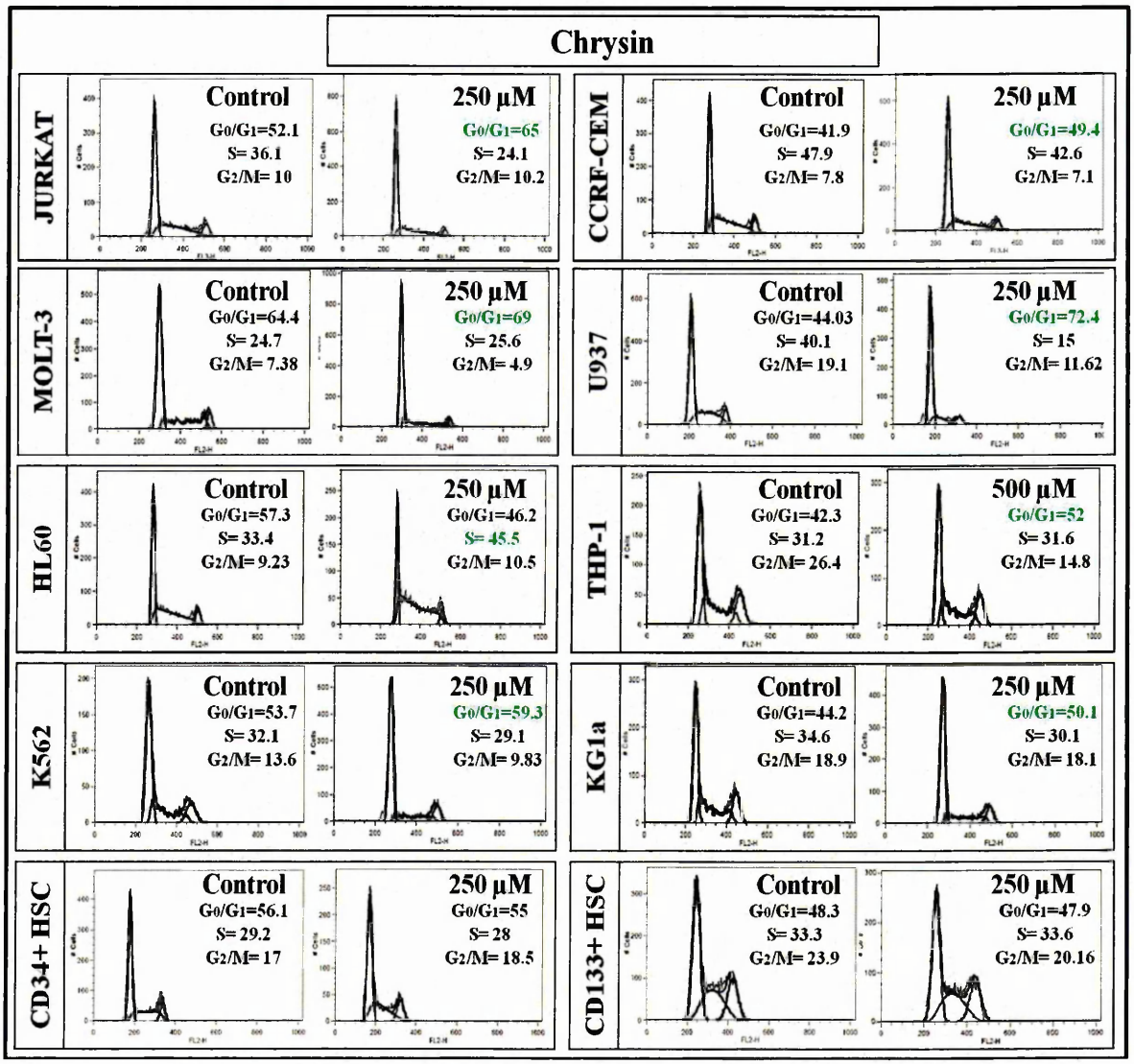


Figure 2.20: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for chrysin treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

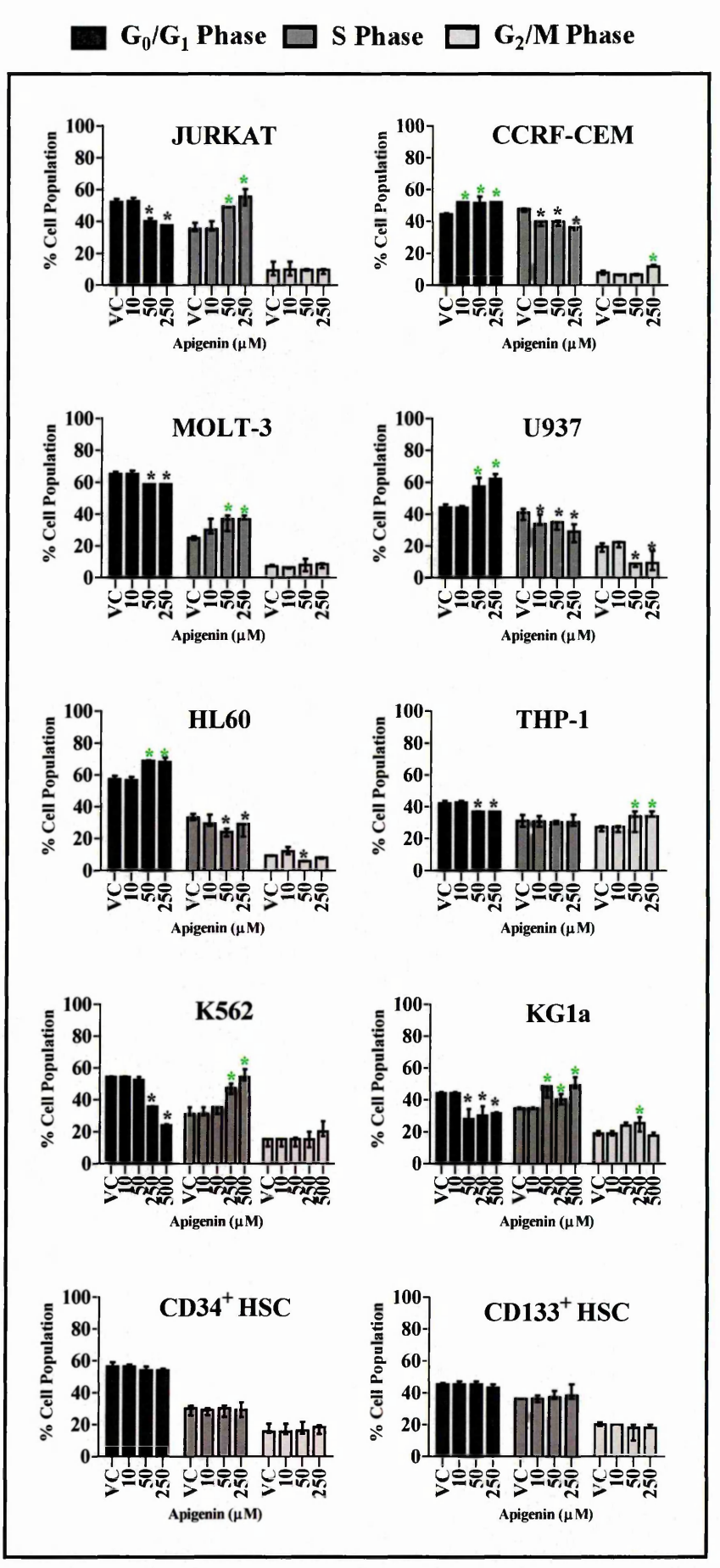

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Apigenin (μM)
VC
10
50
250
500

Figure 2.21: Effect of apigenin on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with apigenin at the range of IC_{25} and IC_{50} doses (at 10, 50 250, 500 µM for K562 and KG1a cell lines, and at 10, 50, 250 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

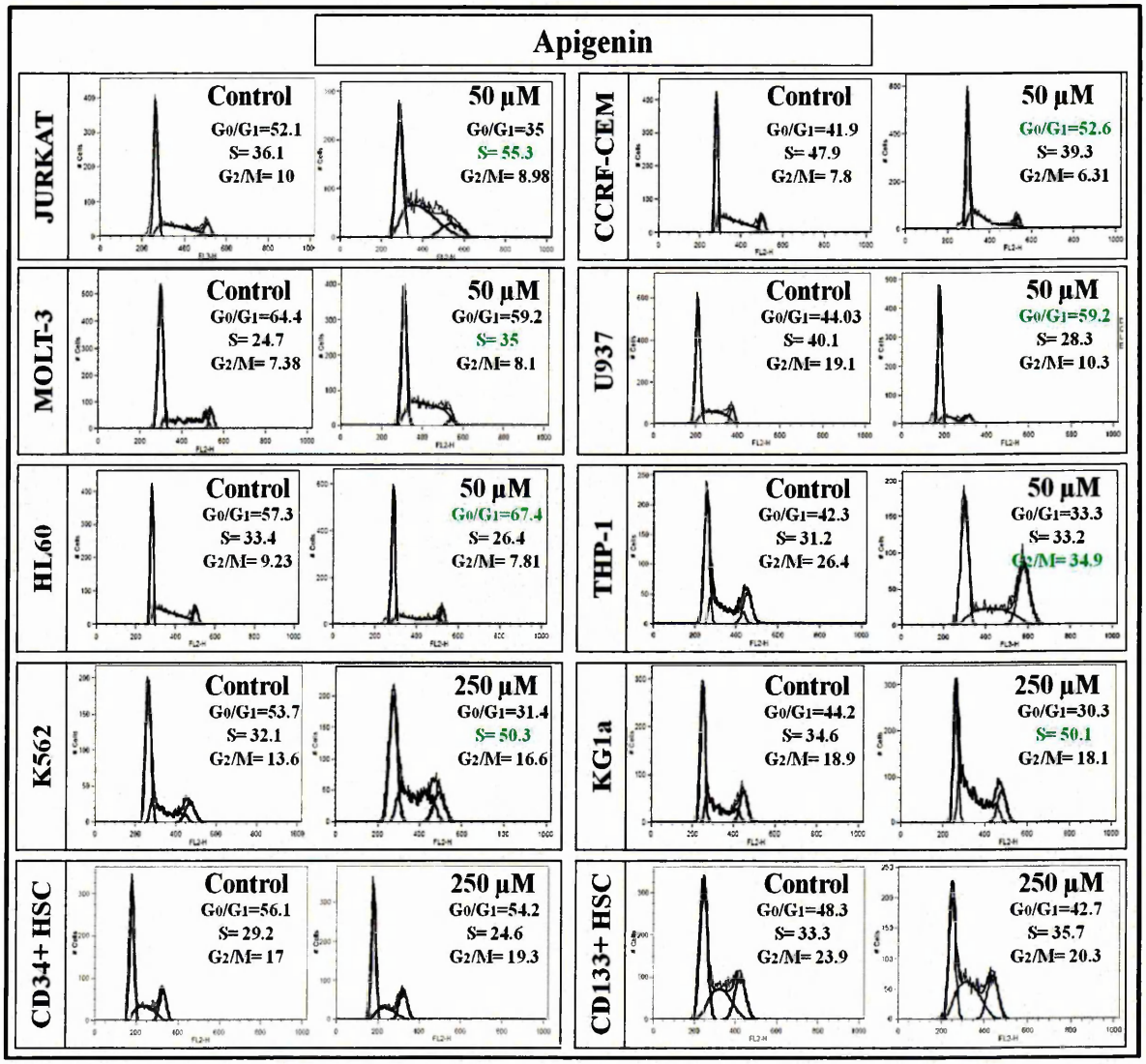


Figure 2.22: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for apigenin treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

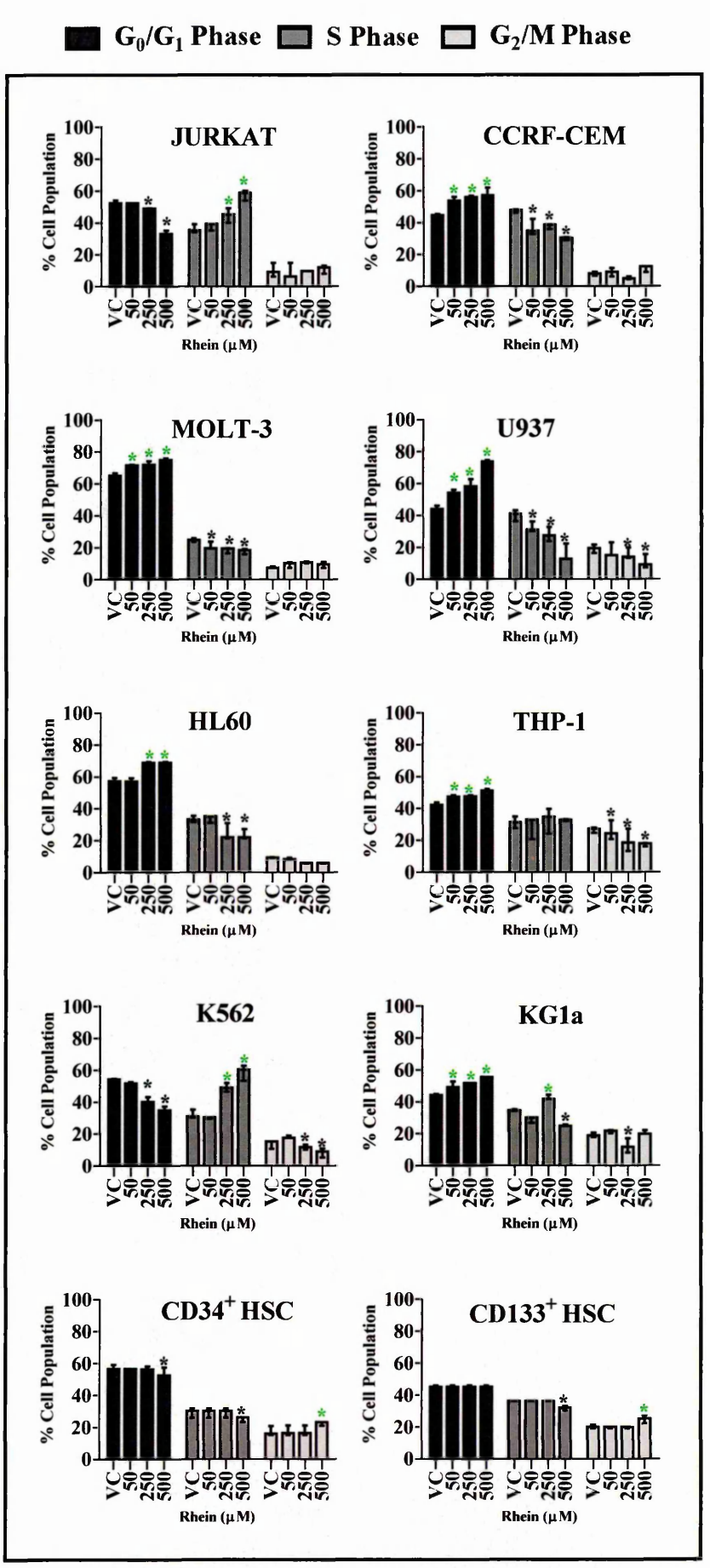

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Rhein (μM)
VC
50
250
500

Figure 2.23: Effect of rhein on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with rhein at the range of IC_{25} and IC_{50} doses (at 50, 250, 500 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

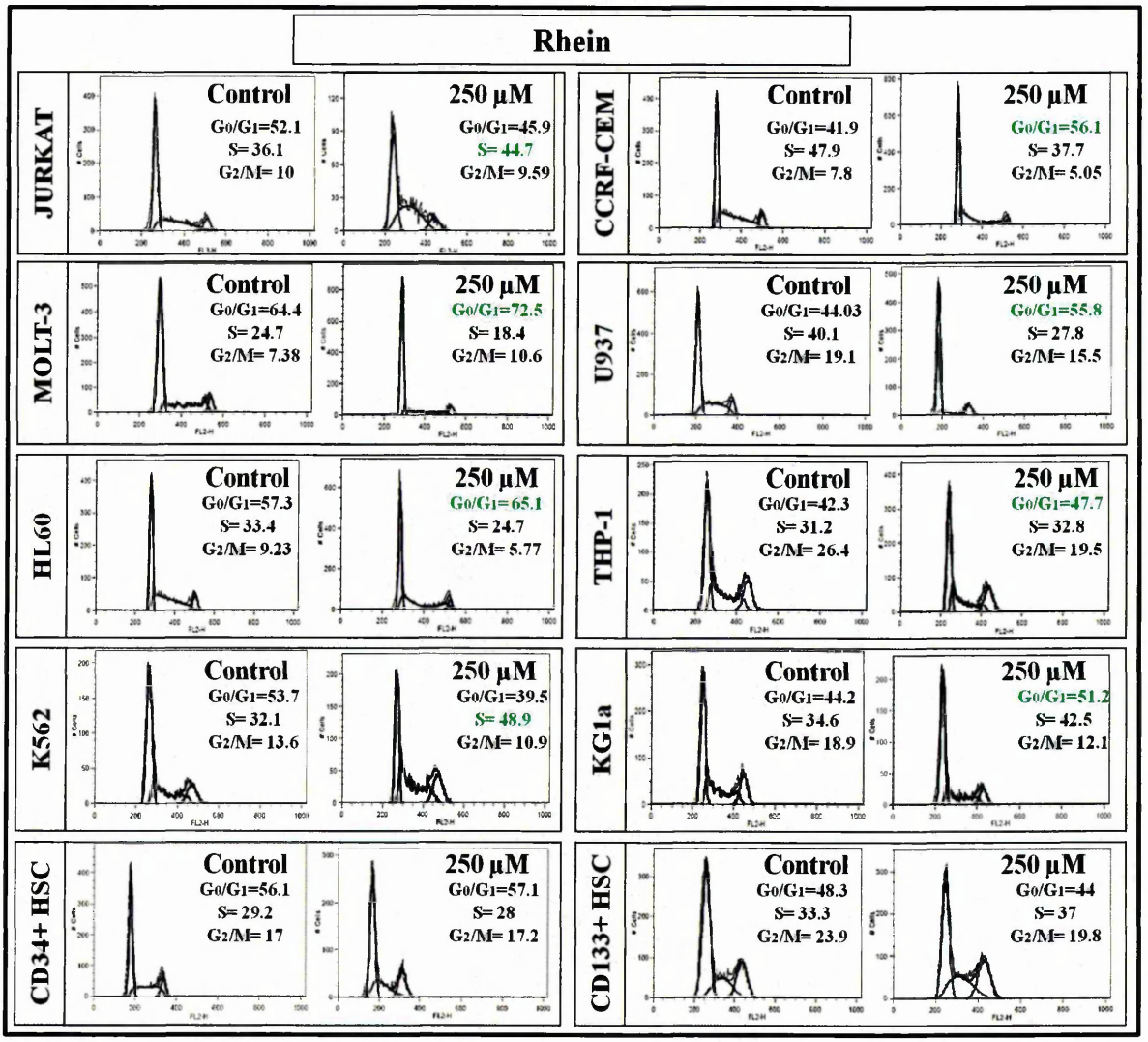


Figure 2.24: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for rhein treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

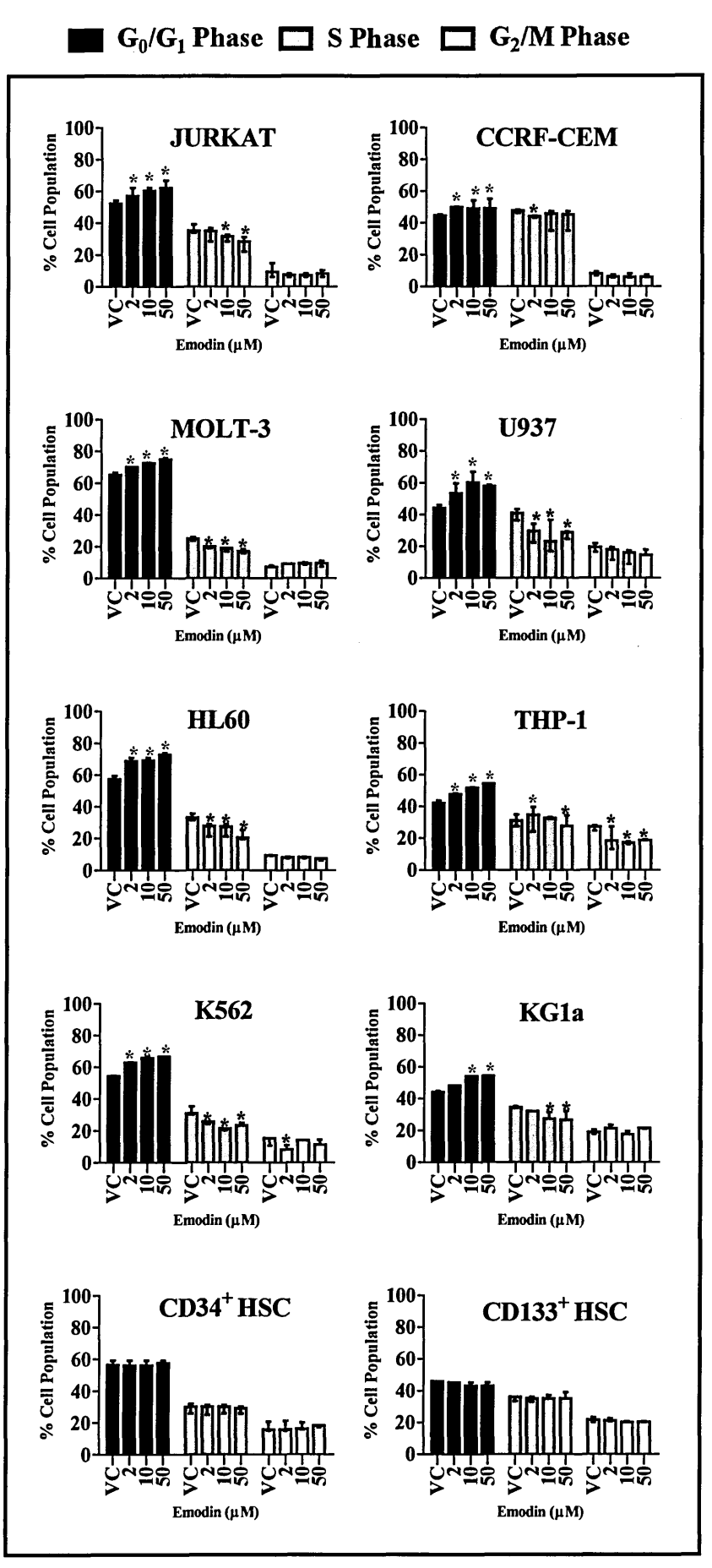

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Emodin (μM)
VC
2
10
50

Figure 2.25: Effect of emodin on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with emodin at the range of IC_{25} and IC_{50} doses (at 2, 10, 50 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

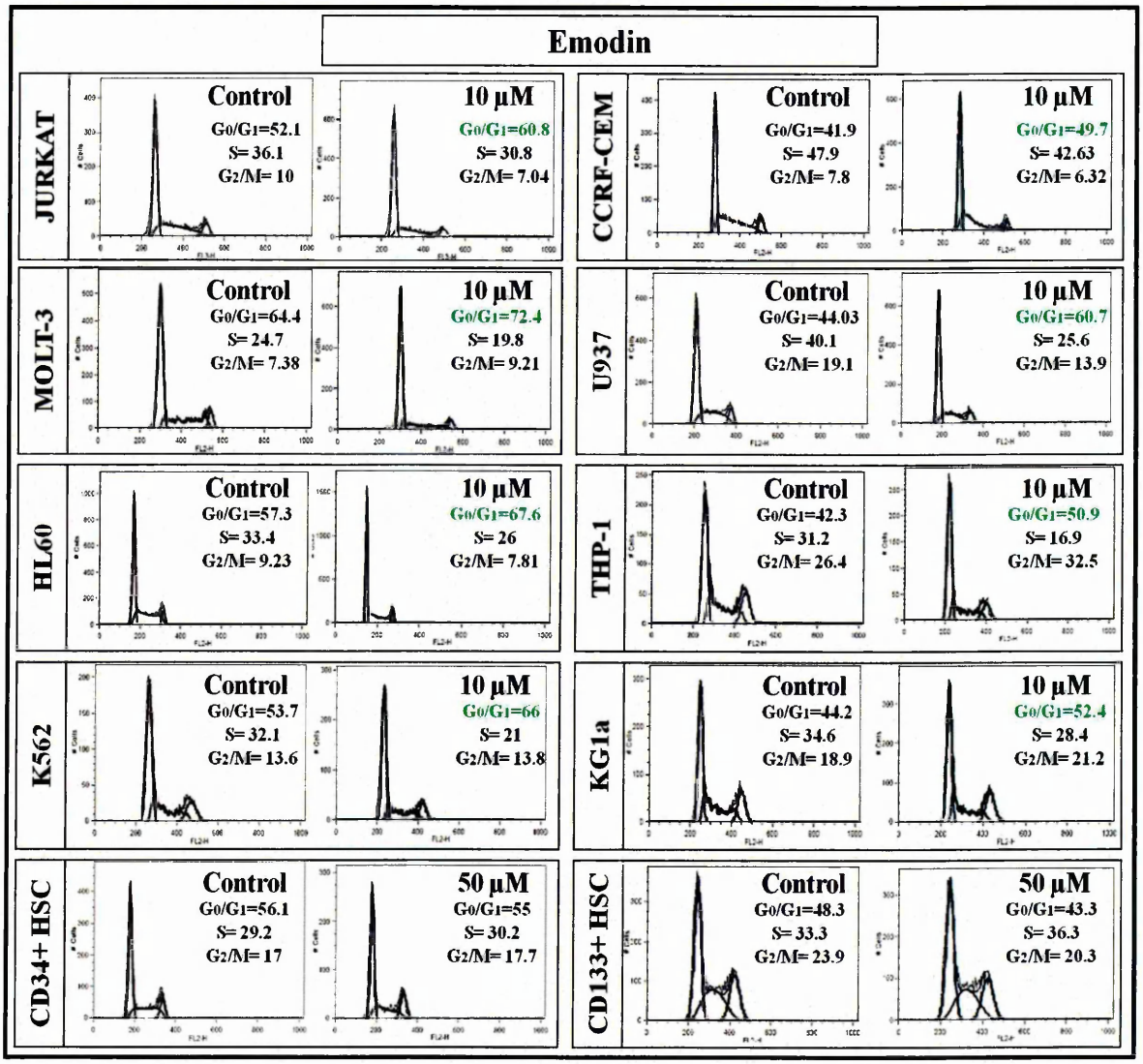


Figure 2.26: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for emodin treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

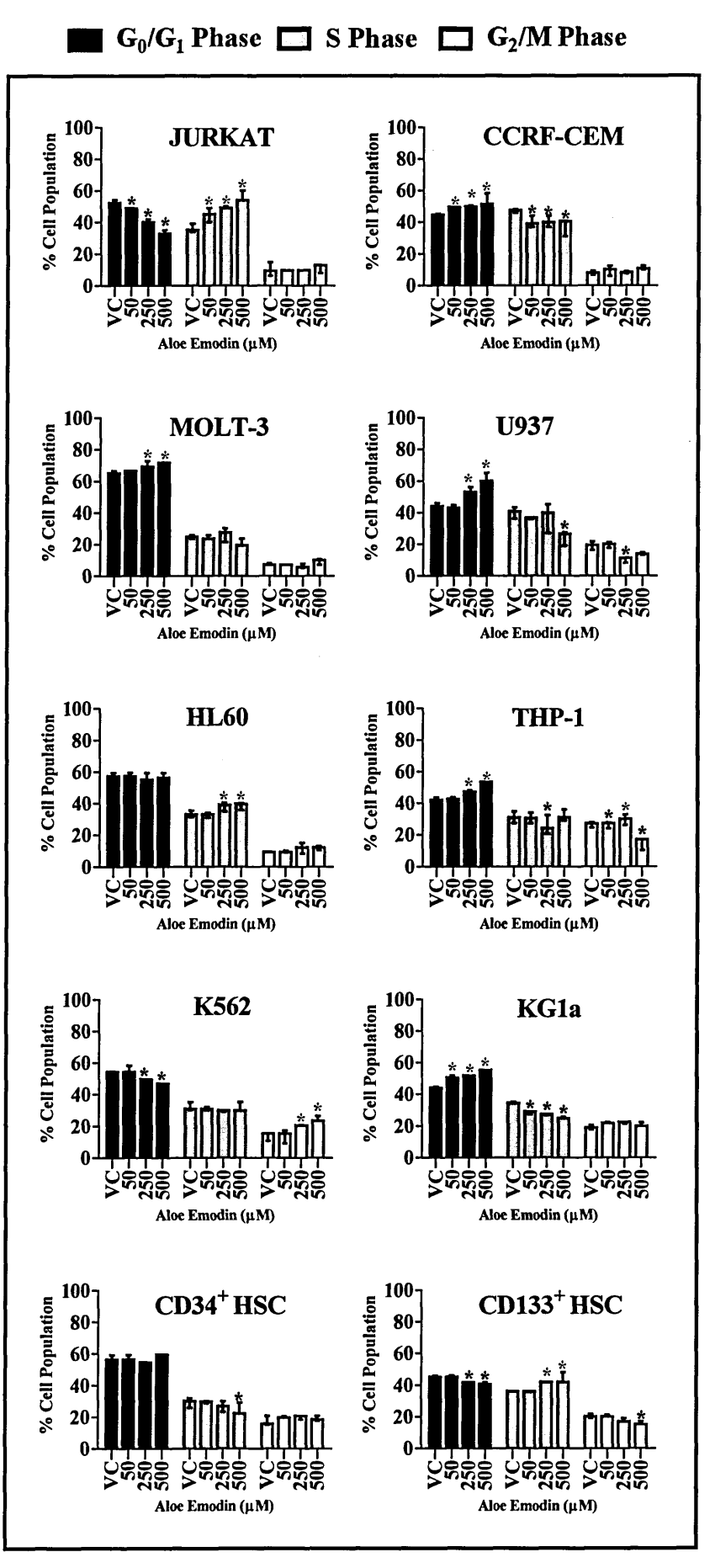

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Aloe Emodin (μM)
VC
50
250
500

Figure 2.27: Effect of aloe-emodin on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with aloe-emodin at the range of IC_{25} and IC_{50} doses (at 50, 250, 500 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

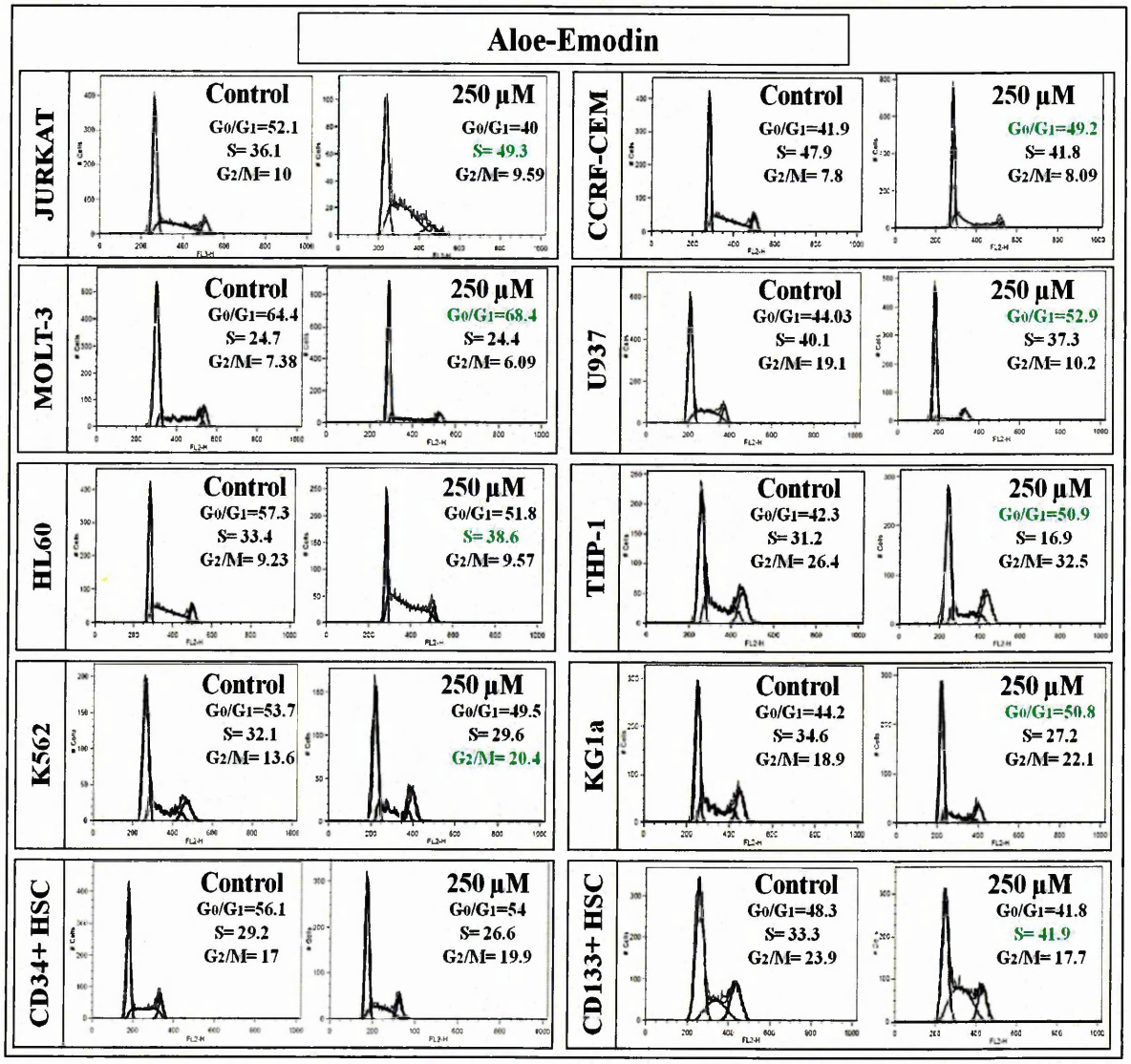


Figure 2.28: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for aloe-emodin treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

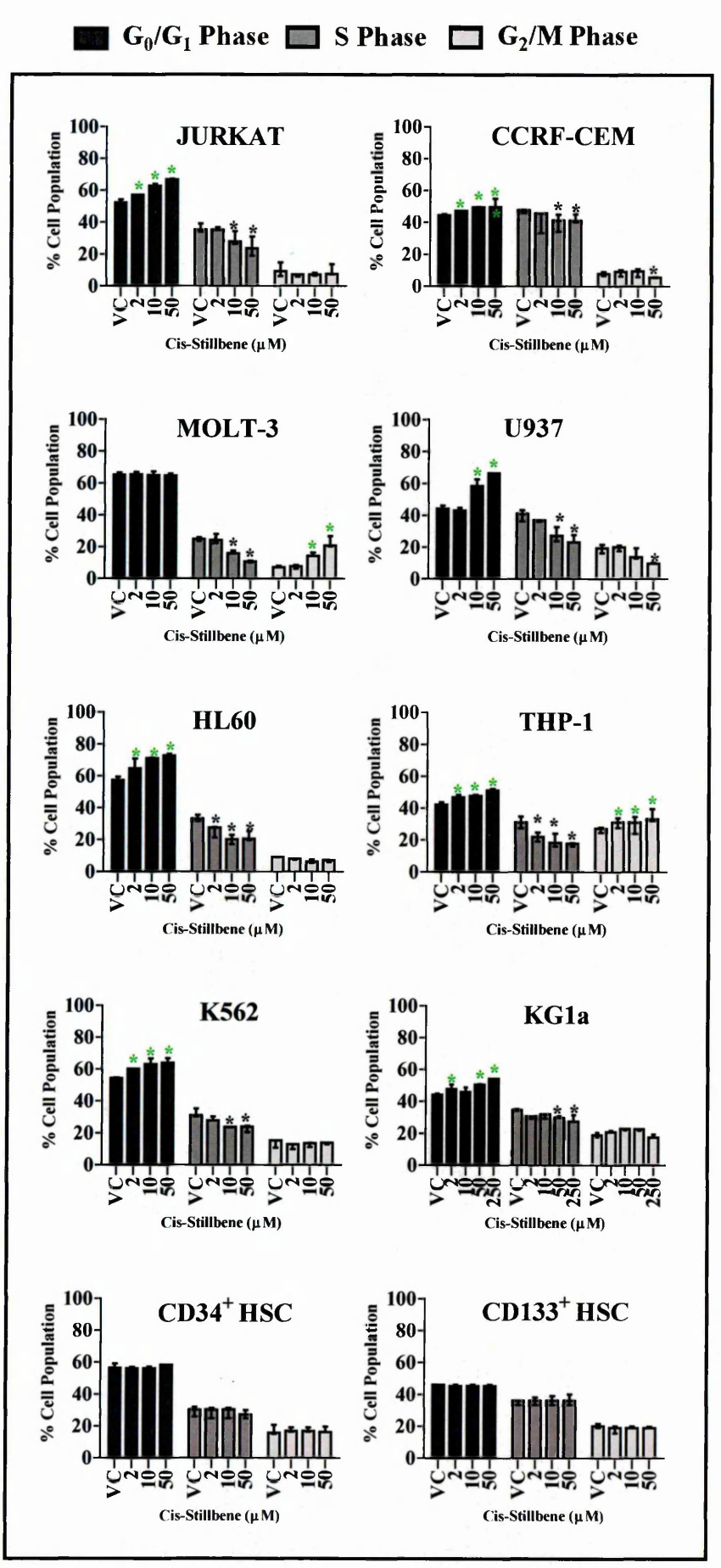

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Cis-Stillbene (μM)
VC
2
10
50
250

Figure 2.29: Effect of *cis*-stilbene on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with *cis*-stilbene at the range of IC_{25} and IC_{50} doses (at 2, 10, 50, 250 µM for KG1a cell lines, and at 2, 10, 50 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

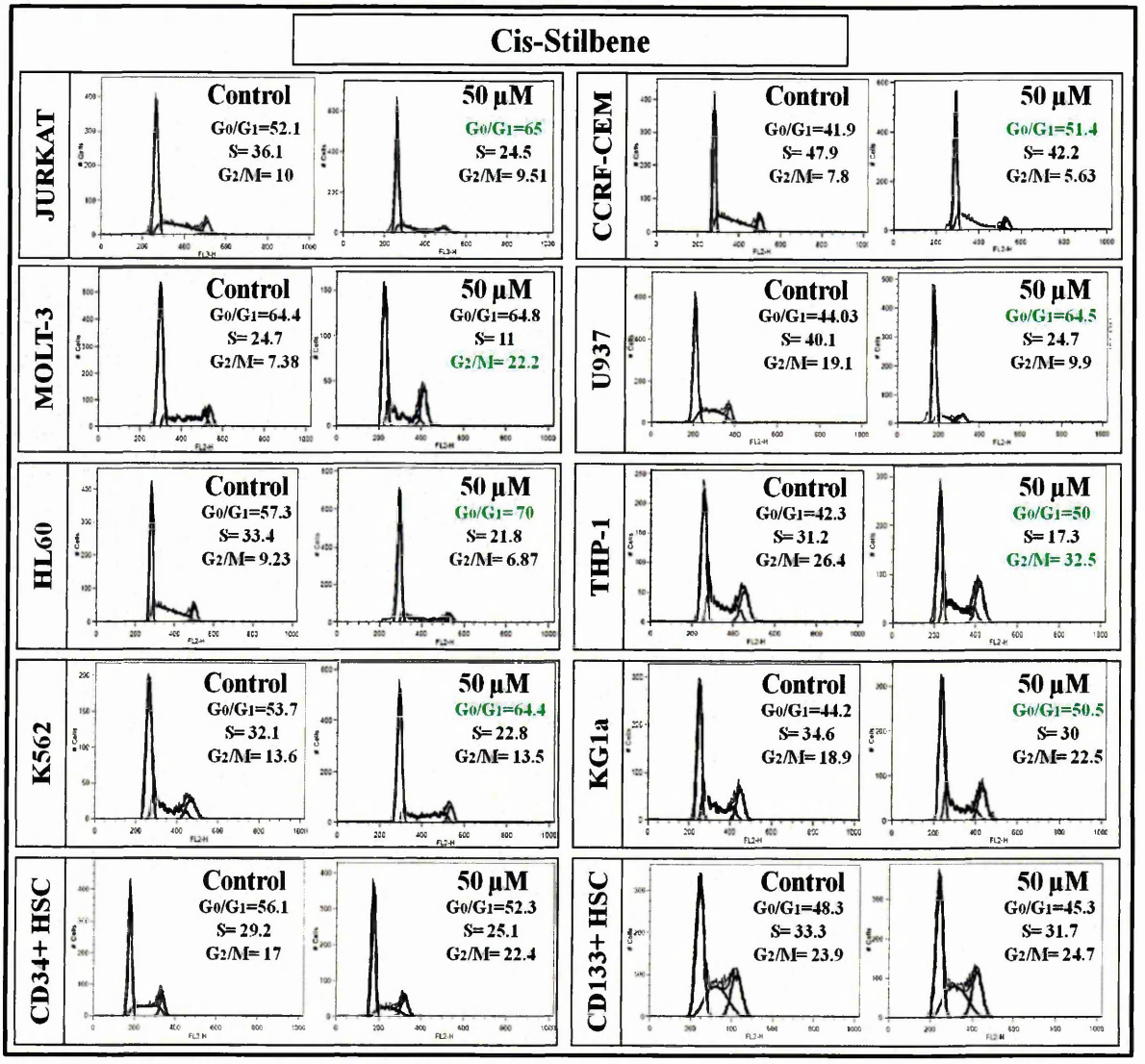


Figure 2.30: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for *cis*-stilbene treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

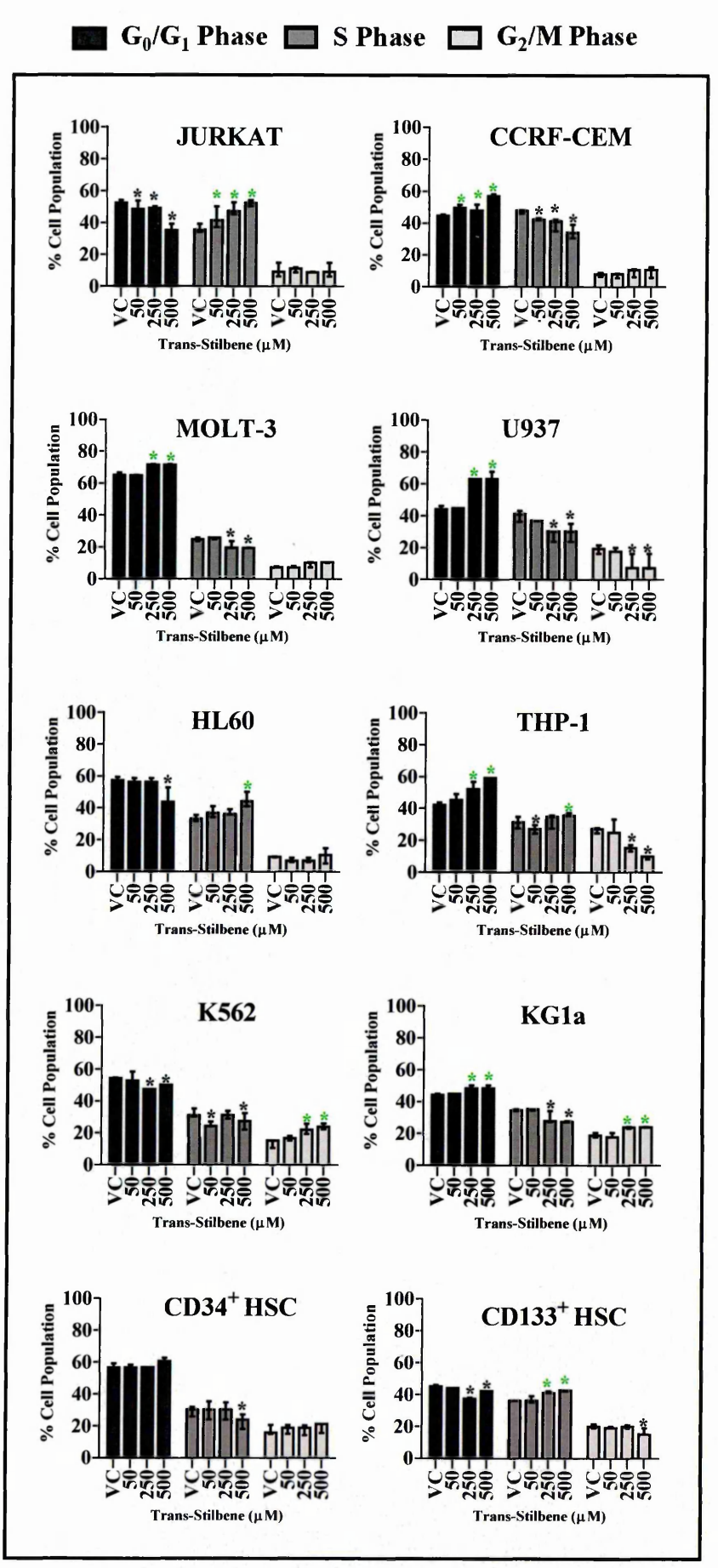

G0/G1 Phase
S Phase
G2/M Phase
JURKAT
CCRF-CEM
MOLT-3
U937
HL60
THP-1
K562
KG1a
CD34+ HSC
CD133+ HSC
% Cell Population
Trans-Stilbene (µM)
VC
50
250
500

Figure 2.31: Effect of *trans*-stilbene on cell cycle progression of three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). This was analysed by propidium iodide staining and flow cytometry. Cell were treated with *trans*-stilbene at the range of IC_{25} and IC_{50} doses (at 50, 250, 500 µM for all other cell lines) as determined by CellTiter-Glo® assay for 24 h. The percentage of cells in each phase was analysed with Flow Jo software using Waston pragmatic model. The data is expressed as median with range (each in triplicate). The statistical significance was determined by comparison with the vehicle control; statistical significance was set at $p \leq 0.05$. The green star (*) indicates where there is a significant increase in the accumulation of cells in a phase of the cell cycle; while the black star (*) indicates a significant decrease in the accumulation of cells in a phase of the cell cycle. The effects of all polyphenols on the cell cycle progression in all the cell lines are summarised in table 2.10.

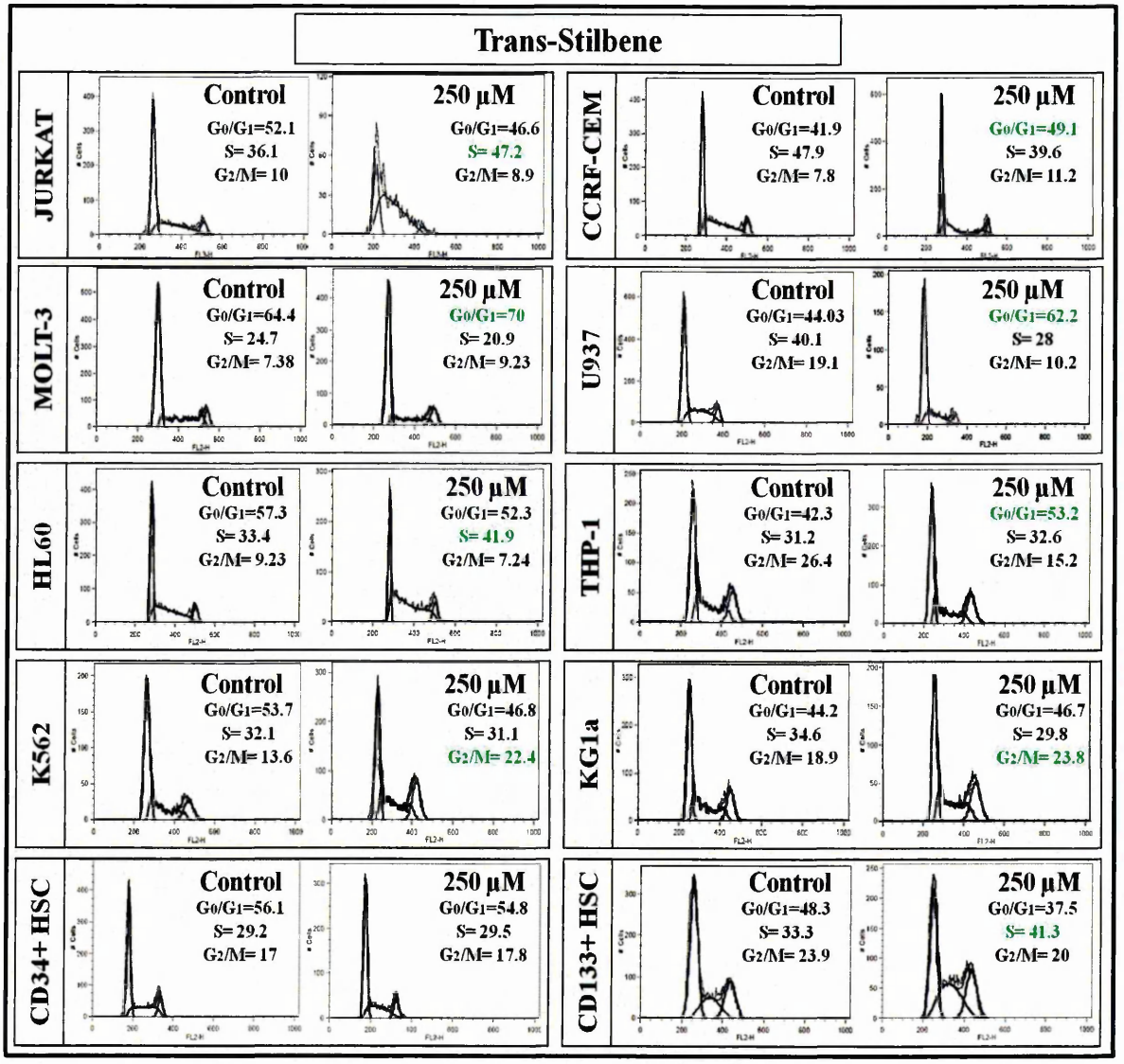


Figure 2.32: An example of histograms for cell cycle phases (G_0/G_1, S, G_2/M) for *trans*-stilbene treatment at the IC_{50} doses at 24 h in three lymphoid leukaemia (JURKAT, CCRF-CEM, MOLT-3), one histocytic lymphoma (U937), four myeloid leukaemia cell lines (HL-60, THP-1, K562, KG1a), and two non-tumour normal progenitor cells ($CD34^+$ HSC, $CD133^+$ HSC). The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The statistical significance was set at $p \leq 0.05$. The phase with green text is indicated for significant increase of cells accumulation compared to vehicle control.

Cell lines		The Phases of Cell Cycle at Which There Was a Significant Accumulation of Cells Following 24h IC_{25} and IC_{50} Polyphenol Treatment							
		Quercetin	Apigenin	Chrysin	Rhein	Emodin	Aloe - Emodin	Cis-Stilbene	Trans-Stilbene
Lymphoid Leukaemia	JURKAT	S	S	G_0/G_1	S	G_0/G_1	S	G_0/G_1	S
	CCRF-CEM	S	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1
	MOLT-3	G_2/M	S	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_2/M	G_0/G_1
Myeloid Leukaemia	HL60	G_0/G_1	G_0/G_1	S	G_0/G_1	G_0/G_1	S	G_0/G_1	S
	THP-1	G_0/G_1	G_2/M	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1
	K562	G_0/G_1	S	G_0/G_1	S	G_0/G_1	G_2/M	G_0/G_1	G_2/M
	KG1a	G_0/G_1	S	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_2/M
Histocytic Lymphoma	U937	G_2/M	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1	G_0/G_1
Non-Tumour Control Cells	CD34+ HSC	No Arrest <50μM	No Arrest ≤250μM	No Arrest <500μM	No Arrest <250μM	No Arrest <50μM	No Arrest <500μM	No Arrest <50μM	No Arrest ≤500μM
	CD133+ HSC	No Arrest <50μM	No Arrest ≤500μM	No Arrest <500μM	No Arrest <250μ	No Arrest <50μM	S	No Arrest <50μM	S

Table 2.10: A summary of the cell cycle phases in which there was significantly accumulation of cells following IC_{25} and IC_{50} polyphenol treatments for 24 h in eight leukaemia cell lines and two non-tumour control cells. The cell cycle was assessed using propidium iodide (PI) staining and flow cytometric. The percentage of cells in each phase was analysed using the Flow Jo software with the Waston pragmatic model. The phase of cell cycle accumulation varied according to polyphenol treatment and cell line. While in general, polyphenols appeared to cause G_0/G_1 phase accumulation in most of leukaemic cell lines. No significant arrest in cell cycle was observed in the non-tumour progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC) with most polyphenols within the IC_{25} and IC_{50} ranges that used to treat the leukaemia cell lines, the only exception was observed with aloe-emodin and trans-stilbene for $CD133^+$ HSC normal cells.

2.4 Discussion

Over the past 10 years, researchers have confirmed that polyphenols are capable of inhibiting cell proliferation, inducing cell cycle arrest and apoptosis in a number of solid tumour cell lines (Dai *et al*, 2013; Han *et al*, 2007; Jaganathan *et al*, 2009; Patel *et al,* 2007; Sharif *et al*, 2010; Shukla *et al*, 2010), however there has not been a direct comparison of the effect of polyphenols on leukaemia cell lines and non-tumour cells. Here, we directly compared the effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*stilbene and *trans*-stilbene) on four lymphoid and three myeloid leukaemia cell lines; one histocytic leukaemia cell line; and two non-tumour blood progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC). In particular, we studied the effect of polyphenols on cell ATP levels as indication of cell viability at 24, 48 and 72 h. From this data, it was able to determine the IC_{50} values for each polyphenol in each cell line at each time point (Tables 2.2, 2.4, 2.6). A

comparison of this data shows that there is little variation between the time points. With this in mind all further assessment including, cell cycle, caspase 3 activity, and Hoechst 33342 nuclear stain of apoptosis was completed at 24 h. So for this discussion, we used the IC_{50} values that determined at 24 h only (Tables 2.2) to determine effectiveness of polyphenols on leukaemia cell lines in comparison with other assays (Table 2.9, 2.10). The effects of these polyphenols were shown to be greater in leukaemia cells compared to non-tumour blood progenitor cells ($CD34^+$ HSC and $CD133^+$ HSC), indicating no effect on their viable cells. When non-tumour cells were treated with quercetin and *cis*–stilbene, chrysin, apigenin and aloe-emodin, there was no significant decrease on ATP levels until the treatment concentration increased to 250-500 μM. There was a significant decrease on ATP levels of non-tumour cells when treated with ≥250 μM of emodin, rhein and *trans*-stilbene; however this is 5-10 times higher than the IC_{50} values reported for all leukaemia cells (Tables 2.2). Consequently, we have shown that each of the polyphenols caused a decrease in ATP levels and hence cell viability in all leukaemia cell lines and can be ranked according to their effectiveness: emodin > quercetin > *cis*-stilbene > apigenin ≥ rhein > aloe-emodin ≥ *trans*-stilbene ≥ chrysin. However, it is important to note that this ranking did vary between individual cell lines (Table 2.2).

Emodin was the most effective polyphenol at reducing ATP levels. It was by far the most effective of the anthraquinones investigated. The structural differences between the anthraquinones are slight and, indeed, emodin and aloe-emodin have the same structural formula ($C_{15}H_{10}O_5$), although the orientations of the functional groups vary. The IC_{50} values for emodin (5-22 μM) were the lowest of all the studied polyphenols; and were comparable with those previously reported in squamous cell carcinoma cell lines (SCC-4) cells (Chen *et al*, 2010). Emodin was shown to consistently induce accumulation of cells at G_0/G_1 phase in all leukaemia cell lines, and induced 50% apoptosis in 5 of the 8 leukaemia cell lines (JURKAT, MOLT3, HL-60, THP-1 and U937). This is consistent with previous studies in which emodin induced apoptosis in HL-60 (Chen *et al*, 2002) and SCC-4 (Chen *et al*, 2010) cell lines.

Quercetin was also a potent polyphenol, with IC_{50} value ranging between 8-33 μM and induction of apoptosis with AP_{50} value ranging between 19-50 μM. Quercetin was the most effective of the flavonoids tested and was routinely 5-10 times more potent than apigenin and chrysin. The IC_{50} values noted are at the lower end of values previously reported (20-278 μM), in cancer cell lines of breast (MDA-MB-231 and MDA-MB-453) (Chien *et al*, 2009; Choi and Kim, 2009) and (MCF-7) (Chou *et al*, 2010); cervical (HeLa) (Huang *et al*, 2009; Vidya *et al*, 2010; Zhang *et al*, 2009), liver (HepG2) (Granado *et al*, 2006), lung (A-549) (Robaszkiewicz *et al*, 2007); and two leukaemia cell lines (HL-60 and K562) (Kang *et al*, 1997;Csokay *et al*, 1997). Lymphoid cell lines were more susceptible to quercetin treatment than myeloid

leukaemia cells. The only exception being the promyelocytic leukaemia cells (HL-60), which showed the same level of sensitivity as lymphoid cells. Quercetin demonstrated a differential induction of apoptosis in each leukaemia cell line although the AP_{50} values were consistently low. Previously, quercetin has been reported to induce apoptosis in a range of solid tumours, *via* a caspase 3-dependent mechanism (Chien *et al*, 2009; Chou *et al*, 2010; Choi *et al*, 2008), and in HL-60 cells *via* decreased PI3K/AKT pathway activity (Yuan *et al*, 2012). However, there are no reported AP_{50} values for these studies. Quercetin was found to have a differential effect on the cell cycle in myeloid and lymphoid cell lines. Inducing accumulation of cells at G_0/G_1 phase in all myeloid cell lines, and either S phase (JURKAT and CCRF-CEM) or G_2/M phase (U937 and MOLT3) accumulation in the lymphoid cell lines (Table 2.10). This varied effect of quercetin has been previously observed, where it induced accumulation in S phase in breast cancer cell lines (MCF-7) (Chou *et al*, 2010) and in G_2/M phase in cervical cancer cell lines (HeLa) (Huang *et al*, 2009; Vidya *et al*, 2010). Together with our study, this suggests that quercetin causes differential effects on cell cycle dependant on cell type, even in comparatively similar leukaemia cell lines. This may reflect expression of different molecular targets in myeloid and lymphoid cell lines; or a differential effect on the same pathway in different cell lineages.

Cis-stilbene was much more effective than its isomer *trans*-stilbene in all leukaemia cell lines. This is reflected in IC_{50} values for *cis*-stilbene (25-85 μM) and *trans*-stilbene (109-500 μM); however, these values were considerably higher than those previously reported in solid tumours (Shankar *et al*, 2007; Yang *et al*, 2002). Very few studies have investigated the effects of stilbenoids on cell cycle. *Cis*-stilbene has been reported to induce cells accumulation in G_2/M phase in the lung cancer cell lines (A549) (Lee *et al*, 2004), and in S phase in one leukaemia cell lines (HL-60) (Saiko *et al*, 2006). Our results have shown that *cis*-stilbene consistently caused cell accumulation at G_0/G_1 phase in 7 of the 8 cell lines including the HL-60 cell line. This contrasts with the finding that HL-60 cells when treated with the stilbene derivate 3, 3', 4, 4', 5, 5'-hexahydroxystilbene induced S phase accumulation (Saiko *et al*, 2006). A less consistent effect was found with *trans*-stilbene treatment, which caused cell accumulation at different phases of cell cycle in all leukaemia cell lines. Both *cis*- and *trans*-stilbene induced caspase 3 activity of early apoptosis and morphological changes characteristic of late apoptosis in the majority of leukaemia cell lines. *Cis*-stilbene was able to induce apoptosis in three lymphoid cell lines (JURKAT, MOLT3, and U937) with AP_{50} values ranging between 20-50 μM, the remaining cell lines were more resistant, and did not reach 50% apoptosis even when treated with a maximal treatment dose (500 μM). Similarly, *trans*-stilbene induced apoptosis in the same three lymphoid cell lines, however the AP_{50} values were much higher (40-460 μM), there was also a similar resistance to treatment in the remaining

lymphoid cell lines. *Cis*-stilbene did not show any significant effect on ATP levels of the non-tumour cells, however, *trans*-stilbene did, but only at high treatment concentration in excess of 250 μM. Previous work has shown that stilbenoids can inhibit cell proliferation and induce apoptosis in cancer cell lines of lung (A549) (Lee *et al*, 2004; Weng *et al*, 2009), prostate (DU145 and PC3), breast (BT-549), colon (HT-29) (Shankar *et al*, 2007; Yang *et al*, 2002) and one leukaemia (HL-60) (Saiko *et al*, 2006; Simoni *et al*, 2006). *Trans*-stilbene had a reported IC_{50} values of 25-98 μM at 24 h in two lung cancer cell lines (A549 and CH27) (Weng *et al*, 2009). A direct comparison of *cis*-stilbene and *trans*-stilbene in cancer cell lines of lung (A549) (Lee *et al*, 2004) and leukaemia (HL-60) (Saiko *et al*, 2006); demonstrated that *cis*-stilbene was more effective than *trans*-stilbene with IC_{50} values of 0.03 μM and 6.25 μM, respectively, at 24 h (Saiko *et al*, 2006; Lee *et al*, 2004). This supports the finding of this study that *cis*-stilbene is more potent than *trans*-stilbene in the treatment of leukaemia cells. However, the reason for this difference is not clear, but may be related to the stability of the *trans*- and *cis*-isomers in culture.

A moderate effect was seen in leukaemia cells treated with apigenin, with IC_{50} values between 100-500 μM. However, in other cell types lower IC_{50} values have been reported, including 36 μM in human cervical cancer cells (HeLa) (Zheng *et al*, 2005) and 70 μM in colorectal cancer cells (SW480, HT-29 and Caco-2) following 24 h treatments (Wang *et al*, 2000) suggesting differential activity within tumour types. In addition, apigenin induced variable effects on cell cycle, which was dependant on the cell lines investigated. This phenomenon has also been seen in solid tumour cell lines, where apigenin induced G_0/G_1 arrest in human cervical cancer cell lines (HeLa) (Zheng *et al*, 2005) and G_2/M arrest in human colon cancer cell lines (SW480, HT-29 and Caco-2) (Wang *et al*, 2000). Apigenin was shown to induce apoptosis in all leukaemia cell lines, with AP_{50} values ranging between 35-130 μM in lymphoid cell lines and 84-235 μM in the myeloid cell lines. In contrast to quercetin, apigenin was capable of inducing both an increase in caspase 3 activity indicating early apoptosis, plus morphological evidence of late apoptosis, in all leukaemia cells lines; including the KG-1a and K562 cells which were resistant to emodin, quercetin and *cis*-stilbene treatment. This pro-apopotic action of apigenin has been previous demonstrated in MDA-MB-453 breast cells (Choi and Kim, 2009). The other polyphenols investigated; rhein, chrysin, aloe-emodin demonstrated a low potency and thus are unlikely to be of clinical use in leukaemia treatment. Similar low potency has also been shown in solid tumours, for example the reported IC_{50} for chrysin in solid tumour cell lines are between 40 and 100μM (Li *et al*, 2011; Khoo *et al*, 2010; Parajuli *et al*, 2009).

Within all the polyphenols agents tested, the leukaemia cells were more sensitive than the non-tumour cell lines ($CD34^+$ HSC and $CD133^+$ HSC). Interestingly, the ATP levels and percentages of G_0/G_1 population were comparable in all untreated leukaemia and non-tumour

control cells, suggesting sensitivity rates were not related to ATP levels and hence cell viability. The order of sensitivity within the leukaemia cells was shown to be dependent on the polyphenol investigated. For example, U937 cells were one of the most affected cell lines when treated with quercetin, emodin and *cis*-stilbene; however they were least affected cell line when treated with apigenin. This demonstrates that no single polyphenol is active on all cell lines and that specific polyphenols should be selected for each type of leukaemia.

The cell cycle arrest data showed predominately G_0/G_1 arrest, however some treatments arrested cells in S phase and G_2/M. It is well known that cell cycle is regulated by the coordinated activity of family of protein kinases: cyclin-dependent kinase (CDKs), cyclins and CDK inhibitors (CDKIs) (Malumbres and Barbacid, 2009). Cell-cycle can be arrested *via* protein kinase inhibitors (CDKIs), such as p21wafl and p27kip1, upon binding to cyclins and CDK complexes and indeed modulation of their activities could be possible targets for the polyphenols. The stage of cell cycle arrest induced by phenolic agents can indicate the molecular mechanisms of action. For example it is well known that cells arrested in G_1 phase can be *via* inhibition of CDK4 and/or CDK6 (Malumbres and Barbacid, 2009). S phase arrest can be caused by inhibition of Cyclin A and Cyclin E through the activation of p21 (*via* p53 in the presence of DNA damage) and p27 (induced by Transforming Growth Factor of β (TGF-β)) (Malumbres and Barbacid, 2009). Arrest of cells in G_2/M phase can be caused by inactivation of cyclin B1 with Cdc2 kinase activity through p53-mediated induction of p21 (Malumbres and Barbacid, 2009).

Here, we demonstrated that the majority of polyphenols investigated induce G_0/G_1 arrest, suggesting that they may inhibit CDK4 and/or -6, however this requires confirmation. Hur, *et.al* (2004) showed that JURKAT cells and T lymphocytes stimulated with rosmarinic acid induce p56*lck* (Lck) protein kinase-dependant apoptosis, through the mitochondrial pathway (Hur *et al*, 2004). P56lck is a lymphoid-specific protein tyrosine kinase and is usually expressed on T lymphocytes (Hur *et al*, 2004). This may explain why the lymphoid cell lines were more sensitive than myeloid cell lines. In addition, recent investigations showed that polyphenols such as the flavonoids (apigenin and quercetin) can act as a p56*lck* (Lck) protein kinase inhibitors (Hur *et al*, 2004; Fassihi and Sabet, 2008). As p56lck is an essential regulator of the cell cycle; modulation of this kinase could lead to the G_0/G_1 arrest. However, further investigation is essential to determine the molecular mechanisms of each polyphenol. It is well established that tumour suppressor gene p53 has a role in the regulation of the cell cycle, as well as in the initiation of apoptosis. However the majority of our cell lines were either null or mutated for p53, with the exception of MOLT-3 which express wild type p53 (An *et al*, 2000; Durland and Reisman, 2002; Geley *et al*, 1997; Cai *et al*, 2001). MOLT-3 cells however, display PTEN mutations, which results in constitutive activity of AKT (Medyouf *et al*, 2010).

p53 induces Bax, which leads to activation of the intrinsic apoptotic pathway. AKT promotes pro-apoptotic Bad to be sequestered. Therefore a lack of p53 or PTEN both lead to an insensitivity to apoptosis with respect to the intrinsic pathway (Deininger *et al*, 2000). This suggests that the p53 status does not influence the effect of polyphenol treatment in this study.

To determine whether the effects of these polyphenols *in vitro* are relevant to their clinical use, it is essential also to consider their bioavailability and whether these treatment concentrations are achievable in plasma. It has been suggested that physiological concentrations of plasma metabolites will not exceed 10 μM (D'Archivio *et al*, 2010; Hollman *et a*l, 1997; Manach *et al*, 2004). Our study has shown that quercetin, emodin and *cis*- stilbene induced significant affects at low doses (between 2 to 10 μM) following 24 h of treatment in most of leukaemia cell lines. The data available on bioavailability of polyphenols however is still limited, but there is evidence that quercetin obtained from plant products can result in micromolar concentrations in blood plasma (Hollman *et a*l, 1997; Mendoza *et al*, 2011), supporting the idea that *in vivo* effects may be possible, through diet. However, quercetin has a reported plasma half-life of 11–28 h; with a 50-100 mg dose causing a plasma concentration of 0.75–1.5 μM in plasma (D'Archivio *et al*, 2010; Hollman *et a*l, 1997; Manach *et al*, 2004; Mendoza *et al*, 2011). This is further complicated as abundant dietary polyphenols do not necessarily have the best bioavailability profile (D'Archivio *et al*, 2010; Manach *et al*, 2004) and they are extensively metabolized by intestinal and hepatic enzymes and microflora (D'Archivio *et al*, 2010; Manach *et al*, 2004). The absorption of polyphenols depends primarily on their chemical structure, and molecular size as well as the degree of glycosylation, esterification, and polymerization with other polyphenols (D'Archivio *et al*, 2010; Manach *et al*, 2004; Mendoza *et al*, 2011; Manach *et al*, 2004; Pérez-Jiménez *et al*, 2011).

2.5 Conclusion

In conclusion, we have shown that the effectiveness of polyphenols varied depending on the leukaemia cell lineage (lymphoid vs. myeloid) and in some cases within the cell lines from the same lineage. We have shown that myeloid cell lines (K562 and KG-1a) were particularly resistant even to the most active polyphenols. This suggests that the molecular mechanism of action of the polyphenols may vary in each cell line and this requires further investigation. Furthermore, we have demonstrated that polyphenols with similar molecular structures such as emodin and aloe-emodin, and even *cis*- and *trans*-stilbene do not have the same effect on leukaemia cells. These findings suggest that polyphenols have anti-tumour activity against leukaemia cells with differential effects. The observed differential sensitivity between leukaemia and normal cells suggests that polyphenols have potential in treatment of leukaemia. The most potent polyphenols are emodin, quercetin, and *cis*-stilbene; these polyphenols may have potential in treating leukaemia.

Chapter 3

Modulation of Chemotherapeutic Activity by Polyphenols.

3.1 Introduction

Chemotherapy is the major treatment for leukaemia, which is targeted according to specific diagnosis, genetic abnormalities identified, the type of leukaemia, age and overall health of the patient (Pokhare, 2012; Cancer Research UK, 2014; Leukaemia & lymphoma Research, 2014). Chemotherapeutic agents are classified according to the chemical structure and mechanisms of action (Sak, 2012; Mohan *et al*, 2013). The major classes of chemotherapeutic agents are: (1) Topoisomerase inhibitor agents such as etoposide (ETP) (Section 1.7.1.1) (Hande, 1998; Baldwin *et al,* 2005; Thakur, 2011) and doxorubicin (DOX) (Section 1.7.1.2) (Thorn et al, 2011; Tacar *et al,* 2013); (2) Alkylating agents such as cyclophosphamide (CYCLO) (Section 1.7.2.1) (Shanafelt *et al,* 2007; Huttunen *et al,* 2011), chlorambucil (CLB) (Section 1.7.2.2) (Rai *et al,* 2000) and cisplatin (CSP) (Section 1.7.2.3) (Siddik, 2003; Florea and Büsselberg, 2011; Praveen et al, 2013); (3) Anti-metabolite agents such as methotrexate (MTX) (Section 1.7.3.1) (Kaye, 1998; Sorbello and Brtino, 2001; Rossi, 2013), 6-mercaptopurine (6-MP) (Section 1.7.3.2) (Sahasranaman *et al,* 2008), and 5-fluorouracil (5-FLU); and (4) Tyrosine kinase inhibitor agents such as imatinib (IM) (Section 1.7.5.1) (Dolly and Apostolia, 2009). These chemotherapeutic agents are further sub-divided due to their effect on the cell cycle (Lamson and Brgnall, 1999; Wu, 2006). They can be classed as: (1) cell cycle-specific drugs (e.g. MTX, 6-MP, 5-FLU are S-phase specific, whilst ETP is G_2/M-phase specific) or (2) non-cell cycle-specific drugs (e.g. CYCLO, CLB, CSP, DOX, and IM all of which can arrest cell cycle at any phase) (Section 1.7) (Lamson and Brignall, 1999; Wu, 2006). Each chemotherapy drug has a unique target and mechanism of action (Section 1.7), although they all interfere with biochemical process controlling cellular replication and ultimately lead to arrest of cell cycle and induction of apoptosis (Sak, 2012;Wu, 2006).

All these agents are currently used clinically and in most cases providing temporary prolongation of patient's life and relief from symptoms, although some can result in complete remission (Gerber, 2008; Ramos, 2008; Mohan *et al*, 2013). However, they are expensive and many are themselves mutagenic, carcinogenic or teratogenic (Gerber, 2008; Ramos, 2008; Mohan *et al*, 2013). Many are cytotoxic to normal cells especially liable cells: haematopoietic, keratinocytes and intestinal epithelial cells (Gerber, 2008; Ramos, 2008; Mohan *et al*, 2013). These chemotherapeutic agents induce considerable side effects including: nausea; vomiting; fatigue; diarrhoea; alopecia; skin rashes and myelosuppression; which can be so severe, that patients withdraw themselves from treatment, which can result in development of drug resistance and remission (Gerber, 2008; Mohan *et al*, 2013).

Drug resistance is considered as a major issue for cancer treatments and can occur through multiple pathways (Ferreira et al, 2006; Mohan *et al*, 2013). Some chemotherapies can alter

membrane transportation and expression of multidrug resistance (MDR1) gene (Ferreira et al, 2006; Mohan *et al*, 2013). These genes mediated by P-glycoprotein (P-gp), which can increase the glutathione (GSH) levels resulting in increased drug efflux and reduced intracellular drug accumulation and loss of therapeutic efficacy (Ferreira *et al*, 2006; Mohan *et al*, 2013). Drug resistance is found commonly in ALL patients when treated with etoposide or doxorubicin, as well as in CML patients when treated with imatinib (Ferreira et al, 2006; Mohan *et al*, 2013). Alternatively chemotherapeutic agents can also activate NF-kB transcription factor gene, which leads to up regulation of pro-survival factors such as Bcl-2 and Bcl-xl (Mohan *et al*, 2013) or induce mutations in cell cycle proteins such as p53 leading to increased cell cycle and resistance to cell death (Florea and Büsselberg, 2011; Mohan *et al*, 2013). Increased DNA repair in cells via activation of nucleotide excision repair (NER) mechanisms has also been shown to lead to drug resistance (Mohan *et al*, 2013).

In order to overcome some of these problems with chemotherapy treatments, combination treatments are under investigation. Where, multiple anti-cancer agents are used, which target different anti-cancer mechanisms. These agents have been used in combination at a reduced treatment concentration to avoid side effects. The use of combination treatments can enhance the efficacy of standard chemotherapy agents, and decrease development of drug resistance, toxicity and side effects (Florea and Büsselberg, 2011; Sandeep *et al,* 2012; Mohan *et al*, 2013). For example: cyclophosphamide has been used successfully in combination with doxorubicin or rituximab in the treatment of lymphoblastic leukaemia and non-Hodgkin lymphoma (Sandeep *et al,* 2012; Mohan *et al*, 2013). Demonstrating the combination treatment was more effective than each treatment alone; there was also reduced drug resistance, and fewer side effects (Sandeep *et al,* 2012; Mohan *et al*, 2013). It is for this reason, that it is important to develop new combination treatments that could improve the response to chemotherapy, clinical outcome and patient survival rates.

Previous studies have shown that polyphenols possess a broad range of biological properties (Ramos, 2008; Mohan *et al*, 2013). They can inhibit cell proliferation; induce apoptosis; cause cell cycle arrest and inhibit several protein kinases (e.g. PKC and tyrosine kinases). They have also been shown to have anti-oxidant, anti-inflammatory, anti-viral and anti-cancer activities (Han *et al*, 2007; Dai *et al*, 2013). Due to these properties, polyphenols have been under investigation as potential chemotherapeutic agents (Han *et al*, 2007; Dai *et al*, 2013; Mohan *et al,* 2013). Work to date has shown that polyphenols have potential in the treatment of leukaemia, with quercetin (QUE), apigenin (AP), emodin (EMO), rhein (RH) and cis-stilbene (CIS) shown to induce apoptosis and arrest cell cycle particularly in lymphoid leukaemia cells lines; with limited effects seen in normal hematopoietic cells (Mahbub *et al,* 2013; Chapter 2). These agents have also been shown to interact with the actions of chemotherapeutic agents,

quercetin potentiated the induction of apoptosis by cisplatin and doxorubicin in human laryngeal carcinoma (Hep-2) cells (Kuhar et al, 2007), human cervical cancer (HeLa) cells (Zhang *et al,* 2008) and human breast cancer (MCF7) cells (Staedler *et al,* 2011). Combination treatments were found to activate caspase 9 and caspase 3, and reduce anti-apoptotic proteins Bcl-2 and Bcl-x (Kuhar et al, 2007; Zhang *et al,* 2008; Staedler *et al,* 2011). In addition, quercetin when used in combined with cisplatin or doxorubicin depleted levels of glutathione (GSH) and inhibited the expression of nuclear translocation and multidrug resistance protein (MDR), leading to the reduction of drug resistance and increase the efficacy of therapy (Kuhar et al, 2007; Zhang *et al,* 2008; Staedler *et al,* 2011; Mohan *et al*, 2013). Also, it showed that quercetin, emodin and resveratrol can inhibit the activation of NFkB, which can aid chemotherapy agents by the modification of drug resistance pathways in cancer cells (Mohan *et al*, 2013).

There are however two contradictory studies, showing that polyphenols can block the activity of standard chemotherapeutic agents (C˘ipak *et al*, 2003; Kim *et al,* 2009). C˘ipak *et al,* 2003 reported that apigenin, galangin and chrysin inhibited the activity of cisplatin and doxorubicin, producing an antagonistic effect on cytotoxicity and induced of apoptosis in murine leukaemia L1210 cells (C˘ipak *et al*, 2003). Similarly, Kim *et al,* 2009 reported that rutin hydrate, quercetin dehydrate, hydrocaffeic acid, gallic acid and tannic acid antagonised the induction of apoptosis caused by bortezomib in multiple myeloma cell lines (MC/CAR, RPMI8226 and U266) (Kim *et al,* 2009). It was suggested that the structure of the polyphenols was a key factor responsible for their antagonistic effect on bortezomib (Kim *et al,* 2009).

These findings suggested that polyphenols can differentially modulate the efficacy of some chemotherapeutics agents. Still there is no clear study on combination effects of polyphenols and a variety of standard chemotherapy agents used in the treatment of leukaemia. Thus, it is essential to determine the possible impacts of polyphenols on chemotherapeutic agents. Here, chemotherapeutic agents were selected to represent the different classes of chemotherapies, and were used to identify any synergistic or indeed inhibitory actions between polyphenols and chemotherapy agents.

3.1.1 Hypothesis

This study tested the hypothesis that: polyphenols have a synergistic effect when combined with standard chemotherapeutic agents in leukaemia cell lines.

3.1.2 Aims

The major aims of this study were to examine the effects of the most active polyphenols selected from Chapter 2 and chemotherapeutic agents alone or in combination on: (1) ATP

levels using CellTiter-Glo® luminescent cell viability assay; (2) apoptosis using the NucView caspase 3 activity assay and flow cytometry; and morphological assessment with Hoechst 33342/PI stained cells and (3) progression of the cell cycle using PI staining and flow cytometry in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1 and KG1a). Additionally this study aimed to determine the nature of interactions of the polyphenols with the standard chemotherapeutic agents.

3.2 Methods and Materials

3.2.1 Experimental Design

Four human leukaemia cell lines were used for this study: two lymphoid leukaemia cell lines (JURKAT (peripheral blood T cell leukaemia) and CCRF-CEM (acute lymphoblastic leukaemia)) (Section 1.3.1) which were previously shown to have high sensitivity to polyphenols treatment (Chapter 2); and two myeloid leukaemia cell lines (THP-1 (acute monocytic leukaemia) and KG-1a (acute myelogenous leukaemia)) (Section 1.3.2) that were the most resistant cell lines to polyphenols treatment (Chapter 2). All cell lines were tested regularly for mycoplasma contamination using the MycoAlert TM mycoplasma detection kit (Lonza) (Section 2.2.2.1) and were all tested negative throughout the study. Cells were cultured as previously described (Chapter 2; Section 2.2.2) and incubated at 37°C with 5% CO_2 in a humidified incubator.

Polyphenols which were the most effective at reducing cell ATP levels and induction of apoptosis were selected to determine interaction with chemotherapeutic agents (Chapter 2). These included: quercetin (QUE), apigenin (AP), emodin (EMO), rhein (RH) and *cis*-stilbene (CIS) (Sigma), which were prepared as described in Chapter 2 and Section 2.2.1.

Nine standard chemotherapy agents were selected for this polyphenol combination study: cyclophosphamide (CYCLO), chlorambucil (CLB), 5-fluorouracil (5-FLU), doxorubicin (DOX), imatinib (IM), cisplatin (CSP), etoposide, (ETP), methotrexate (MTX) and mercaptopurine (6-MP). These are commonly used for leukaemia treatment. Each selected chemotherapy agent was chosen as each had a different chemical structure, target and mechanism of action (Chapter 1; Section 1.7).

3.2.2 Treatment Regimes

Chemotherapeutic agents: cyclophosphamide (CYCLO), chlorambucil (CLB), 5-fluorouracil (5-FLU), doxorubicin (DOX) and imatinib (IM) were dissolved in water. Cisplatin (CSP) and etoposide, (ETP) were dissolved in 1:1 v/v water: ethanol; and finally methotrexate (MTX) and mercaptopurine (6-MP) were dissolved in 0.1M of NaOH alkylating solution. All

chemotherapy drugs were supplied by Sigma. A stock solution of 25mM was prepared with 10% (v/v) ethanol (Sigma) in serum free media (Invitrogen) to generate treatment concentrations of 0.005, 0.01, 0.4, 2, 10, 50 μM.

For the combination study, a non-significant dose (NSD) and a lowest significant dose (LSD) which reduced ATP levels and induced apoptosis were determined for each standard chemotherapy agent and each polyphenol, following 24 h of treatment.

- **The NSD was defined as:** the treatment dose that did not cause a significant reduction in ATP levels or induction of apoptosis when compared to the vehicle control.
- **The LSD was defined as:** the lowest treatment dose that caused a significant reduction in ATP levels or induction of apoptosis that was significantly greater than that of the vehicle control.

All p-values were set at ≤ 0.05 (Table 3.1; 3.2; 3.3 and 3.4). The NSD and LSD for each polyphenols were determined from the data presented in Chapter 2 and are summarised in Table 3.1 for cell proliferation analysis and Table 3.2 for apoptosis analysis.

Initially the NSD and LSD was determined for each standard chemotherapy agents alone on ATP levels and apoptosis in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1 and KG-1a) (Table 3.3 and 3.4).

Once the NSD and LSD levels were determined for each standard chemotherapy agents, these doses were then used in combination with the NSD and LSD for the polyphenols (Table 3.1 and 3.2) on cells proliferation, apoptosis and cell cycle progression within the four selected leukaemia cell lines.

The Polyphenol Non-Significant Doses (NSD) and Lowest Significant Doses (LSD) (μM) Determined by Reducing the ATP Levels Comparing to the Vehicle Control at 24 h

Polyphenols Classification	Polyphenols	Doses	Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Flavonoids	Quercetin	NSD	0.4	0.4	0.4	0.4
		LSD	2	2	2	2
	Apigenin	NSD	10	2	2	2
		LSD	50	10	10	10
Anthraquinones	Emodin	NSD	0.4	0.4	0.4	0.4
		LSD	2	2	2	2
	Rhein	NSD	10	10	10	10
		LSD	50	50	50	50
Stilbenoid	*Cis*- Stilbene	NSD	0.4	2	0.4	0.4
		LSD	2	10	2	2

Table 3.1: The non-significant doses (NSD) and lowest significant doses (LSD) of selected polyphenols (μM) at which there was non-significant and lowest significant reduction of ATP levels, respectively, compared to the vehicle control at 24 h for two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines (Chapter 2; Figure 2.8). These NSD and LSD were used for combination work with standard chemotherapy agents for analysis of cell proliferation. Statistical significance was set at $p \leq 0.05$.

3.2.3 CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) (Section 2.2.3) was used to measure ATP levels, which are directly proportional to the number of metabolically active cells and hence indicative of cell proliferation. Firstly, this assay was used to examine the effect of eight chemotherapy drugs alone (CYCLO, CLB, CSP, MTX, 6-MP, 5-FLU, DOX, ETP, IM) (Sigma) on the ATP levels of two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1 and KG-1a). The treatment doses used were 0, 0.005, 0.01, 0.4, 2, 10, 50 μMol for 24 h. This wide range of treatment doses were selected so that it would be possible to determine the non-significant doses (NSD) and lowest-significant doses (LSD) that reduction ATP levels, for each chemotherapy agent; in each cell line (Table 3.3). These NSD and LSD (Table 3.3) were then used in subsequent combination work with the NSD and LSD of selected polyphenols (QUE, AP, EMO, RH, CIS) (Sigma) (Table 3.1).

The Polyphenol Non-Significant Doses (NSD) and Lowest Significant Doses (LSD) (μM) Determined by Induction of Capase 3 Activity Comparing to the Vehicle Control at 24 h

Polyphenols Classification	Polyphenols	Doses	Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Flavonoids	Quercetin	NSD	0.4	0.4	2	2
		LSD	2	2	10	10
	Apigenin	NSD	10	2	2	2
		LSD	50	10	10	10
Anthraquinones	Emodin	NSD	0.4	2	0.4	0.4
		LSD	2	10	2	2
	Rhein	NSD	10	10	10	10
		LSD	50	50	50	50
Stilbenoid	*Cis*- Stilbene	NSD	2	10	10	2
		LSD	10	50	50	10

Table 3.2: The non-significant doses (NSD) and lowest significant doses (LSD) of selected polyphenols (μM)) at which there was non-significant and lowest significant induction of apoptosis respectively, compared to the vehicle control at 24 h for two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines (Chapter 2; Figure 2.11). The NSD and LSD were used for combination work with standard chemotherapy agents to analysis the apoptosis. Statistical significance was set at $p \leq 0.05$.

3.2.4 Apoptosis Analysis

3.2.4.1 NucView Caspase 3 Activity Assay by Flow Cytometry

The NucView caspase 3 activity assay and flow cytometry (Section 2.2.4.1) was used to determine the non-significant doses (NSD) and lowest-significant doses (LSD) of each chemotherapy drugs alone (CYCLO, CLB, CSP, MTX, 6-MP, 5-FLU, DOX, ETP, IM) on the induction of caspase 3 activity and early stage apoptosis in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines. The treatment doses used were 0, 0.005, 0.01, 0.4, 2, 10, and 50 μMol for 24 h. These capase 3 NSD and

LSD were then used in subsequent combination studies with the NSD and LSD of selected polyphenols (QUE, AP, EMO, RH, CIS) for 24 h (Table 3.2). From this the LSD for apoptosis determined by NucView caspase 3 activity assay for each polyphenol (Table 3.2) and chemotherapy drugs (Table 3.4) were then used for analysis the nuclear morphological of cells using double staining Hoechst 33342/PI by fluorescence microscopy.

3.2.4.2 Nuclear Morphological Analysis using Double Staining of Hoechst 33342/PI and Fluorescence Microscopy

The effects of the combined chemotherapy agents and polyphenols on apoptosis were further investigated via analysis the nuclear morphological changes of cells using Hoechst 33342 and PI staining and fluorescence microscopy. Using these stains, it is possible to distinguish normal, apoptotic and dead cell populations. Particularly, Hoechst 33342 (a blue fluorescence dye) stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells; whilst PI (a red fluorescence dye) only permeates late apoptotic and dead cells. Hence normal cells appear round and pale blue; apoptotic cells appear bright blue with the characteristic feature of apoptosis and late apoptotic and dead cells appear red. This nuclear morphological analysis was used to confirm the results of caspase 3 activity assay.

The two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines were seeded in 12 well plates 0.5 x 10^6 cells per ml and treated for 24 h with each chemotherapy drug (CYCLO, CLB, CSP, MTX, 6-MP, 5-FLU, DOX, ETP, IM) and each polyphenol (QUE, AP, EMO, RH, CIS) (Sigma) alone and in combination at their LSD (Table 3.2 and 3.4). An ethanol vehicle controls at 0.1 % (v/v) was also included. Following 24 h of treatments, 100 µl of cells was transferred from each culture well of 12 well plate to labelled 96-well plates (Fisher Scientific). Then, 10 µl of 2 µg/ml Hoescht 33342 dye (Sigma) was added to each well and incubated for 5 min in the dark place. Next 10 µl of 2 µg/ml PI dye (Sigma) was added to each well and was incubated for a further 15 min in the dark. Plates were examined using inverted fluorescence microscope (Olympus). Two hundred cells (live and apoptotic) were counted and the percentage of apoptotic nuclei determined for each sample. Images were captured using Cell-F software, Olympus).

3.2.5 Cell Cycle Analysis using Propidium Iodide (PI) and Flow Cytometry

The effects of the combined standard chemotherapy agents and polyphenols were also examined on the progression of cell cycle using Propidium Iodide (PI) staining and flow cytometric analysis as described previously (Section 2.2.5). Two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines were treated for 24 h with each chemotherapy drugs (CYCLO, CLB, CSP, MTX, 6-MP, 5-FLU,

DOX, ETP, IM) and each polyphenols (QUE, AP, EMO, RH, CIS) (Sigma) alone and in combined at their LSD (Table 3.1 and 3.3).

3.2.6 Statistical Analysis

The median with range were calculated for CellTiter-Glo®, NucView Caspase 3 Activity, Double Staining of Hoechst 33342/PI and Cell Cycle analysis. The Stats Direct software (Stats Direct Ltd, England) was used to test whether data followed a normal distribution using a Shapiro-Wilke test; which was used to determine whether the data was parametric or non-parametric. As the data was non-parametric, a Kruskal-Wallis and Conover-Inman post hoc test was used to determine statistical significance of the data. Results were considered statistically significant when $p \leq 0.05$.

3.2.6.1 Analysis of Effect of Combination Treatments on ATP level and Apoptosis

The effects of the combination treatments were categorised using the following criteria:

- Reduction of ATP levels or induction of apoptosis by a polyphenol = **X**
- Reduction of ATP levels or induction of apoptosis by chemotherapy agent = **Y**
- Reduction of ATP levels or induction of apoptosis by combined polyphenol and chemotherapy agent treatment = **Z**
- **X+Y** were calculated and described as our **Expected Value**

The effect of combination chemotherapy and polyphenols treatments was classified as: **Additive** or **Synergistic** or **Competitive Antagonistic** or **Antagonistic** according to their statistical analysis. The combination effects were classified and defined as follows:

1. **Additive (ADD):** the effect of combination treatments (Z) is equal to the sum of the effect of the two treatments alone. This is an Additive Response if: the combined effect (Z) is significantly greater than the effect of each treatment X and Y alone, as well as, significantly greater than the control, but not significant greater than the Expected value (X + Y).
2. **Synergistic (SYN):** the effect of combination treatments is higher than the sum of the effect of the two treatments alone. A Synergist Response is seen if: the combined effect (Z) is significantly greater than the control, each drug alone (X alone, Y alone) and the Expected Value (X+Y).
3. **Competitive Antagonistic (C-ANTG):** the effect of combination treatments is equal to the effect of one of two treatments. A Competitive Antagonistic Response is seen if: the combined effect (Z) is significantly lower than the Expected Value (X+Y) and has

a similar response to the effect of one treatment alone (X or Y alone) with no significant difference.

4. **Antagonistic (ANTG):** the effect of combination treatments is lower than the sum of the effect of the two treatments and individual treatments. An Antagonistic Response seen if: the combined effect (Z) is significantly lower than the effect of each treatment alone and the Expected Values (X alone, Y alone and X+Y).

3.2.6.2 Analysis of Effect of Combination Treatments on Cell Cycle

The percentage of cells in each phase was analysed with FlowJo software using the Watson pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs was determined firstly in comparison to the vehicle control. Then, the statistical significance of combined drugs was determined in comparison to the vehicle control and individual treatments. The effect of combination treatments on cell cycle was classified either having an Interactive, Non- interactive or Antagonistic effect. These classifications are defined as:

1. **Interactive Effect:** the combination treatments induce a highly significant increase of cell accumulation in any phase of cell cycle, when compared to the vehicle control and effect of the individual treatment alone ($p<0.05$)
2. **Non-Interactive Effect:** the combination treatments induced a significant increase of cell accumulation in any phase of cell cycle when compared to the vehicle control; but this was not significant greater than that seen with one or both of treatments when used alone ($p<0.05$)
3. **Antagonistic Effect:** the combination treatments had no significant differences compared to the vehicle control ($p<0.05$) and also had a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$).

3.3 Results

3.3.1 Effect of Chemotherapy Agents Treatment on ATP levels and Apoptosis in Leukaemia Cell Lines following 24 h treatments

The most effective chemotherapy agents at reducing ATP levels and induction of apoptosis in all leukaemia cell lines were: DOX, ETP, CSP, IM (Figure 3.1 and 3.2). The least effective agents were CYCLO, MTX, 6-MP and 5-FLU (Figure 3.1 and 3.2). The NSD and LSD for each chemotherapy agents in each cell line are shown in Table 3.3 and 3.4. These NSD and LSD which were used in subsequent polyphenols: chemotherapy combination studies (Table 3.1 and 3.2).

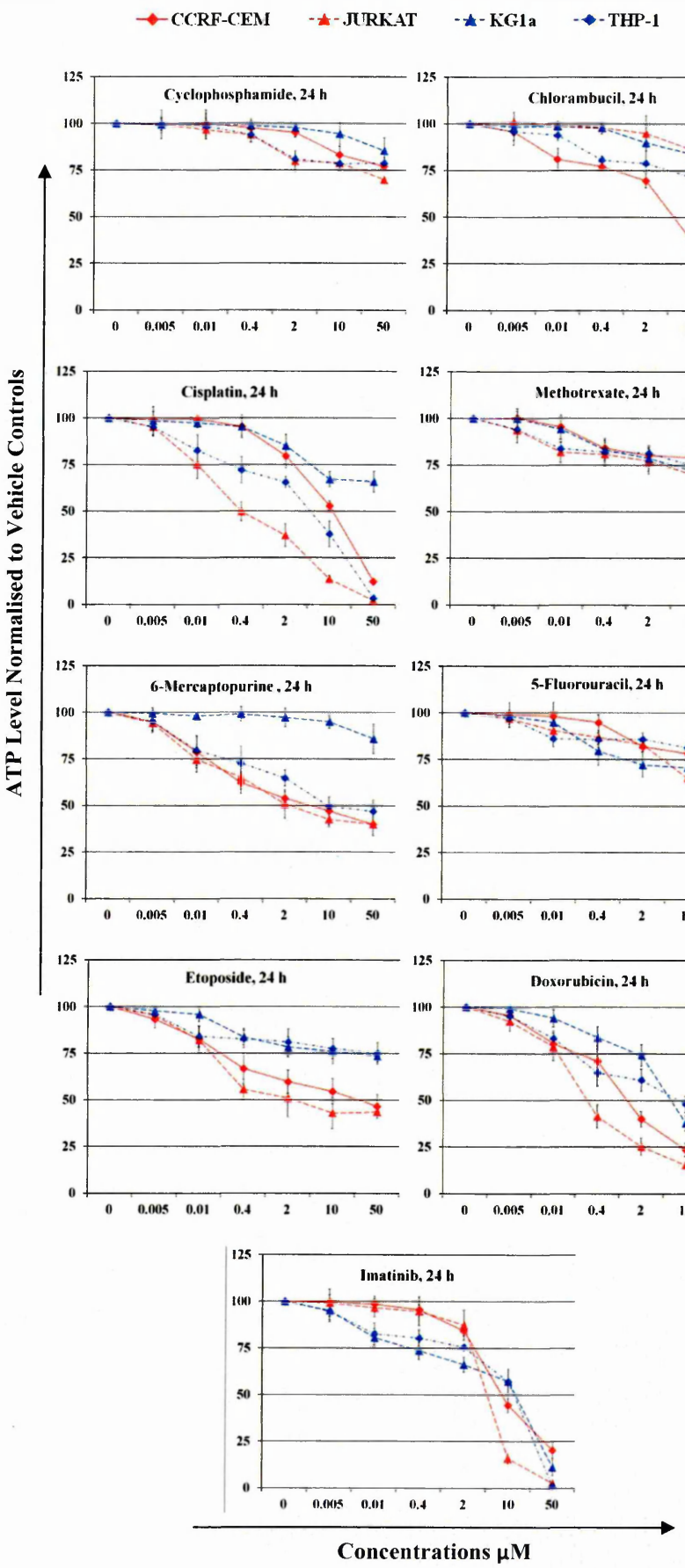

CCRF-CEM
JURKAT
KG1a
THP-1
Cyclophosphamide, 24 h
Chlorambucil, 24 h
Cisplatin, 24 h
Methotrexate, 24 h
6-Mercaptopurine , 24 h
5-Fluorouracil, 24 h
Etoposide, 24 h
Doxorubicin, 24 h
Imatinib, 24 h
ATP Level Normalised to Vehicle Controls
Concentrations µM
0
25
50
75
100
125
0
0.005
0.01
0.4
2
10
50

Figure 3.1: Effect of nine chemotherapy agents on ATP levels in two lymphoid leukaemia (CCRF-CEM and JURKAT; red lines), and two myeloid leukaemia (THP-1 and KG-1a; blue lines) cell lines. Cells were treated with each chemotherapy agent at 0, 0.005, 0.01, 0.4, 2, 10, 50 μM for 24 h. All data was normalised to the vehicle-only control, which was assigned 100% cell viability, and each data was in triplicate. Non-significant dose (NSD) and lowest significant dose (LSD) that reduced ATP levels for each chemotherapy drug in each cell lines are determined (Table 3.3). Statistical significance was set at $p \leq 0.05$ compared to vehicle control.

The Chemotherapy Non-Significant Doses (NSD) and Lowest Significant Doses (LSD) (μM) Determined by Reducing the ATP Level Comparing to the Vehicle Control at 24 h.

Chemotherapy Classification	Chemotherapy Agents	Doses	Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Alkylating Agents	Cyclophosphamide	NSD	0.4	2	0.4	10
		LSD	2	10	2	50
	Chlorambucil	NSD	2	0.005	0.01	2
		LSD	10	0.01	0.4	10
	Cisplatin	NSD	0.005	0.4	0.005	0.4
		LSD	0.01	2	0.01	2
Anti-metabolites Agents	Methotrexate	NSD	0.005	0.01	0.005	0.01
		LSD	0.01	0.4	0.01	0.4
	Mercaptopurine	NSD	0.005	0.005	0.005	10
		LSD	0.01	0.01	0.01	50
	5-Fluorouracil	NSD	0.005	0.4	0.005	0.01
		LSD	0.01	2	0.01	0.4
Topoisomerase Inhibitors Agents	Etoposide	NSD	0.005	0.005	0.005	0.01
		LSD	0.01	0.01	0.01	0.4
	Doxorubicin	NSD	0.005	0.005	0.005	0.01
		LSD	0.01	0.01	0.01	0.4
Tyrosine Kinase Inhibitors Agent	Imatinib	NSD	0.4	0.4	0.005	0.005
		LSD	2	2	0.01	0.01

Table 3.3: The non-significant doses (NSD) and lowest significant doses (LSD) of each chemotherapy agent (μM) at which there was a non-significant and lowest significant reduction of ATP levels compared to the vehicle control at 24 h for two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines. Statistical significance was set at $p \leq 0.05$.

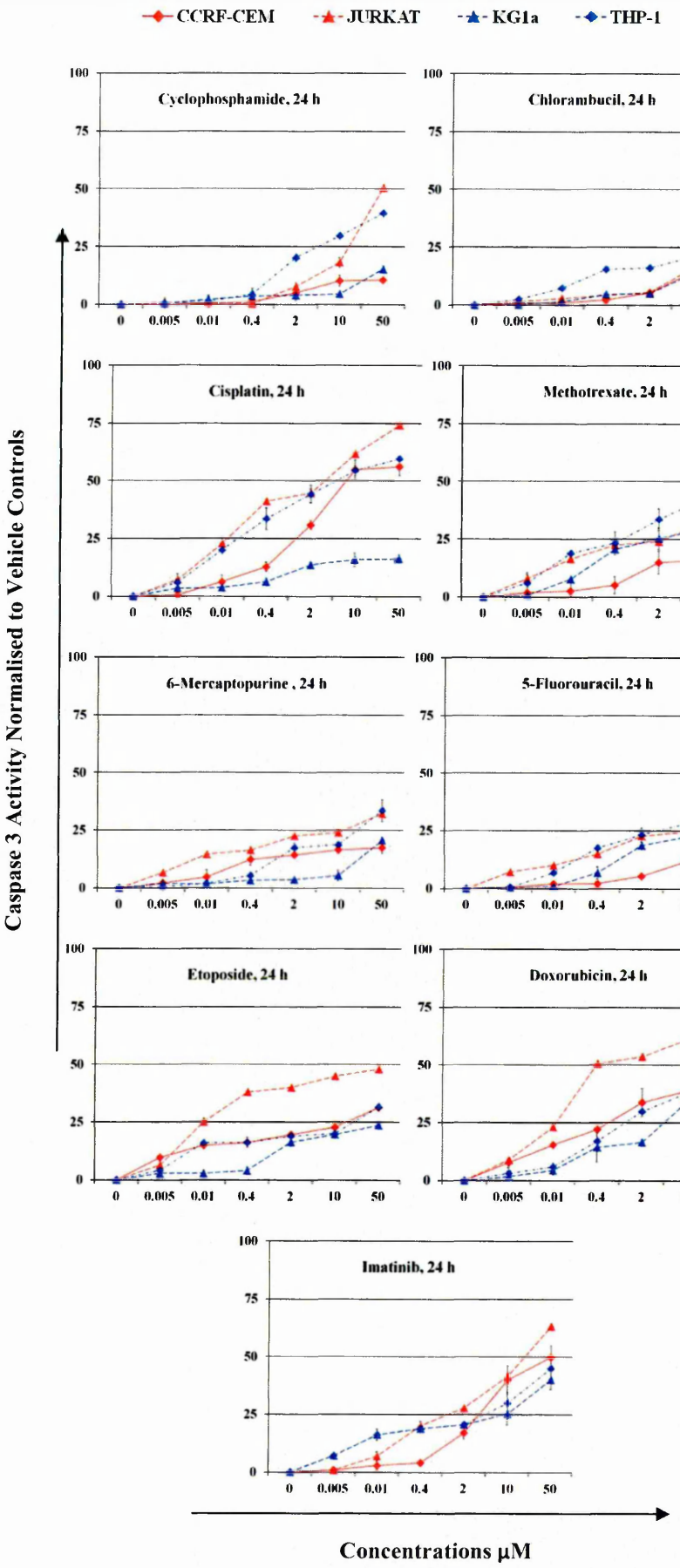

CCRF-CEM
JURKAT
KG1a
THP-1
Cyclophosphamide, 24 h
Chlorambucil, 24 h
Cisplatin, 24 h
Methotrexate, 24 h
6-Mercaptopurine , 24 h
5-Fluorouracil, 24 h
Etoposide, 24 h
Doxorubicin, 24 h
Imatinib, 24 h
Caspase 3 Activity Normalised to Vehicle Controls
Concentrations µM
0
25
50
75
100
0
0.005
0.01
0.4
2
10
50

Figure 3.2: Effect of nine chemotherapy agents on caspase 3 activity in two lymphoid leukaemia (CCRF-CEM and JURKAT; red lines), and two myeloid leukaemia (THP-1 and KG-1a; blue lines) cell lines. Cells were treated with each chemotherapy agent at 0, 0.005, 0.01, 0.4, 2, 10, 50 μM for 24 h. All data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level, and each data was in triplicate. Non-significant dose (NSD) and lowest significant dose (LSD) that induced caspase 3 activity for each chemotherapy in each cell line are determined in (Table 3.4). Statistical significance was set at $p \leq 0.05$ compared to vehicle control.

The Chemotherapy Non-Significant Doses (NSD) and Lowest Significant Doses (LSD) (μM) Determined by Induction of Capase 3 Activity Comparing to the Vehicle Control at 24 h						
Chemotherapy Classification	Chemotherapy Agents	Doses	Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Alkylating Agents	Cyclophosphamide	NSD	2	2	0.4	10
		LSD	10	10	2	50
	Chlorambucil	NSD	2	2	0.01	2
		LSD	10	10	0.4	10
	Cisplatin	NSD	0.005	0.01	0.005	0.4
		LSD	0.01	0.4	0.01	2
Anti-metabolites Agents	Methotrexate	NSD	0.005	0.4	0.005	0.01
		LSD	0.01	2	0.01	0.4
	Mercaptopurine	NSD	0.005	0.01	0.4	10
		LSD	0.01	0.4	2	50
	5-Fluorouracil	NSD	0.005	2	0.01	0.4
		LSD	0.01	10	0.4	2
Topoisomerase Inhibitors Agents	Etoposide	NSD	0.005	0.005	0.005	0.4
		LSD	0.01	0.01	0.01	2
	Doxorubicin	NSD	0.005	0.005	0.01	0.01
		LSD	0.01	0.01	0.4	0.4
Tyrosine Kinase Inhibitors Agent	Imatinib	NSD	0.01	0.4	0.005	0.005
		LSD	0.4	2	0.01	0.01

Table 3.4: The non-significant doses (NSD) and lowest significant doses (LSD) of each chemotherapy agent (μM) at which there was a non-significant and lowest significant increase in apoptosis compared to the vehicle control at 24 h for two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1 and KG-1a) cell lines. Statistical significance was set at $p \leq 0.05$.

3.3.2 Effect of Chemotherapy Agents and Polyphenols Combination Treatment in Leukaemia Cell Lines following 24 h

3.3.2.1 Effects of Topoisomerase Inhibitors Combined with Polyphenols

3.3.2.1.1 Doxorubicin (DOX)

(A) Effects of Doxorubicin (DOX) Combined with Polyphenols on ATP Levels

DOX when used in combination with QUE caused a synergistic reduction of ATP levels ($p<0.05$) in both lymphoid and myeloid cell lines (Figure 3.3 and Table 3.5). Similarly, when DOX was combined with AP there was a synergistic reduction in ATP level in three out of four leukaemia cell lines (CCRF-CEM, JURKAT and THP-1) and an additive effect on KG1a, ($p<0.05$) (Figure 3.3 and Table 3.5). When DOX was used in combination with EMO, RH or CIS it also caused a synergistic reduction in ATP levels, but only in the lymphoid leukaemia cell lines (Figure 3.3 and Table 3.5). In contrast, when DOX was combined with EMO or RH it produced a competitive antagonistic effect, which significantly increasing ATP levels in the myeloid leukaemia cell lines ($p<0.05$) (Figure 3.3 and Table 3.5).

(B) Effects of Doxorubicin (DOX) Combined with Polyphenols on Apoptosis

DOX when used in combination with QUE caused a synergistic effect on induction of apoptosis in all leukaemia cell lines ($P<0.05$) (Figure 3.4, 3.5 and Table 3.5). Similarly, DOX when used in combination with AP synergistically induced apoptosis in three out of four of the leukaemia cell lines (CCRF-CEM, JURKAT and THP-1) (Figure 3.4 and 3.5). Whilst only additive effects were seen in the KG1a myeloid cell lines (Figure 3.4, 3.5 and Table 3.5). DOX when used in combination with EMO, RH or CIS synergistically induced apoptosis in the two lymphoid leukaemia cell lines (Figure 3.4, 3.5 and Table 3.5). However, EMO and RH when used in combination with DOX produced either a competitive antagonistic or antagonistic effect on apoptosis; significantly reducing the levels of caspase 3 activity (Figure 3.4) and percentage of cells undergoing apoptosis (Figure 3.5) in the myeloid leukaemia (THP-1 and KG1a) cell lines ($p<0.05$) (Figure 3.4, 3.5 and Table 3.5). An example of the morphological assessment of apoptosis following DOX and polyphenols (QUE, AP, EMO, RH, CIS) treatment alone and in combination at their LSD for 24 h on THP-1 myeloid leukaemia cell line are showed in Figure 3.6.

(C) Effects of Doxorubicin (DOX) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had a significant increase of cell accumulation in G_2/M phase caused by DOX, in S phase caused by QUE or AP, in G_0/G_1 phase caused by EMO or RH when compared to the vehicle control ($p<0.05$). There was however no significant increase of cell accumulation in any phases of the cell cycle when treated with CIS (Figure 3.7). Treatment of JURKAT cells with DOX in combination with QUE, AP, EMO, RH or CIS

caused an interactive effect on cell cycle; with all combination treatments inducing a significant increase in cell accumulation in the G_2/M phase of the cell cycle, when compared to the vehicle control and individual effects of each treatment ($p<0.05$) (Figure 3.7 and Table 3.5).

CCRF-CEM lymphoid leukaemia cells had a significant increase of cell accumulation in S phase caused by DOX, QUE, EMO and RH, and G_2/M phase caused by AP. However there was no significant increase in accumulation in any phases of cell cycle caused by CIS when compared to the vehicle control ($p<0.05$) (Figure 3.7). Treatment of CCRF-CEM cells with DOX in combination with QUE, AP, EMO, RH or CIS had an interactive effect on cell cycle; with all combination treatments inducing a significant increase of cell accumulation in S phase when compared to the vehicle control and the individual effects if each treatment alone ($p<0.05$) (Figure 3.7 and Table 3.5).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in S and G_2/M phases caused by DOX and AP, in G_2/M phase caused by QUE or CIS; while they had no significant increase of cell accumulation in any phases of cell cycle caused by EMO or RH when compared to the vehicle control ($p<0.05$) (Figure 3.7). Treatment of THP-1 cells with DOX in combination with QUE, AP and CIS had an interactive effect on cell cycle; with the combination treatments inducing either a highly significant increase of cell accumulation in S or G_2/M phases when compared to the effects caused by the vehicle control and individual treatments alone ($p<0.05$) (Figure 3.7 and Table 3.5). However, treatment of THP-1 cells with DOX in combination with EMO or RH had an antagonistic effect on cell cycle, there was no significant differences compared to the vehicle control ($p<0.05$) and but a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.7 and Table 3.5).

KG-1a myeloid leukaemia cells had a significant increase in cells accumulated in S and G2/M phases following treatment with DOX, G_0/G_1 phase caused by AP, while they had no significant increase of cell accumulation in any phases of cell cycle caused by QUE, EMO, RH or CIS when compared to the vehicle control ($p<0.05$) (Figure 3.7). Treatment of KG-1a cells with DOX in combination with QUE, AP and CIS had an interactive effect on cell cycle causing a significant increase of cell accumulation in S and G_2/M phases when compared to the vehicle control; but this was not significant when compared to effect caused by DOX alone ($p<0.05$) (Figure 3.7 and Table 3.5). In contrast, treatment of KG-1a cells with DOX in combination with EMO or RH had an antagonistic effect on cell cycle. These combinations had no significant effect compared to the vehicle control ($p<0.05$) but they did significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.7 and Table 3.5).

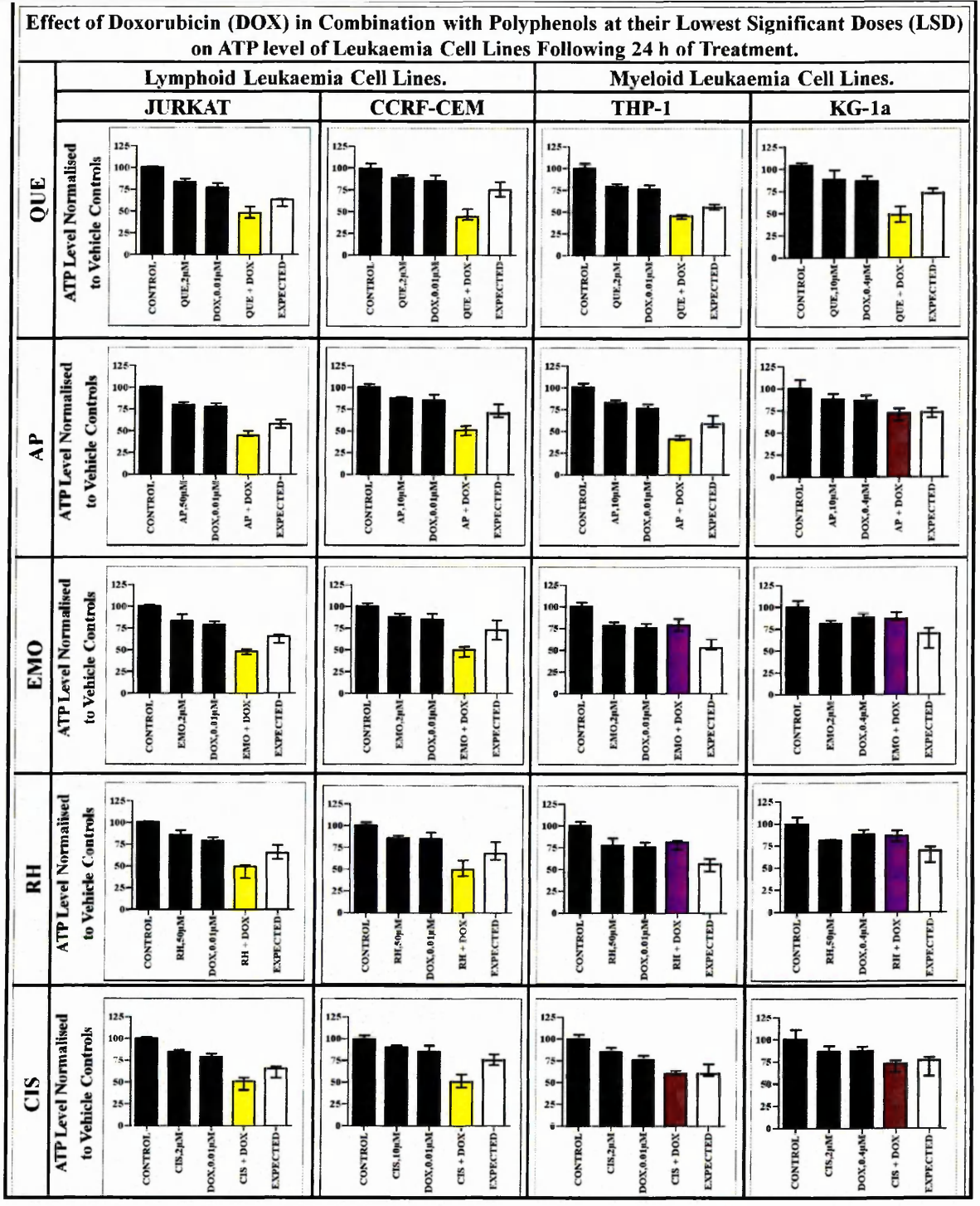


Figure 3.3: The effect of DOX when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with DOX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

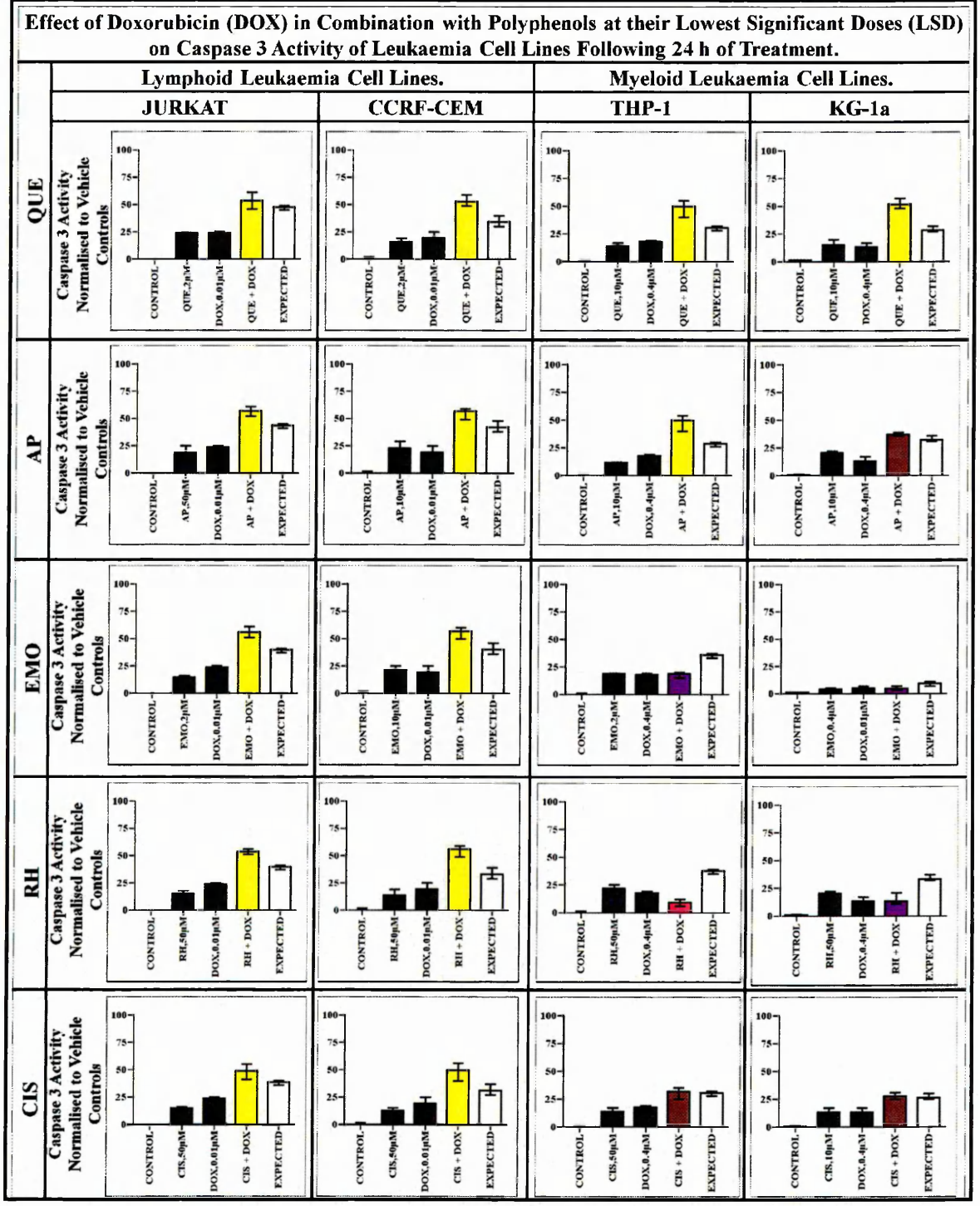


Figure 3.4: The effect of DOX when used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with DOX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

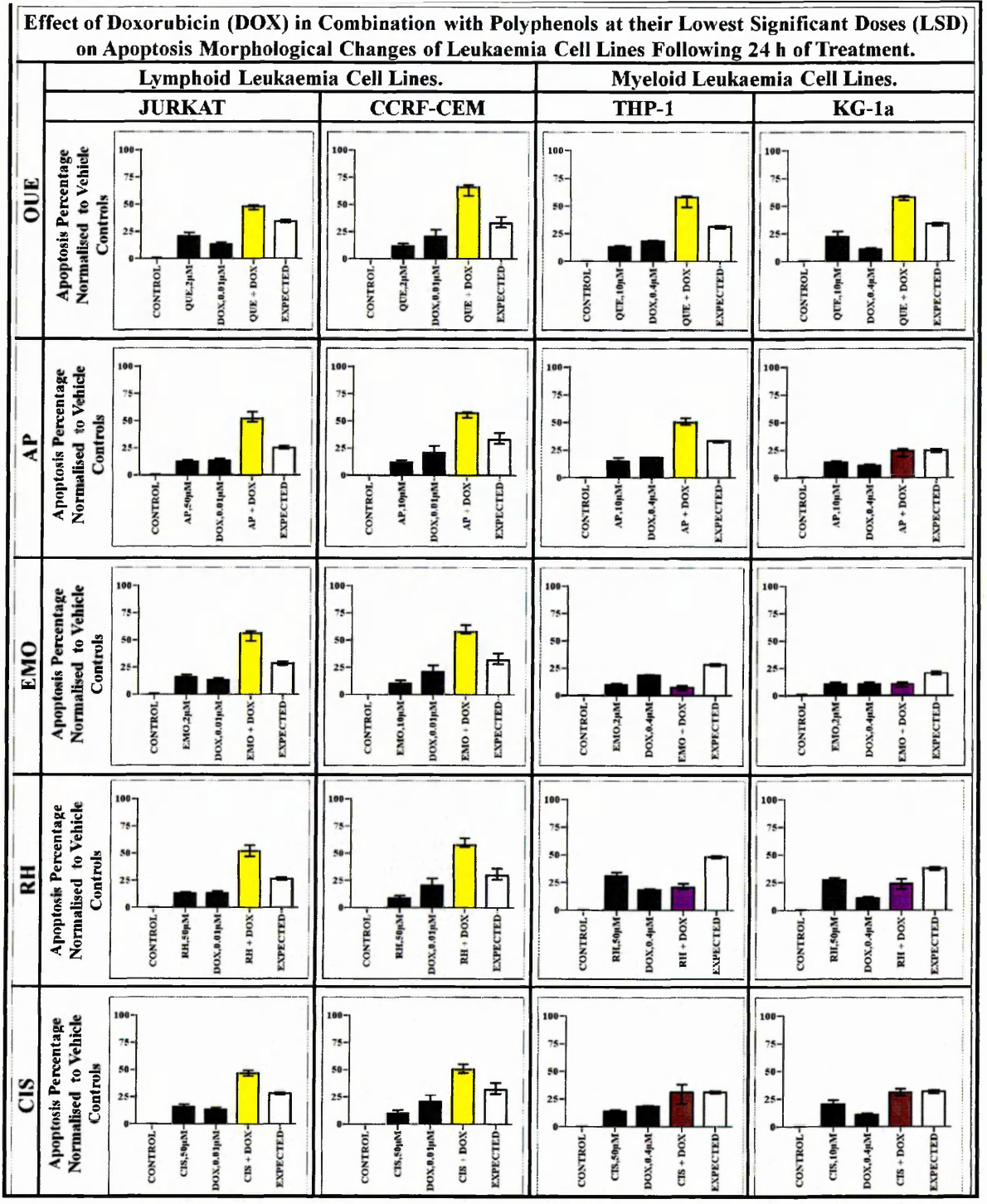


Figure 3.5: The effect of DOX when used in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with DOX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

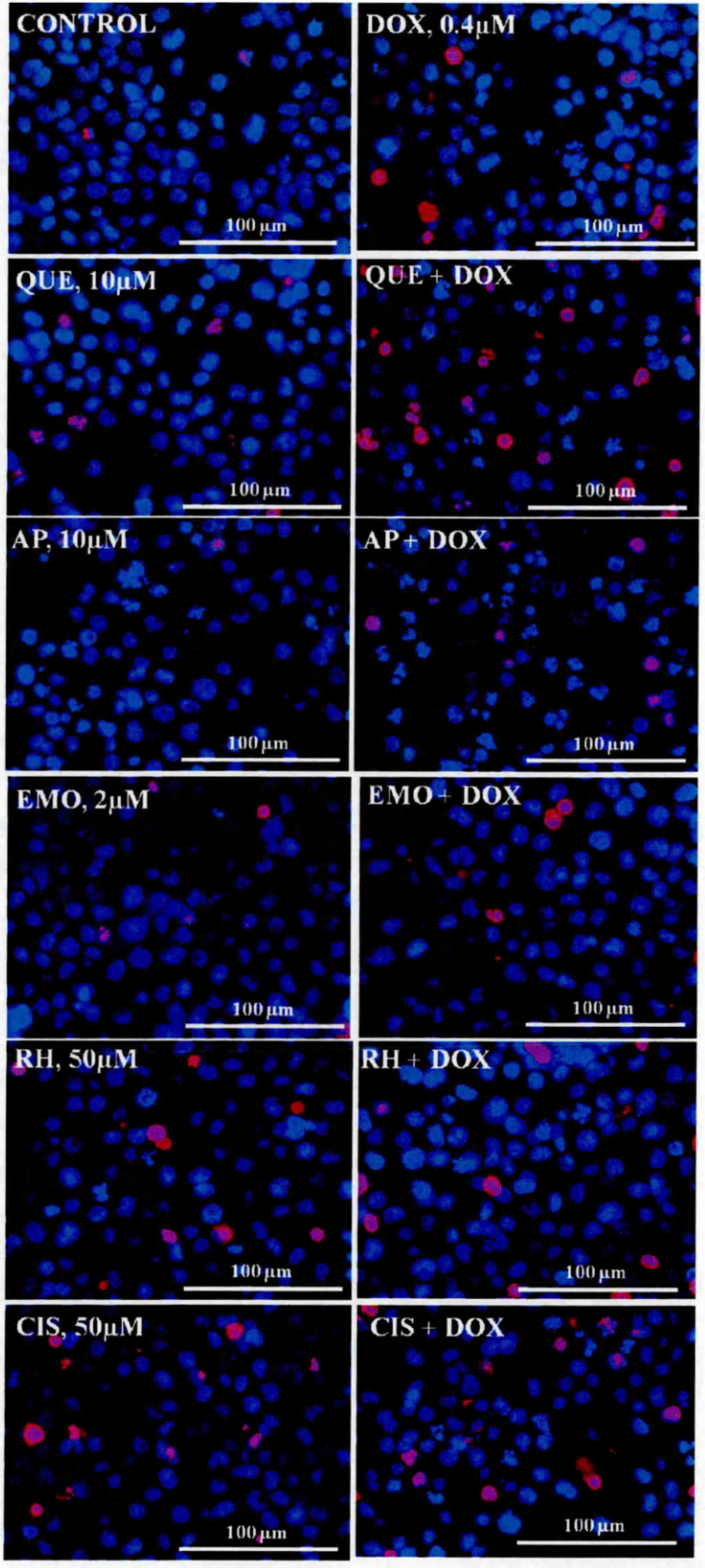
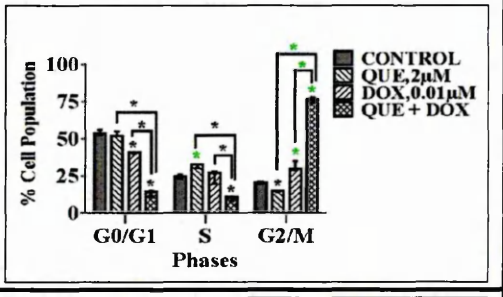
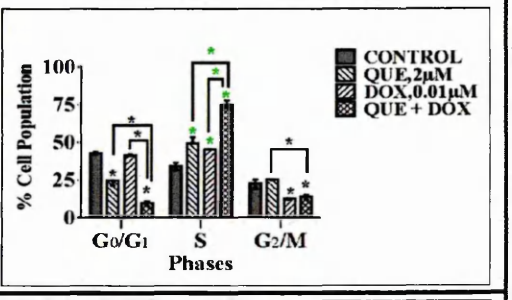
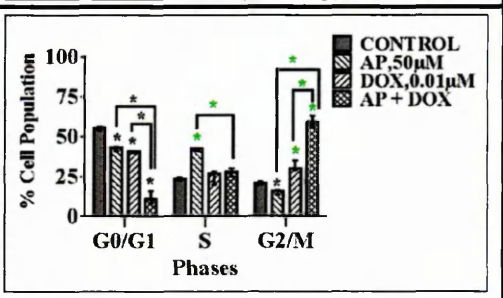
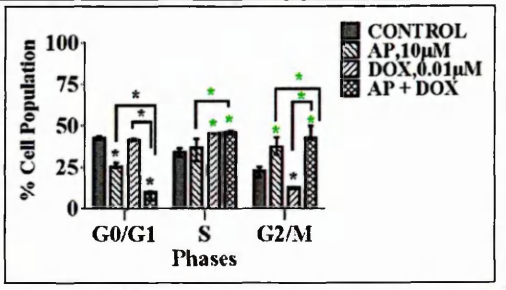
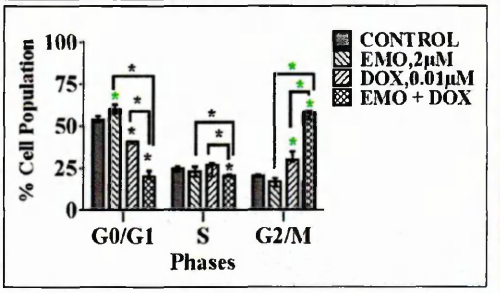
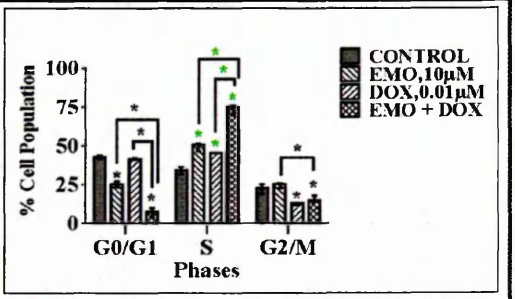
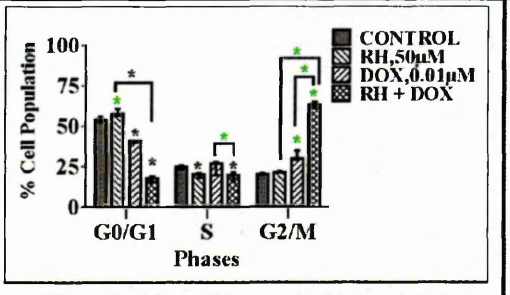
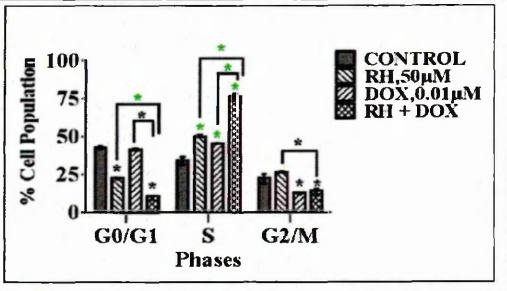
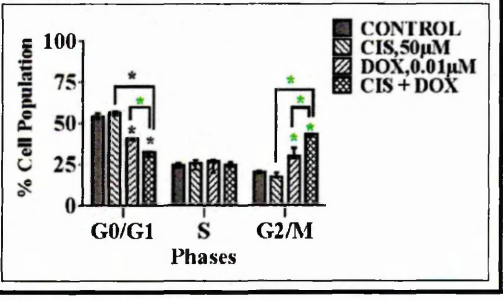
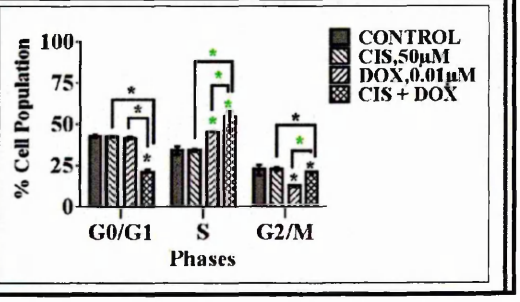


Figure 3.6: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of DOX and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A) Effect of Doxorubicin (DOX) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of Doxorubicin (DOX) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

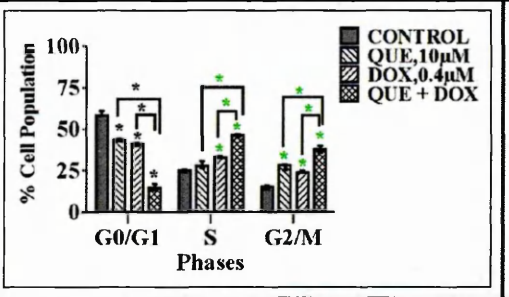
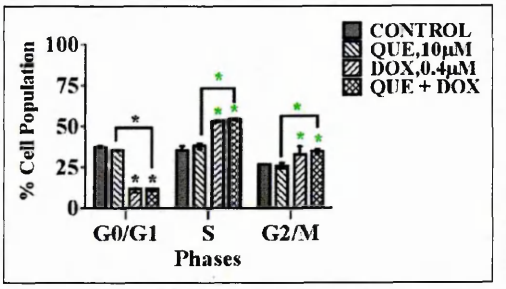
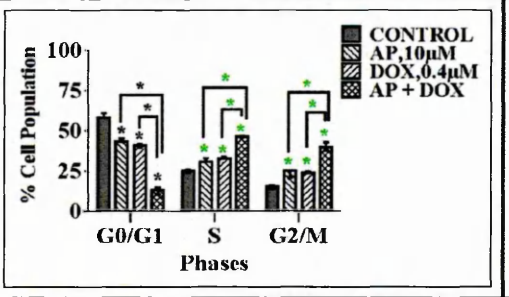
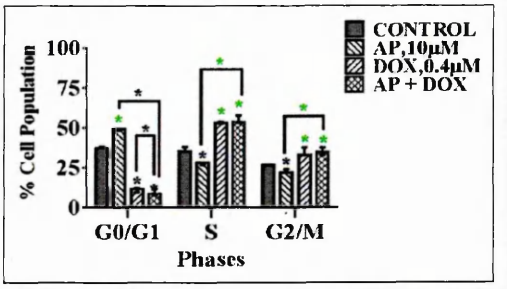
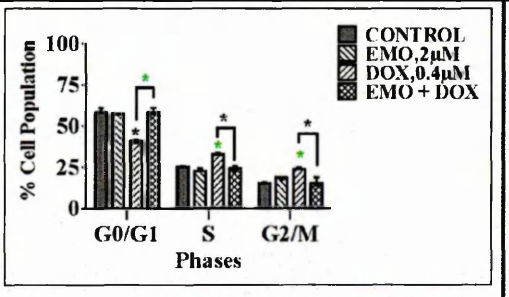
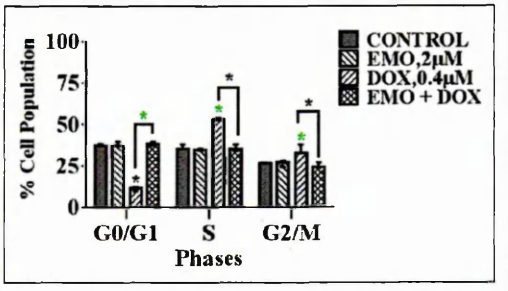
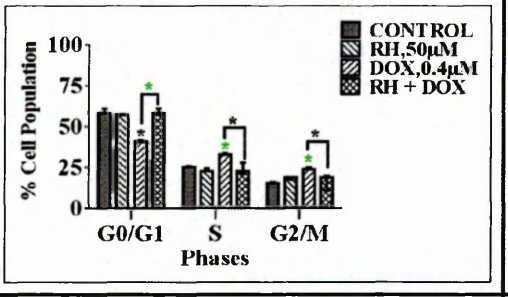
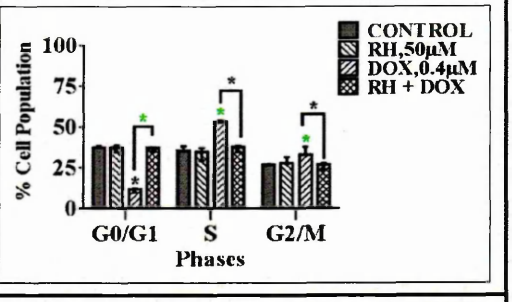
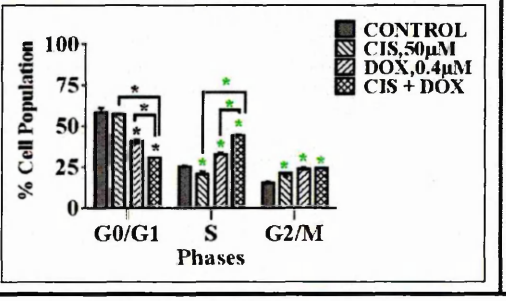
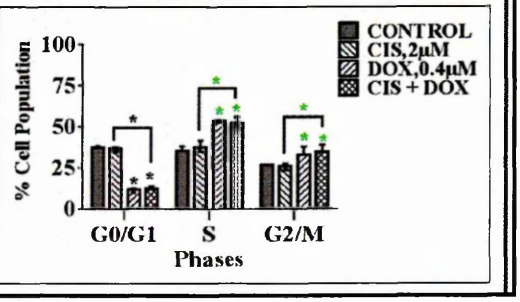
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.7: The effect of DOX when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with DOX and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.5.

Assays	Targets	Combination of Doxorubicin and Polyphenols	The Significant Effects of Doxorubicin (DOX) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	DOX + QUE (NSD)	SYN	SYN	SYN	SYN
		DOX + QUE (LSD)	SYN	SYN	SYN	SYN
Caspase 3 Activity	Apoptosis	DOX + QUE (NSD)	SYN	SYN	SYN	SYN
		DOX + QUE (LSD)	SYN	SYN	SYN	SYN
Hoechst/ PI	Apoptosis	DOX + QUE (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	DOX + QUE (LSD)	Interaction	Interaction	Interaction	Non Interaction
CellTiter-Glow	ATP Level	DOX + AP (NSD)	SYN	SYN	SYN	ADD
		DOX + AP (LSD)	SYN	SYN	SYN	ADD
Caspase 3 Activity	Apoptosis	DOX + AP (NSD)	SYN	SYN	SYN	ADD
		DOX + AP (LSD)	SYN	SYN	SYN	ADD
Hoechst/ PI	Apoptosis	DOX + AP (LSD)	SYN	SYN	SYN	ADD
Cell Cycle/PI	Cell Cycle	DOX + AP (LSD)	Interaction	Interaction	Interaction	Non Interaction
CellTiter-Glow	ATP Level	DOX + EMO (NSD)	SYN	SYN	C-ANTG	C-ANTG
		DOX + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	DOX + EMO (NSD)	SYN	SYN	C-ANTG	C-ANTG
		DOX + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	DOX + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	DOX + EMO (LS)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glow	ATP Level	DOX + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		DOX + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	DOX + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		DOX + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	DOX + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	DOX + RH (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glow	ATP Level	DOX + CIS (NSD)	SYN	SYN	ADD	ADD
		DOX + CIS (LSD)	SYN	SYN	ADD	ADD
Caspase 3 Activity	Apoptosis	DOX + CIS (NSD)	SYN	SYN	ADD	ADD
		DOX + CIS (LSD)	SYN	SYN	ADD	ADD
Hoechst/ PI	Apoptosis	DOX + CIS (LSD)	SYN	SYN	ADD	ADD
Cell Cycle/PI	Cell Cycle	DOX + CIS (LSD)	Interaction	Interaction	Interaction	Non Interaction

Table 3.5: A summary of the effects of doxorubicin (DOX) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.1.2 Etoposide (ETP)

(A) Effect of Etoposide (ETP) Combined with Polyphenols on ATP levels

ETP when used in combination with QUE produced a synergistic reduction of ATP levels ($p<0.05$) in both lymphoid and myeloid cell lines (JURKAT, CCRF-CEM, THP-1, KG1a) (Figure 3.9 and Table 3.6). ETP when used in combination with AP produced an additive effects in most leukaemia cell lines ($p<0.05$), the only exception was seen in THP-1 myeloid cell lines in which there as a synergistic reduction in ATP level ($p<0.05$) (Figure 3.9 and Table 3.6). ETP when used in combination with EMO produced a synergistic reduction in ATP level in the lymphoid leukaemia cell lines only (JURKAT and CCRF-CEM) (Figure 3.8 and Table 3.6); and an additive effects in myeloid leukaemia cell lines (THP-1 and KG1a) ($p<0.05$) (Figure 3.8 and Table 3.6). ETP when used in combination with RH or CIS produced synergistic effects significantly reducing ATP levels in the lymphoid leukaemia cell lines only (JURKAT and CCRF-CEM) (Figure 3.8 and Table 3.6). In the myeloid leukaemia (THP-1 and KG1a) cell lines a competitively antagonistic or antagonistic effects was seen with a significantly increase ATP level ($p<0.05$) (Figure 3.8 and Table 3.6).

(B) Effect of Etoposide (ETP) Combined with Polyphenols on Apoptosis

ETP when used in combination with QUE produced a synergistic increase in caspase 3 activity (Figure 3.9) and the percentage of cells undergoing apoptosis (Figure 3.10) in all cell lines (JURKAT, CCRF-CEM, THP-1, KG1a) ($p<0.05$) (Figure 3.9, 3.10and Table 3.6). ETP when used in combination with AP produced an additive effects in most leukaemia cell lines ($p<0.05$), the only exception was seen in THP-1 myeloid cell lines in which there as a synergistic induction on caspase 3 activity and percentage apoptosis ($p<0.05$) (Figure 3.9, 3.10 and Table 3.6). ETP when used in combination with EMO produced a synergistic effects on caspase 3 activity (Figure 3.9) and percentage apoptosis (Figure 3.10) ($p<0.05$) in the lymphoid leukaemia cell lines only (JURKAT and CCRF-CEM) (Figure 3.9, 3.10 and Table 3.6); and an additive effects in myeloid leukaemia cell lines (THP-1 and KG1a) ($p<0.05$) (Figure 3.9, 3.10 and Table 3.6). Similarly ETP when used in combination with RH or CIS produced a synergistic effects on caspase 3 activity (Figure 3.9) and percentage apoptosis (Figure 3.10) ($p<0.05$) in the lymphoid leukaemia cell lines (Figure 3.9, 3.10 and Table 3.6). However, they were produced either a competitively antagonistic or antagonistic effects on capase 3 activity (Figure 3.9) and percentage apoptosis (Figure 3.10) in the myeloid leukaemia (THP-1 and KG1a) cell lines ($p<0.05$) (Figure 3.9, 3.10 and Table 3.6). An example of the morphological assessment of apoptosis for ETP and polyphenols (QUE, AP, EMO, RH and CIS) alone and in combination at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.11.

(C) Effect of Etoposide (ETP) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had a significant increase of cell accumulation in S and G_2/M phases caused by ETP, in S phase caused by QUE and AP, in G_0/G_1 phase caused by EMO and RH; whilst there was no significant increase of cell accumulation in any phases of cell cycle caused by CIS ($p<0.05$) (Figure 3.12). Treatment of JURKAT cells with ETP in combination with QUE, EMO, RH or CIS had an interactive effect on cell cycle; causing a highly significant increase of cell accumulation in G_2/M phase with all these treatment combinations ($p<0.05$) (Figure 3.12 and Table 3.6). The only variation was seen when JURKAT cells were treated with a combination of ETP and AP, which caused an interactive effect and cell accumulation was seen in S phase ($p<0.05$) (Figure 3.12 and Table 3.6).

CCRF-CEM lymphoid leukaemia cells had a significant increase of cell accumulation in S and G_2/M phases caused by ETP, in S phase caused by QUE, EMO or RH, and in G_2/M phase caused by AP; there was however no significant increase of cell accumulation in any phases of cell cycle caused by CIS ($p<0.05$) (Figure 3.12). Treatment of CCRF-CEM cells with ETP in combination with QUE, AP, EMO, RH or CIS had an interactive effect on cell cycle each inducing a highly significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.12 and Table 3.6). In addition, CCRF-CEM cells when treated with ETP in combination with QUE, AP or EMO also caused a significant increase of cell accumulation in G_2/M phase when compared to the vehicle control and polyphenols alone, but no significant increase of cell accumulation in this phase when compared to effect of ETP alone (Figure 3.12 and Table 3.6).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in S phase caused by ETP, in G_2/M phase caused by QUE and by CIS; and in S and G_2/M phases caused by AP. No significant increase of cell accumulation in any phases of cell cycle was caused by EMO or by RH ($p<0.05$) (Figure 3.12). Treatment of THP-1 cells with ETP in combination with QUE and AP had an interactive effect causing a significant increase of cell accumulation in S and G_2/M phases ($p<0.05$) (Figure 3.12 and Table 3.6). The combination of ETP and EMO had a non-interactive effect on cell cycle; there was an increase of cell accumulation in S and G_2/M phases when compared to the vehicle control ($p<0.05$); but this was not significantly greater that that seen by the individual treatments alone (Figure 3.12 and Table 3.6). In contrast, when THP-1 cells were treated with ETP in combination with RH or CIS there was an antagonistic effect on cell cycle, there was a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.12 and Table 3.6).

KG-1a myeloid leukaemia cells had a significant increase of cells in S and G2/M phases caused by ETP, G_0/G_1 phase caused by AP; while there was no significant increase of cell

accumulation in any phases of cell cycle caused by QUE, EMO, RH or CIS ($p<0.05$) (Figure 3.12). Treatment of KG-1a cells with combination of ETP and AP had an interactive effect on cell cycle and induced a highly significant increase of cell accumulation in G_2/M phase ($p<0.05$) (Figure 3.12 and Table 3.6). In contrast, treatment of KG-1a cells with ETP in combination with QUE, EMO or CIS had a non-interactive effect on cell cycle, there were significant increases in cell accumulation in S and G_2/M phases when compared to the vehicle control; but there not when compared to phases arrested by ETP alone ($p<0.05$) (Figure 3.12 and Table 3.6). Furthermore, when KG-1a cells were treated with ETP in combination with RH or CIS there was an antagonistic effect on cell cycle, there were no significant differences compared to the vehicle control ($p<0.05$); but a significant decrease of cell accumulation caused by the individual treatment alone ($p<0.05$) (Figure 3.12 and Table 3.6).

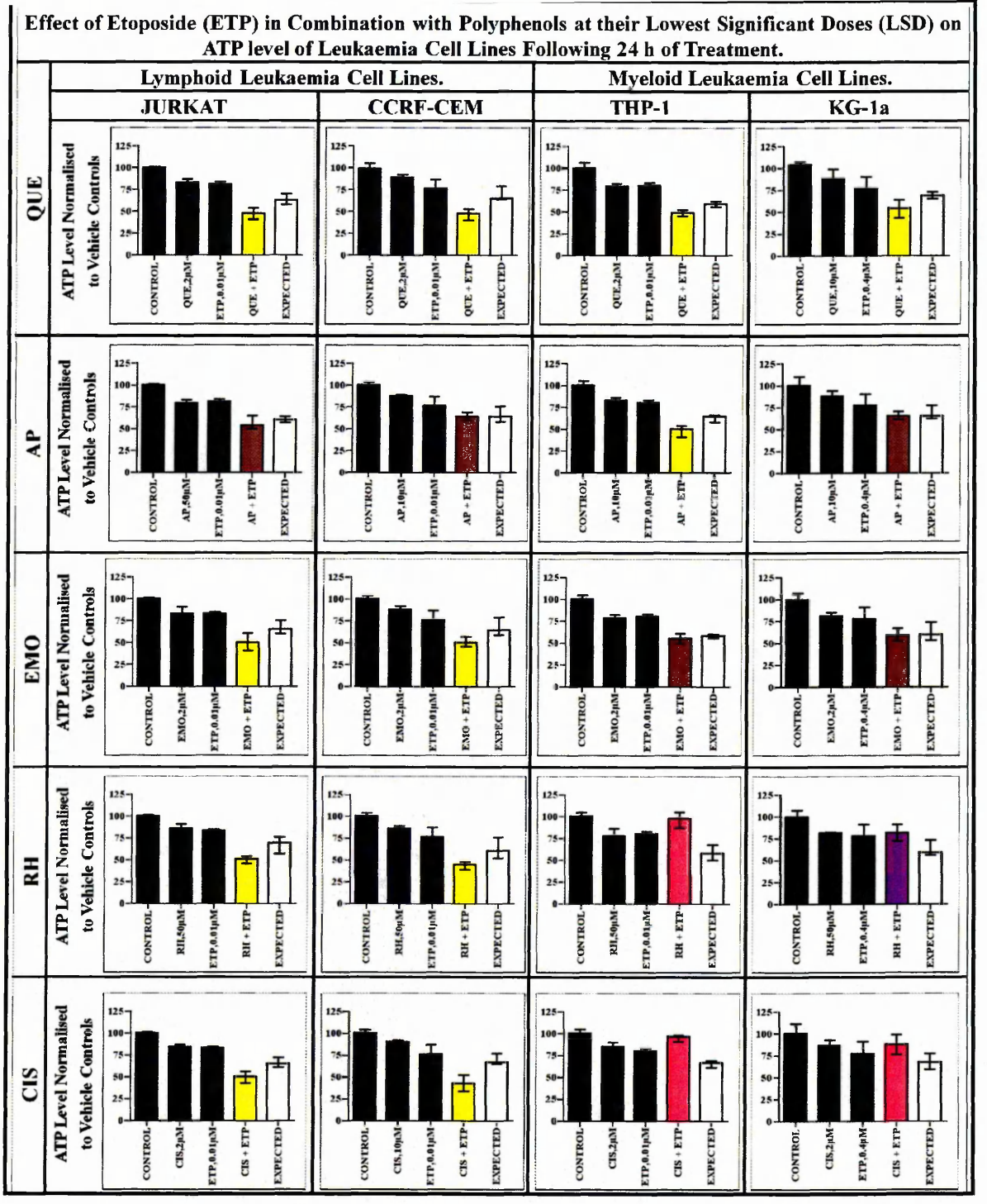


Figure 3.8: The effect of ETP when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

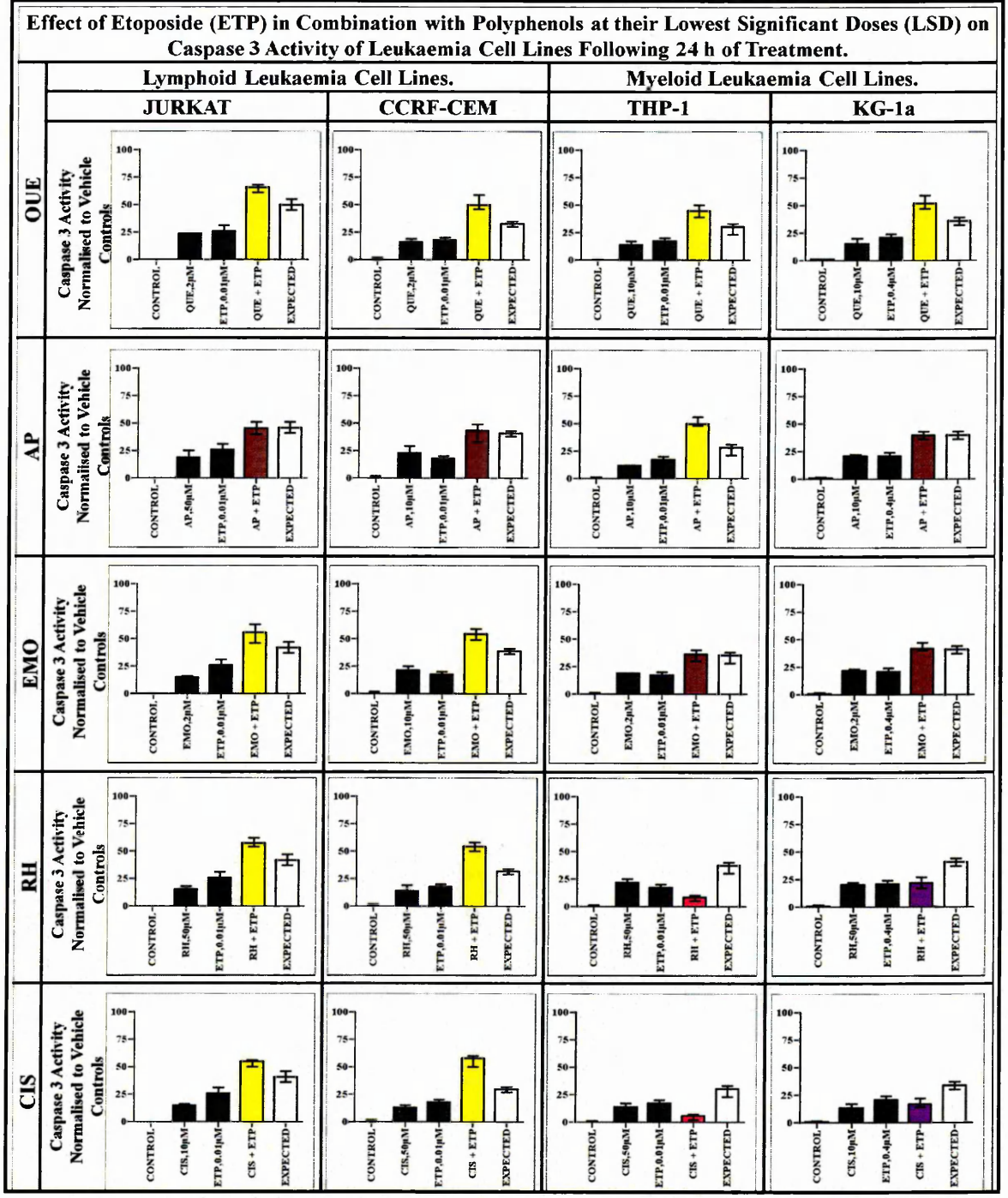


Figure 3.9: The effect of ETP when used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

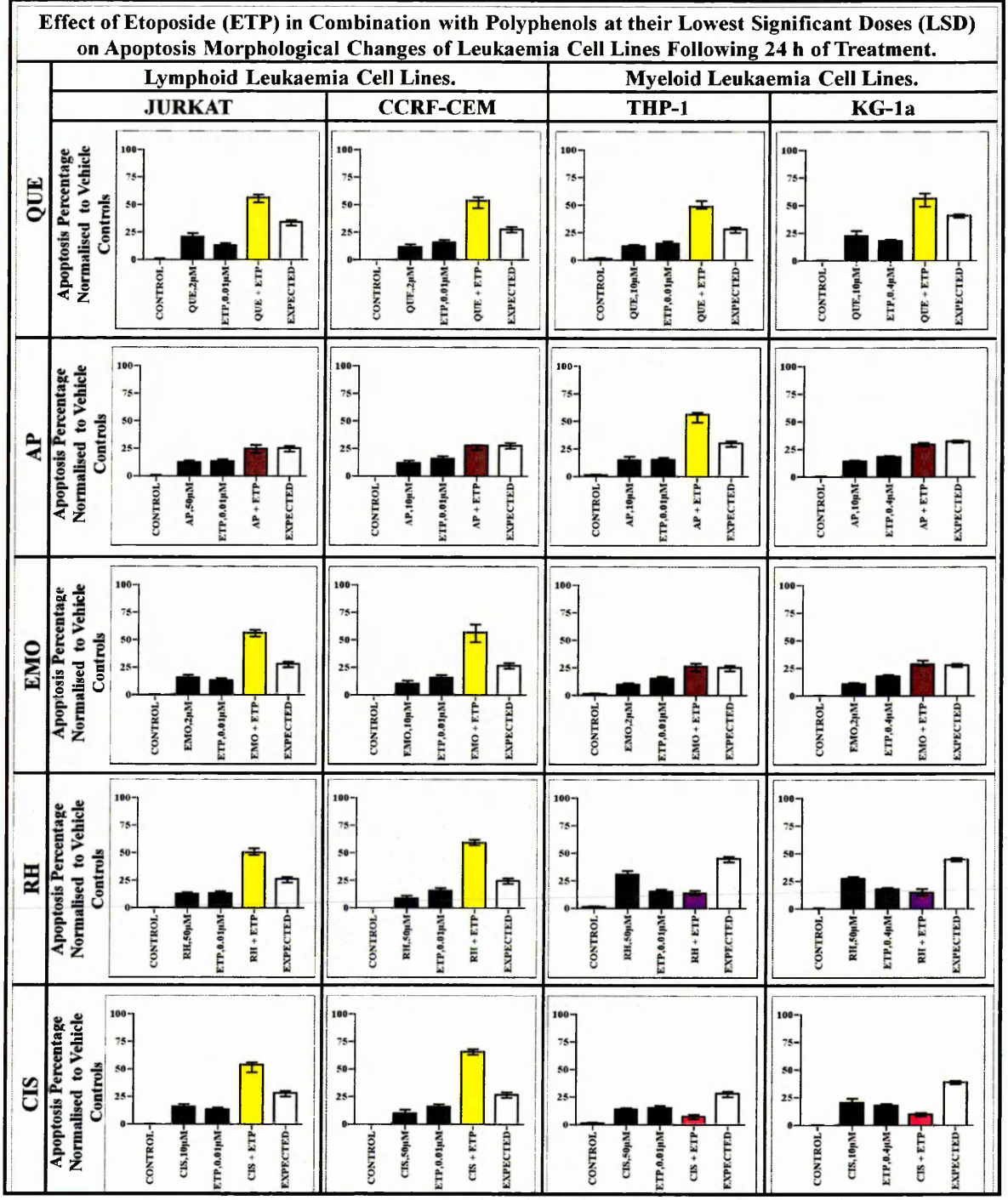


Figure 3.10: The effect of ETP when used combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

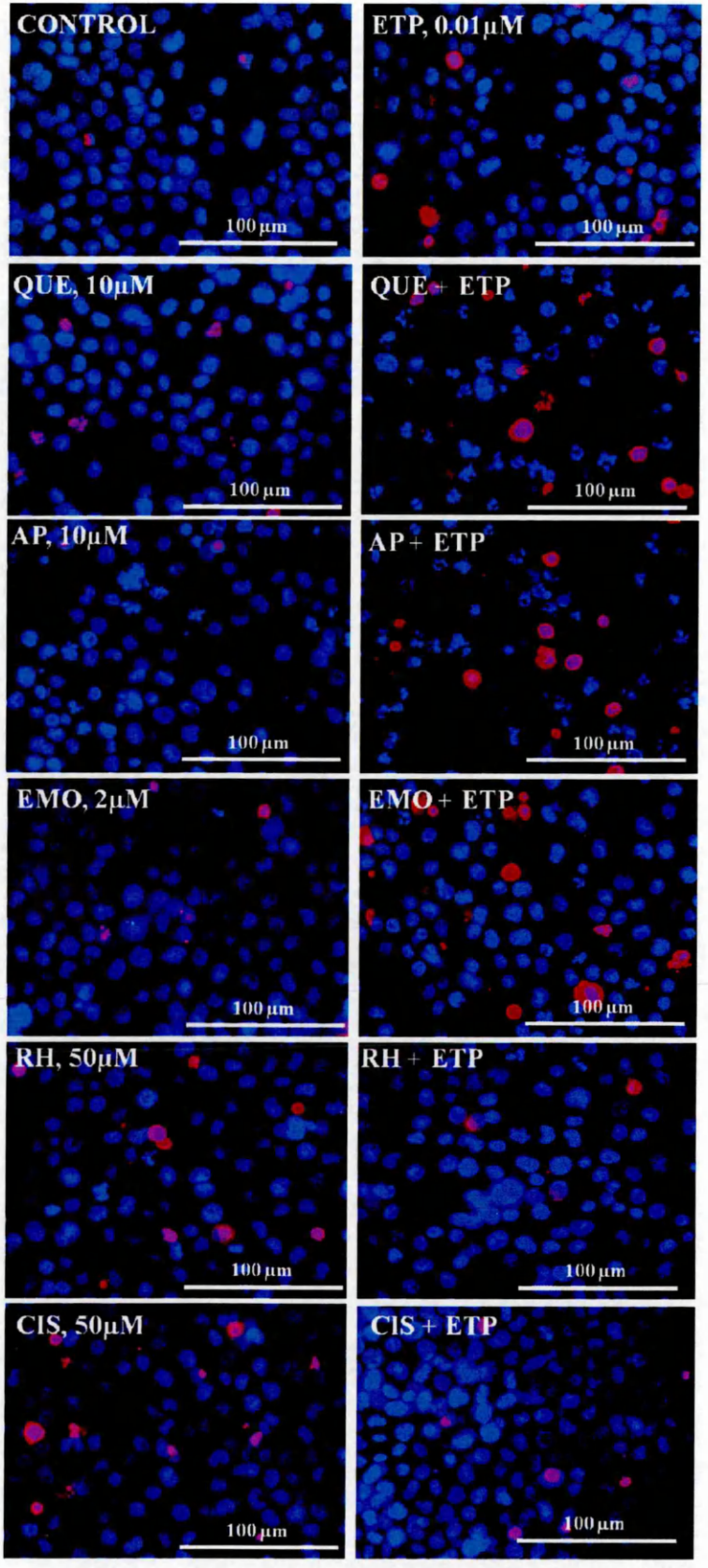
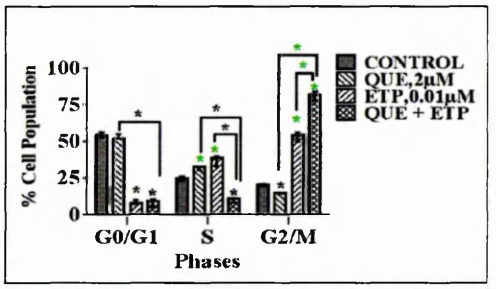
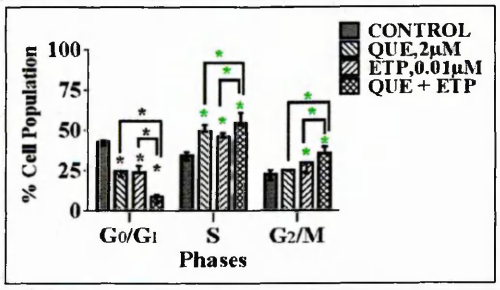
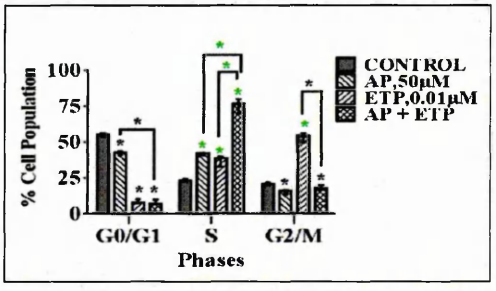
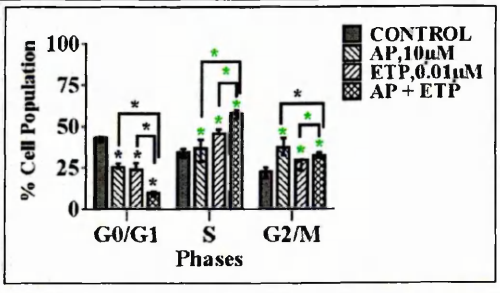
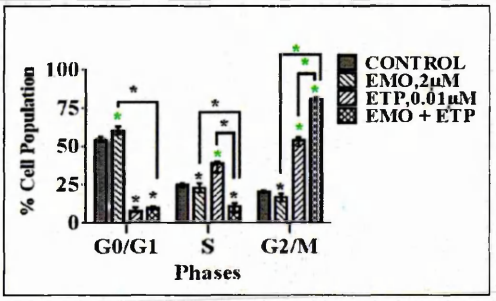
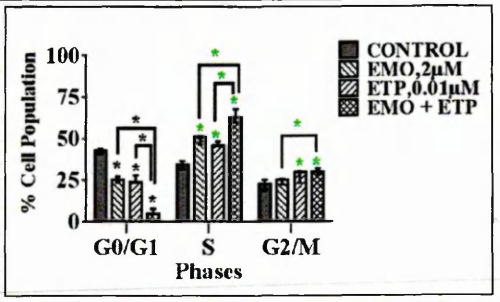
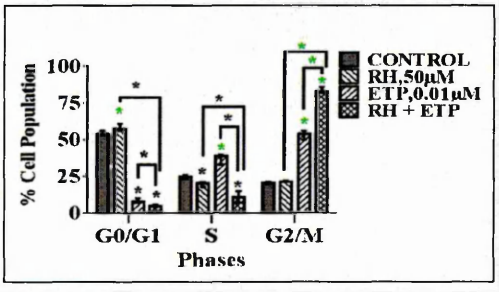
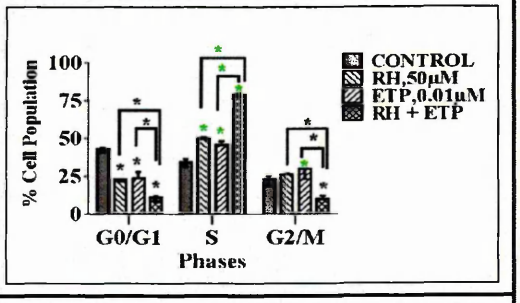
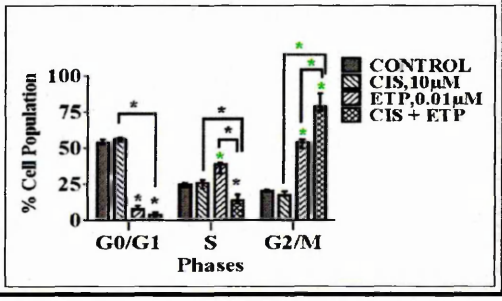
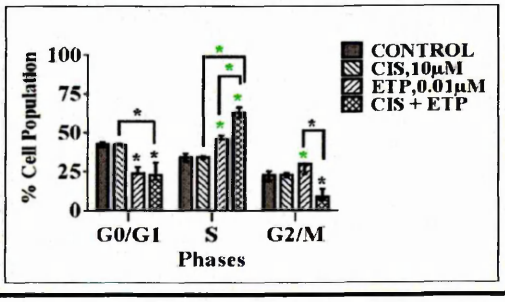


Figure 3.11: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of ETP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A) Effect of Etoposide (ETP) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of Etoposide (ETP) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

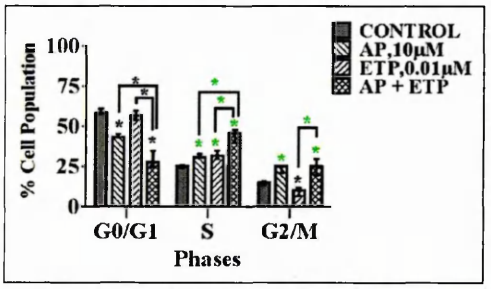
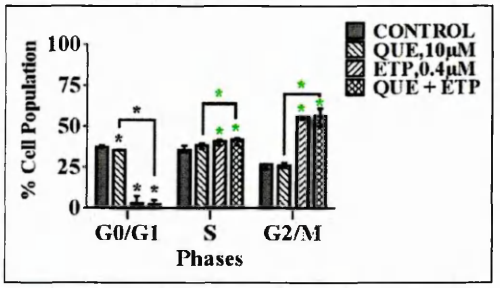
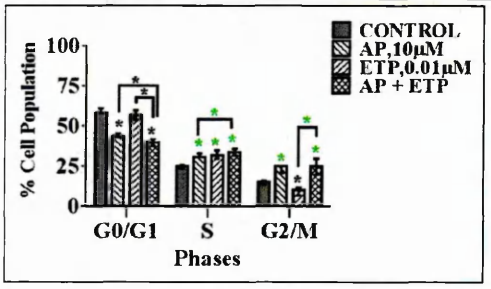
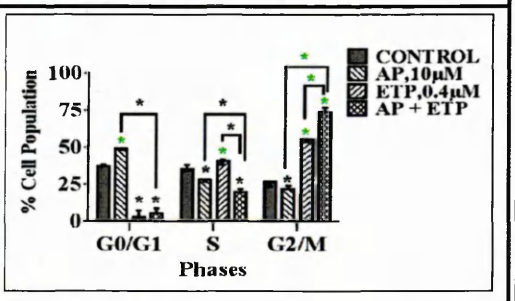
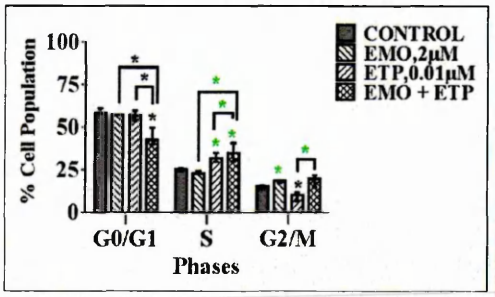
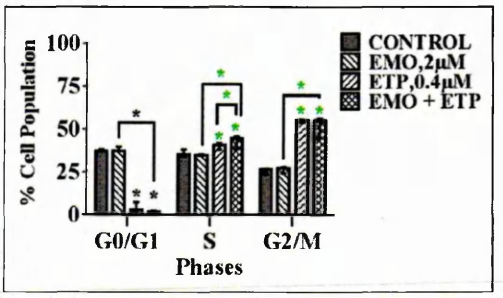
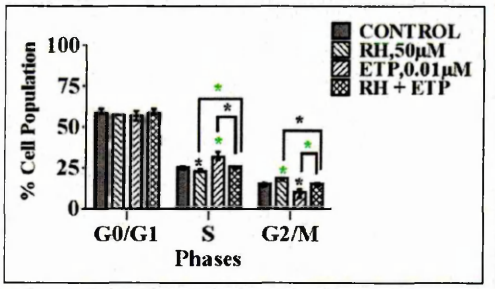
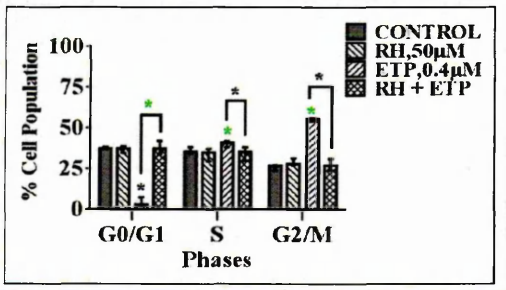
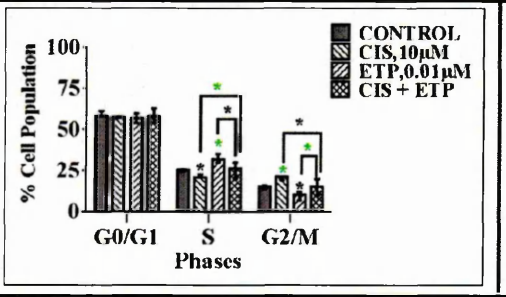
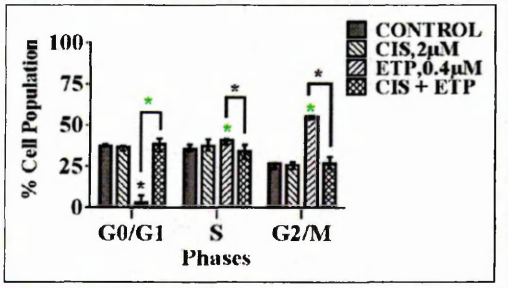
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.12: The effect of ETP when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with ETP and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.6.

Assays	Targets	Combination of Etoposide and Polyphenols	The Significant Effects of Etoposide (ETP) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	ETP + QUE (NSD)	SYN	SYN	SYN	SYN
		ETP + QUE (LSD)	SYN	SYN	SYN	SYN
Caspase 3 Activity	Apoptosis	ETP + QUE (NSD)	SYN	SYN	SYN	SYN
		ETP + QUE (LSD)	SYN	SYN	SYN	SYN
Hoechst/ PI	Apoptosis	ETP + QUE (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	ETP + QUE (LSD)	Interaction	Interaction	Interaction	Non Interaction
CellTiter-Glow	ATP Level	ETP + AP (NSD)	ADD	ADD	SYN	ADD
		ETP + AP (LSD)	ADD	ADD	SYN	ADD
Caspase 3 Activity	Apoptosis	ETP + AP (NSD)	ADD	ADD	SYN	ADD
		ETP + AP (LSD)	ADD	ADD	SYN	ADD
Hoechst/ PI	Apoptosis	ETP + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	ETP + AP (LSD)	Interaction	Interaction	Interaction	Interaction
CellTiter-Glow	ATP Level	ETP + EMO (NSD)	SYN	SYN	ADD	ADD
		ETP + EMO (LSD)	SYN	SYN	ADD	ADD
Caspase 3 Activity	Apoptosis	ETP + EMO (NSD)	SYN	SYN	ADD	ADD
		ETP + EMO (LSD)	SYN	SYN	ADD	ADD
Hoechst/ PI	Apoptosis	ETP + EMO (LSD)	SYN	SYN	ADD	ADD
Cell Cycle/PI	Cell Cycle	ETP + EMO (LSD)	Interaction	Interaction	Non Interaction	Non Interaction
CellTiter-Glow	ATP Level	ETP + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		ETP + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	ETP + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		ETP + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	ETP + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	ETP + RH (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glow	ATP Level	ETP + CIS (NSD)	SYN	SYN	C-ANTG	C-ANTG
		ETP + CIS (LSD)	SYN	SYN	ANTG	ANTG
Caspase 3 Activity	Apoptosis	ETP + CIS (NSD)	SYN	SYN	C-ANTG	C-ANTG
		ETP + CIS (LSD)	SYN	SYN	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	ETP + CIS (LSD)	SYN	SYN	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	ETP + CIS (LSD)	Interaction	Interaction	ANTG	ANTG

Table 3.6: A summary of the effects of etoposide (ETP) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.2 Effects of Alkylating Chemotherapy agents in Combined with Polyphenols

3.3.2.2.1 Cyclophosphamide (CYCLO)

(A) Effect of Cyclophosphamide (CYCLO) Combined with Polyphenols on ATP Levels

CYCLO when used in combination with AP caused a synergistic reduction in ATP levels ($p<0.05$) in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1, KG1a) (Figure 3.13 and Table 3.7). Furthermore, CYCLO when used in combination with QUE, EMO or RH caused a synergistic reduction in ATP levels ($p<0.05$) in lymphoid leukaemia cell lines only (JURKAT, CCRF-CEM) ($p<0.05$) (Figure 3.13 and Table 3.7); whilst they were produced a competitively antagonistic or antagonistic effect and significantly increased ATP levels in the myeloid leukaemia cell lines (THP-1, KG1a) ($p<0.05$) (Figure 3.13 and Table 3.7). In contrast, CYCLO when used in combination with CIS produced a competitive antagonistic or antagonistic effect and significantly increased the ATP levels in all lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.13 and Table 3.7).

(B) Effect of Cyclophosphamide (CYCLO) Combined with Polyphenols on Apoptosis

CYCLO when used in combination with AP produced a synergistic increases in caspase 3 activity (Figure 3.14) and percentage apoptosis (Figure 3.15) ($p<0.05$) in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG1a) (Figure 3.14 and Figure 3.15). In addition, CYCLO when used in combination with QUE, EMO or RH produced a synergistic increases in caspase 3 activity (Figure 3.14) and percentage apoptosis (Figure 3.15) in the lymphoid leukaemia cell lines only (JURKAT and CCRF-CEM) ($p<0.05$); whilst they were produced a competitively antagonistic or antagonistic effects and significantly reduced caspase 3 activity (Figure 3.14) and percentage apoptosis (Figure 3.15) in the myeloid leukaemia cell lines (THP-1 and KG1a) ($p<0.05$) (Figure 3.14, 3.15 and Table 3.7). On the other hand, when CYCLO was used in combination with CIS it produced a competitive antagonistic or antagonistic effects and significantly decreased caspase 3 activity (Figure 3.14) and the level of apoptosis (Figure 3.15); this time in both the lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.14, 3.15 and Table 3.7). An example of the morphological assessment of apoptosis for CYCLO and the polyphenols (QUE, AP, EMO, RH and CIS) alone and in combined at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.16.

(C) Effect of Cyclophosphamide (CYCLO) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had no significant increase of cell accumulation in any phases of cell cycle following CYCLO treatment. However there was a significant increase of cell accumulation in S phase caused by QUE, and AP, in G_0/G_1 phase caused by EMO, in G_0/G_1 phase caused by RH; and there was no significant increase of cell accumulation in any

phases of cell cycle caused by CIS ($p<0.05$) (Figure 3.17). Treatment of JURKAT cells with CYCLO in combination with QUE or AP had an interactive effect on cell cycle causing a highly significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.17 and Table 3.7). In addition, treatment of JURKAT cells with CYCLO in combination with EMO or RH had an interactive effect causing a significant increase of cell accumulation in G_0/G_1 phase ($p<0.05$) (Figure 3.17 and Table 3.7). However, treatment of JURKAT cells with CYCLO in combination with CIS had an antagonistic effect on the cell cycle, however this was not significantly different from that of the vehicle control and individual drugs alone ($p<0.05$) (Figure 3.17 and Table 3.7). CCRF-CEM lymphoid leukaemia cells had a significant increase of cell accumulation in S phase caused by CYCLO, QUE, EMO and RH; in G_2/M phase caused by AP; whilst there was no significant increase of cell accumulation in any phases of cell cycle caused by CIS ($p<0.05$) (Figure 3.17). Treatment of CCRF-CEM cells with CYCLO in combination with QUE, AP, EMO or RH had an interactive effect on cell cycle causing a significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.17 and Table 3.7). However, treatment of CCRF-CEM cells with CYCLO in combination with CIS had an antagonistic effect on cell cycle, this was however not significantly different from that of the vehicle control ($p<0.05$), but was significantly less when compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.17 and Table 3.7).

THP-1 myeloid leukaemia cells had no significant increase of cell accumulation caused by CYCLO, EMO or RH; while they had a significant increase of cell accumulation in G_2/M phase caused by QUE, in S and G_2/M phases caused by AP and in G_2/M phase caused by CIS ($p<0.05$) (Figure 3.17). Treatment of THP-1 cells with CYCLO in combination with AP had an interactive effect on cell cycle causing a highly significant increase of cell accumulation in S and G_2/M phases ($p<0.05$) (Figure 3.17 and Table 3.7). In contrast, treatment of THP-1 cells with CYCLO in combination with QUE, EMO or RH had an antagonistic effect on cell cycle. These combination treatments caused no significant differences compared to the vehicle control ($p<0.05$) but a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.17 and Table 3.7). KG-1a myeloid leukaemia cells had a significant increase of cell accumulation in G_0/G_1 phase caused by CYCLO and AP; while there was no significant increase of cell accumulation in any phases of cell cycle caused by QUE, EMO, RH or CIS ($p<0.05$) (Figure 3.17). Treatment of KG-1a cells with CYCLO in combination with AP showed the only interactive effect on cell cycle, causing a highly significant increase of cell accumulation in G_0/G_1 phase ($p<0.05$) (Figure 3.17 and Table 3.7). Treatment of KG-1a cells with CYCLO in combination with QUE, EMO or RH had an antagonistic effect which was not significantly different from the vehicle control

($p<0.05$) but cell accumulation was significantly less when compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.17 and Table 3.7).

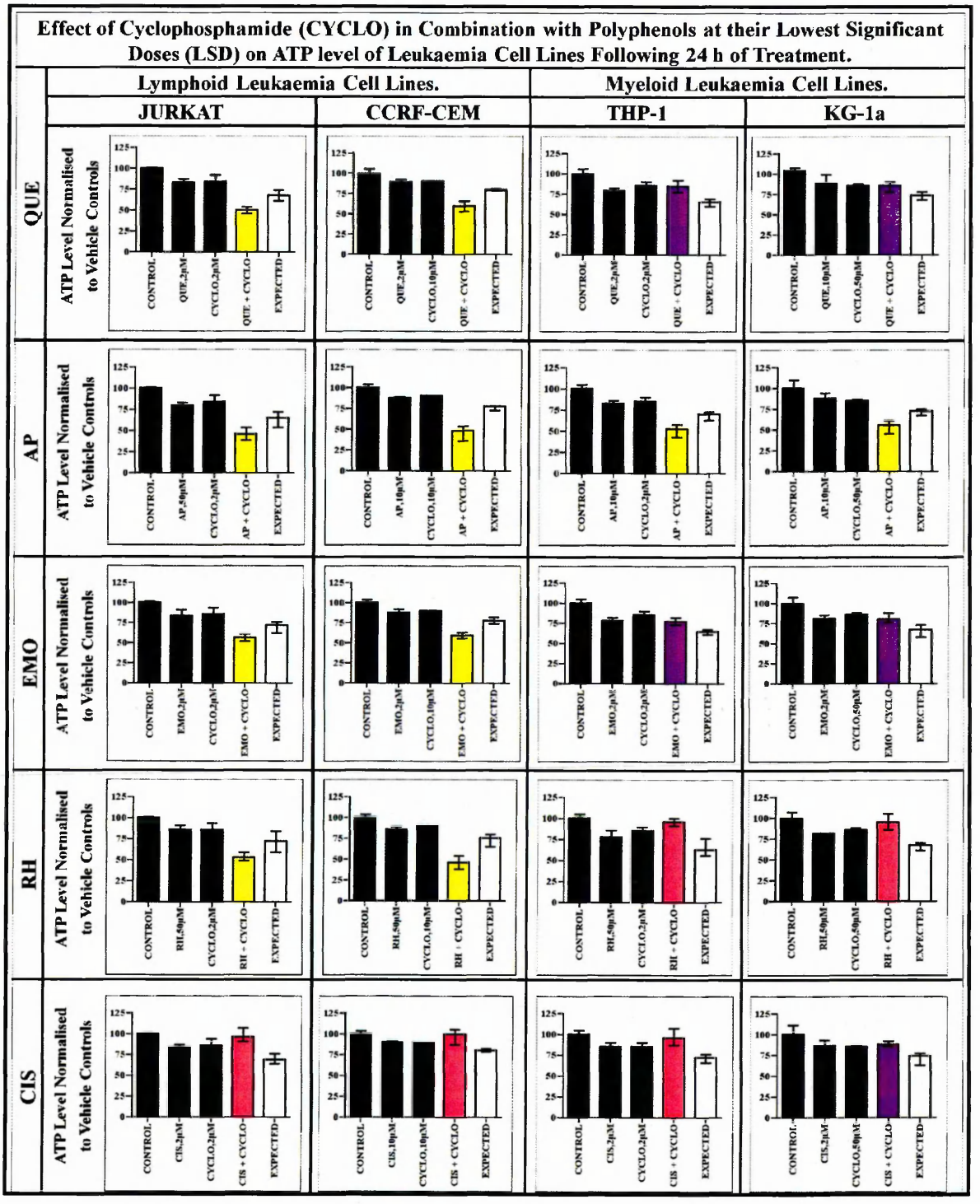


Figure 3.13: The effect of CYCLO when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

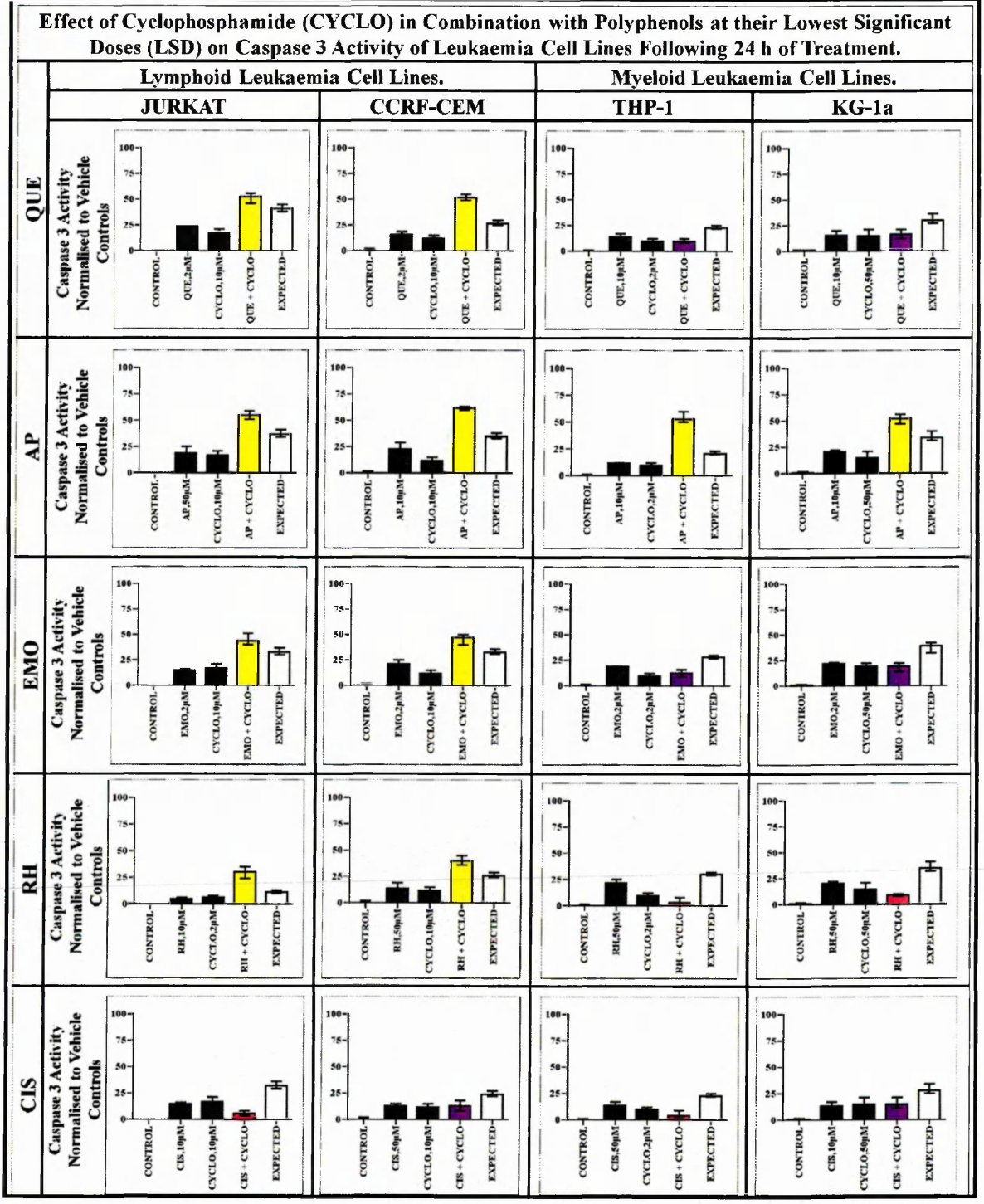


Figure 3.14: The effect of CYCLO when used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone

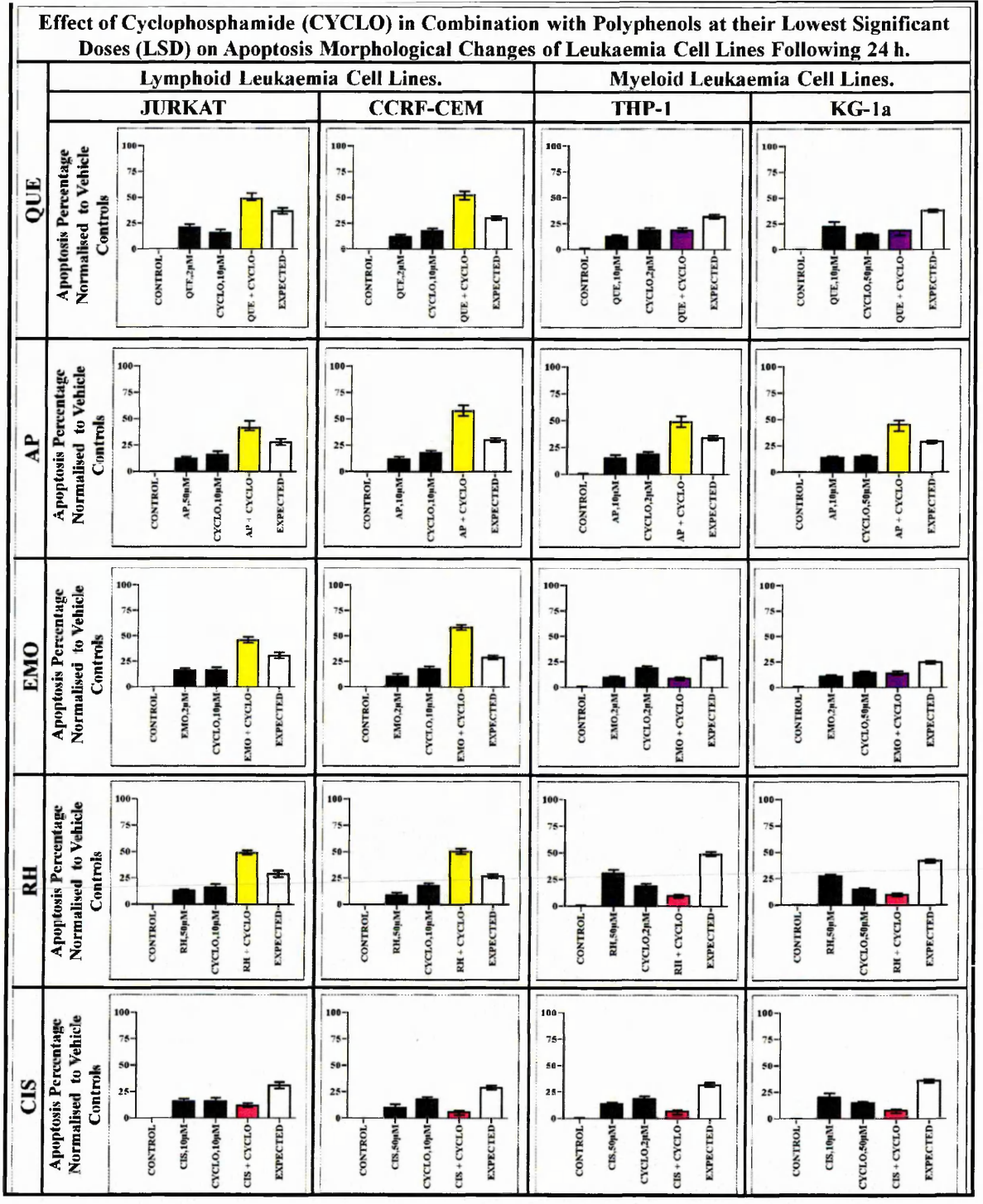


Figure 3.15: The effect of CYCLO when used in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

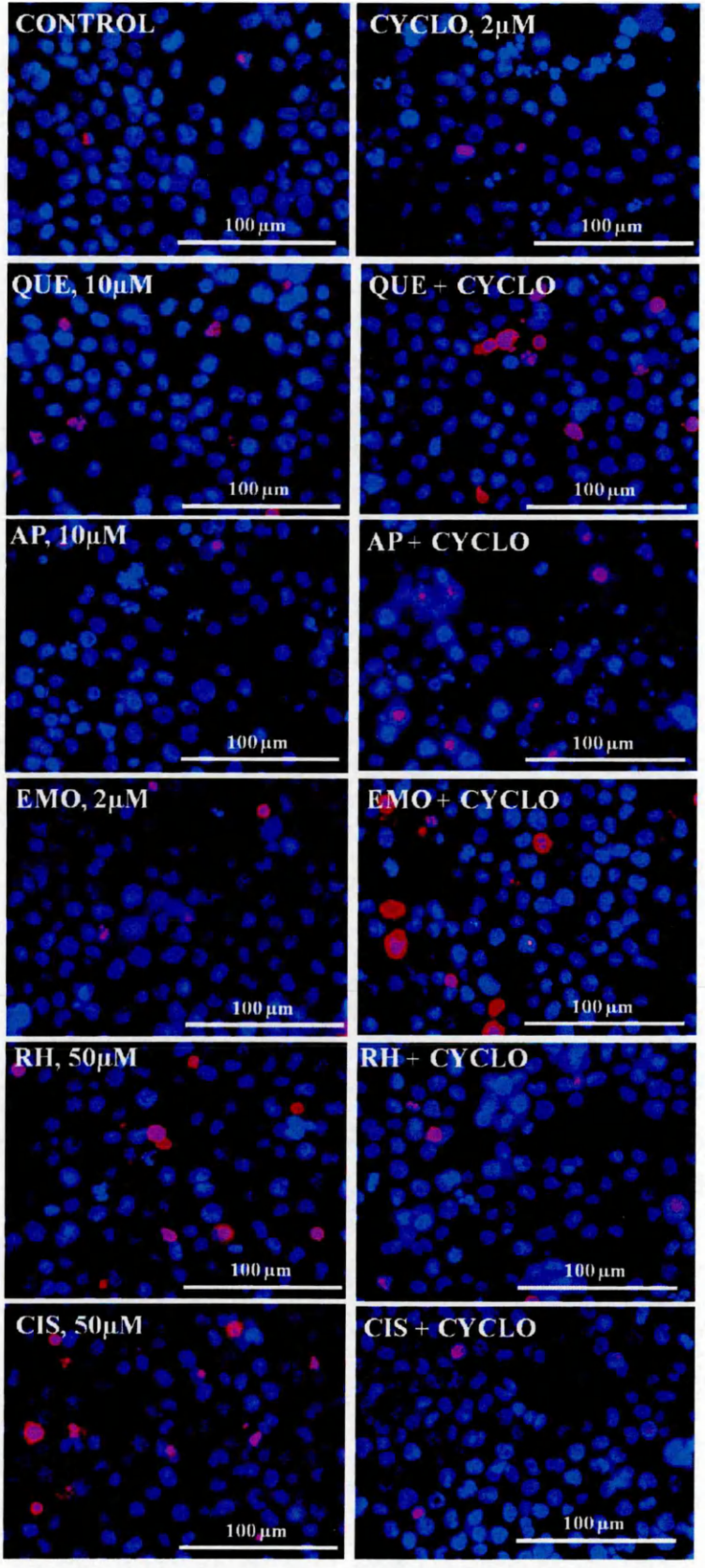
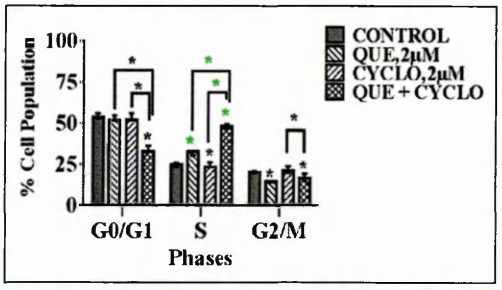
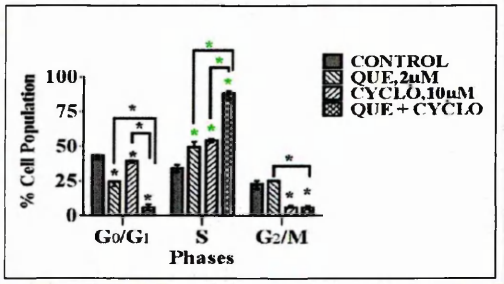
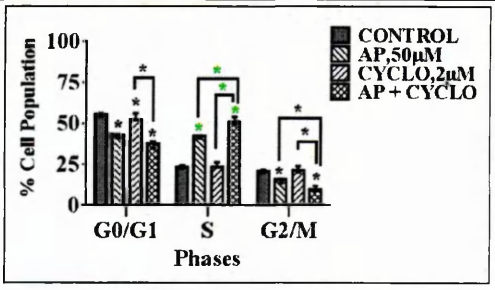
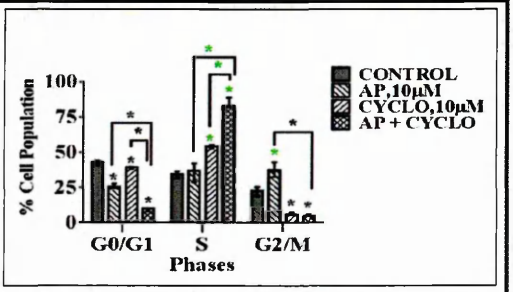
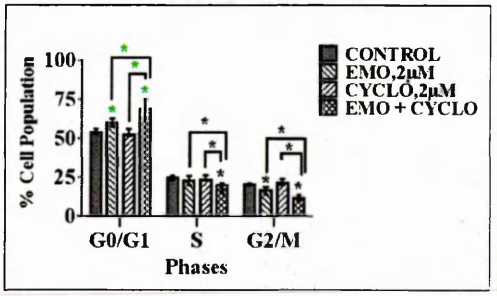
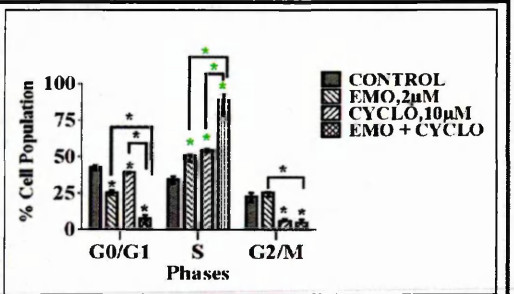
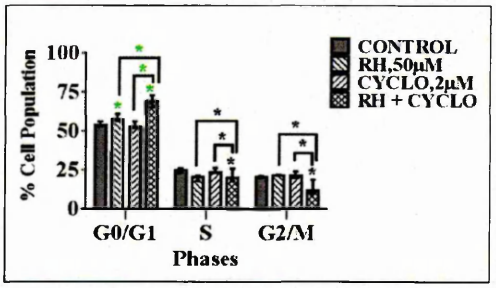
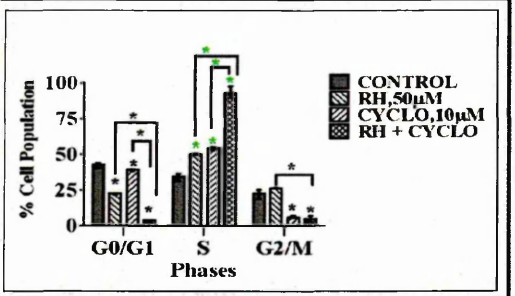
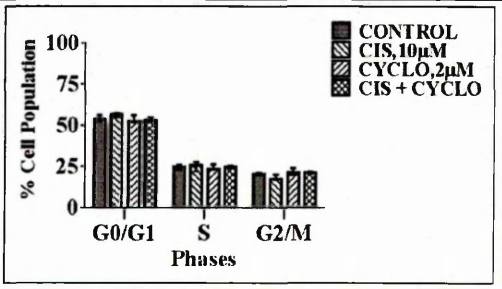
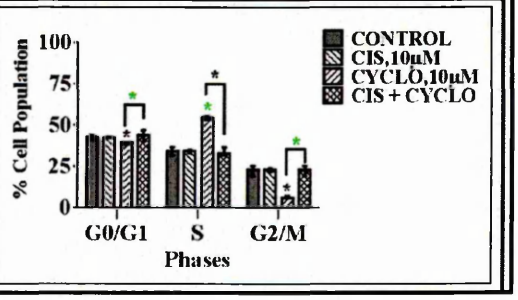


Figure 3.16: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of CYCLO and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A)Effect of Cyclophosphamide (CYCLO) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(A) Effect of Cyclophosphamide (CYCLO) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

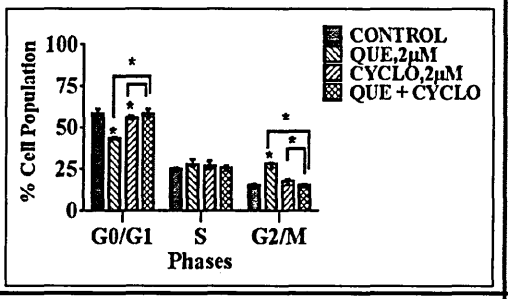
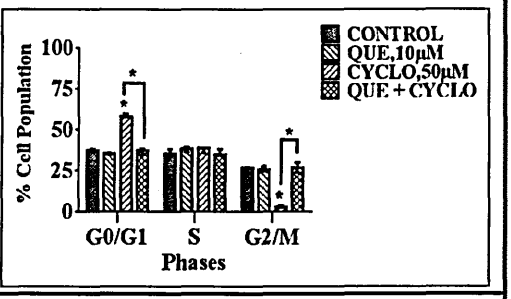
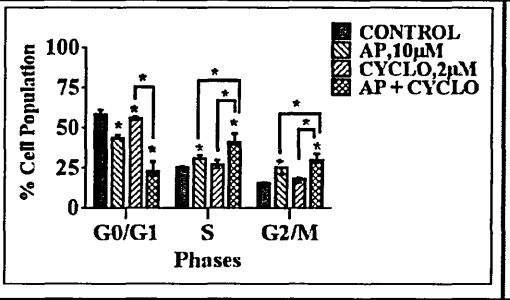
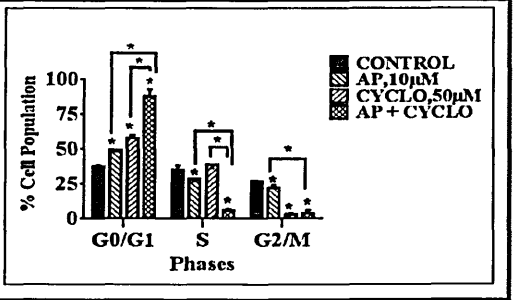
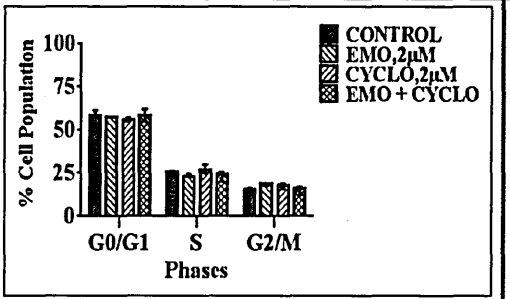
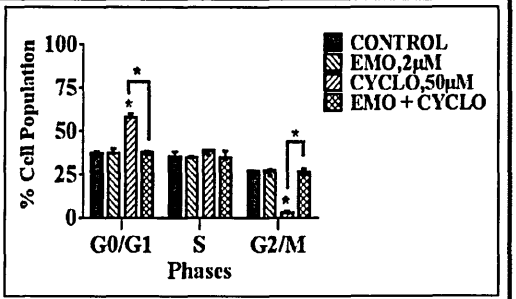
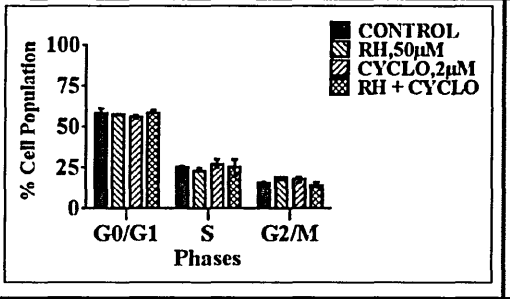
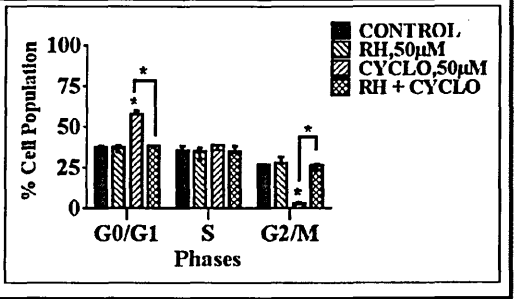
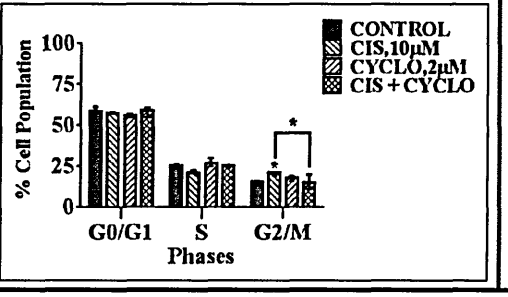
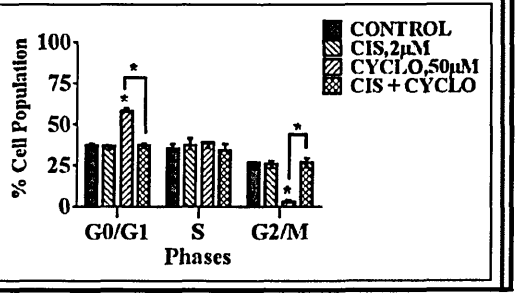
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.17: The effect of CYCLO when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.7.

Assays	Targets	Combination of Cyclophosphamide and Polyphenols	The Significant Effects of Cyclophosphamide (CYCLO) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glo	ATP Level	CYCLO + QUE (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CYCLO + QUE (LSD)	SYN	SYN	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	CYCLO + QUE (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CYCLO + QUE (LSD)	SYN	SYN	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CYCLO + QUE (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	CYCLO + QUE (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glo	ATP Level	CYCLO + AP (NSD)	SYN	SYN	SYN	SYN
		CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
Caspase 3 Activity	Apoptosis	CYCLO + AP (NSD)	SYN	SYN	SYN	SYN
		CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
Hoechst/ PI	Apoptosis	CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	CYCLO + AP (LSD)	Interaction	Interaction	Interaction	Interaction
CellTiter-Glo	ATP Level	CYCLO + EMO (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CYCLO + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	CYCLO + EMO (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CYCLO + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CYCLO + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	CYCLO + EMO (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glo	ATP Level	CYCLO + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CYCLO + RH (LSD)	SYN	SYN	ANTG	ANTG
Caspase 3 Activity	Apoptosis	CYCLO + RH (NSD)	SYN	SYN	ANTG	C-ANTG
		CYCLO + RH (LSD)	SYN	SYN	ANTG	ANTG
Hoechst/ PI	Apoptosis	CYCLO + RH (LSD)	SYN	SYN	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	CYCLO + RH (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glo	ATP Level	CYCLO + CIS (NSD)	C-ANTG	C-ANTG	ANTG	C-ANTG
		CYCLO + CIS (LSD)	ANTG	ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	CYCLO + CIS (NSD)	C-ANTG	C-ANTG	ANTG	C-ANTG
		CYCLO + CIS (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CYCLO + CIS (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	CYCLO + CIS (LSD)	ANTG	ANTG	ANTG	ANTG

Table 3.7: A summary of the effects of cyclophosphamide (CYCLO) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.2.2 Chlorambucil (CLB)

(A) Effect of Chlorambucil (CLB) Combined with Polyphenols on ATP Levels

CLB when used in combination with AP produced a synergistic reduction in ATP level in both lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.18 and Table 3.8). CLB when combined with QUE and EMO produced an additive effects reducing ATP level in both lymphoid and myeloid ($p<0.05$) (Figure 3.18 and Table 3.8). However when CLB was combined with RH, they produced a synergistic reduction in ATP levels in lymphoid leukaemia cell lines (JURKAT, CCRF-CEM) ($p<0.05$) (Figure 3.18 and Table 3.8); whilst produced a competitive antagonistic or antagonistic effects significantly increasing ATP levels in the myeloid leukaemia cell lines ($p<0.05$) (Figure 3.18 and Table 3.8). The later effect was also seen when CLB was combined with CIS; here they produced a competitive antagonistic or antagonistic effects, significantly increasing ATP levels this time in both the lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.18 and Table 3.8).

(B) Effect of Chlorambucil (CLB) Combined with Polyphenols on Apoptosis

CLB interacted with AP producing synergistic effects that significantly increased the caspase 3 activity (Figure 3.19) and the percentage of cells undergoing apoptosis (Figure 3.20) ($p<0.05$) in both lymphoid and myeloid leukaemia cell lines (Figure 3.19, 3.20 and Table 3.8). CLB when used in combination with QUE and EMO caused an additive effects on apoptosis in both lymphoid and myeloid cell lines ($p<0.05$) (Figure 3.19, 3.20 and Table 3.8). However when CLB was used in combination with RH, there was a synergistic increased the caspase 3 activity (Figure 3.19) and percentage apoptosis (Figure 3.20) in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) ($p<0.05$) (Table 3.8); but a competitive antagonistic or antagonistic effects and significantly decreased the caspase 3 activity (Figure 3.19) and the level of apoptosis (Figure 3.20) in myeloid leukaemia cell lines (THP-1 and KG1a) ($p<0.05$) (Table 3.8). Likewise, when CLB was combined with CIS they also produced a competitive antagonistic or antagonistic effects on apoptosis this time in both the lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.19, 3.20 and Table 3.8). An example of the morphological assessment of apoptosis for CLB and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.21.

(C) Effect of Chlorambucil (CLB) Combined with Polyphenols on Cell Cycle

Neither CLB or CIS induced cell cycle arrest in any phase in the JURKAT lymphoid leukaemia cells; however there was a significant increase of cell accumulation in S phase caused by QUE and AP, in G_0/G_1 phase caused by EMO and RH ($p<0.05$) (Figure 3.22).

Treatment of JURKAT cells with CLB in combination with AP or RH had an interactive effect on cell cycle inducing a highly significant increase of cell accumulation in S phase and G0/G1 phase ($p<0.05$) (Figure 3.22 and Table 3.8). JURKAT cells when treated with CLB in combination with QUE or EMO had a limited interactive effect on cell cycle; these treatment combinations induced a significant increase of cell accumulation in S phase and G_0/G_1 phase, respectively, when compared to the vehicle control and CLB alone, but this was not significantly different from the QUE or EMO treatment alone ($p<0.05$) (Figure 3.22 and Table 3.8). In contrast, treatment of JURKAT cells with combination of (CLB and CIS) had non-interactive effect on cell cycle ($p<0.05$) (Figure 3.22 and Table 3.8).

In CCRF-CEM lymphoid leukaemia cells, CLB, EMO, RH, and QUE when used alone caused a significant increase of cell accumulation in S phase of the cell cycle; whilst there was G_2/M phase accumulation caused by AP. There was however no significant increase of cell accumulation in any phases of cell cycle caused by CIS ($p<0.05$) (Figure 3.22). Treatment of CCRF-CEM cells with CLB in combination with QUE, AP, EMO or RH had an interactive effect on cell cycle each inducing a significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.22 and Table 3.8). In contrast, treatment of CCRF-CEM cells with combination of CLB and CIS had an antagonistic effect which was not significantly different to the vehicle control ($p<0.05$) but was significantly less than the effect of the individual treatments alone ($p<0.05$) (Figure 3.22 and Table 3.8).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in G2/M phase caused by CLB, QUE and CIS, in S and G_2/M phases caused by AP; and in S phase caused by EMO, and RH ($p<0.05$) (Figure 3.22). Treatment of THP-1 cells with CLB in combination with QUE, AP or EMO had an interactive effect on cell cycle causing a highly significant increase of cell accumulation in G_2/M phase ($p<0.05$) (Figure 3.22 and Table 3.8). In contrast, treatment of THP-1 cells with CLB in combination with RH or CIS had an antagonistic effect on cell cycle, which was not significantly different from the vehicle control ($p<0.05$), but was significantly reduced compared to levels of cell cycle arrested caused by individual treatments alone ($p<0.05$) (Figure 3.22 and Table 3.8).

KG-1a myeloid leukaemia cells had no significant increase of cell accumulation in any phases of the cell cycle when treated with by CLB, QUE, EMO, RH or CIS. However, there was a significant increase of cell accumulation in G_0/G_1 phase caused by AP ($p<0.05$) (Figure 3.22). Treatment of KG-1a cells with CLB in combination with QUE or AP had an interactive effect causing a highly significant increase of cell accumulation in S phase and G_0/G_1 phase, respectively, ($p<0.05$) (Figure 3.22 and Table 3.8). In contrast, treatment of KG-1a cells with CLB in combination with EMO, RH or CIS had a non-interactive effect on the cell cycle; there

were no significant different between these combination treatments and the vehicle control ($p<0.05$), and between these combination treatments and the individual treatments alone ($p<0.05$) (Figure 3.22 and Table 3.8).

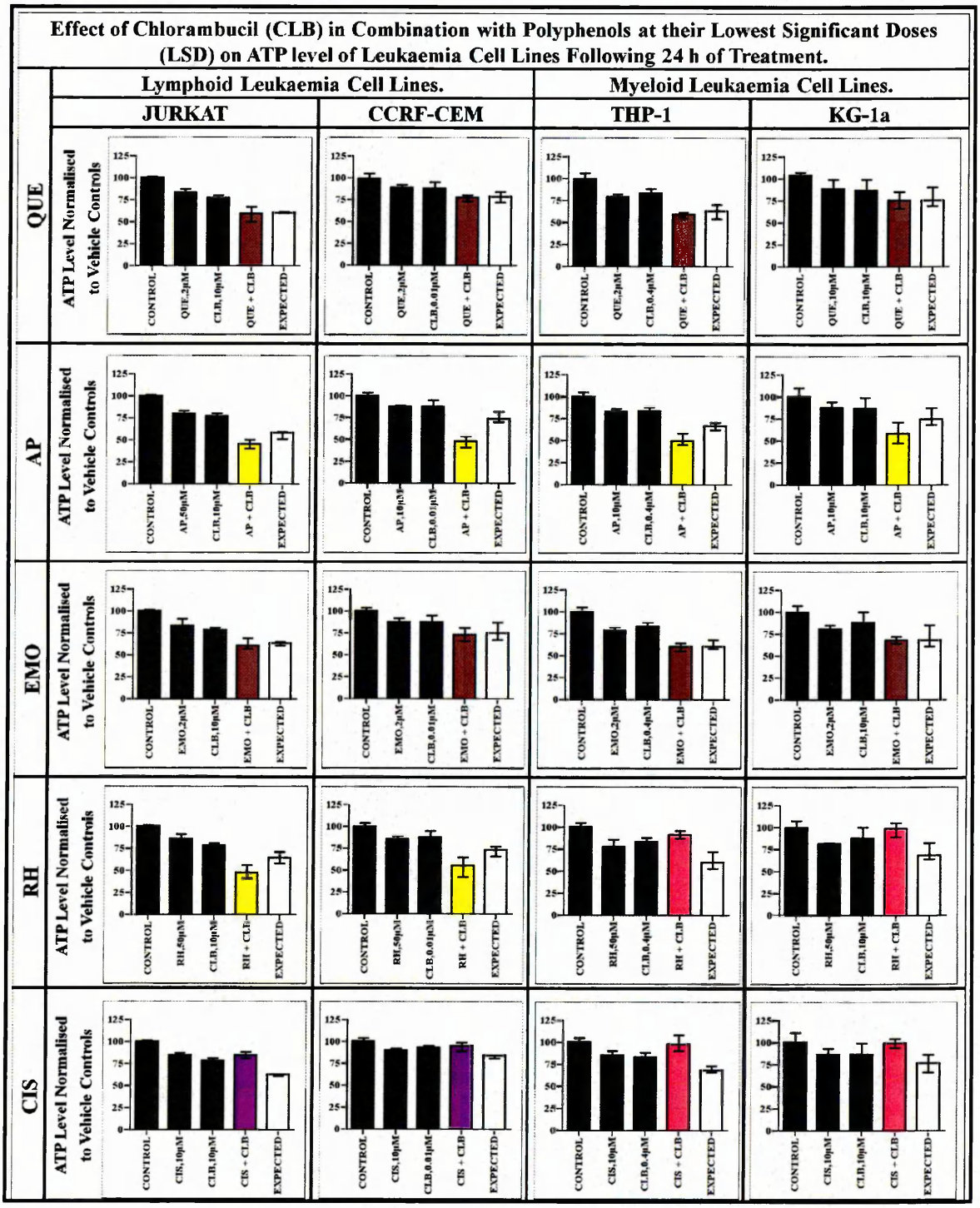


Figure 3.18: The effect of CLB when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with CLB and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p\leq0.05$ compared to vehicle control and drugs alone.

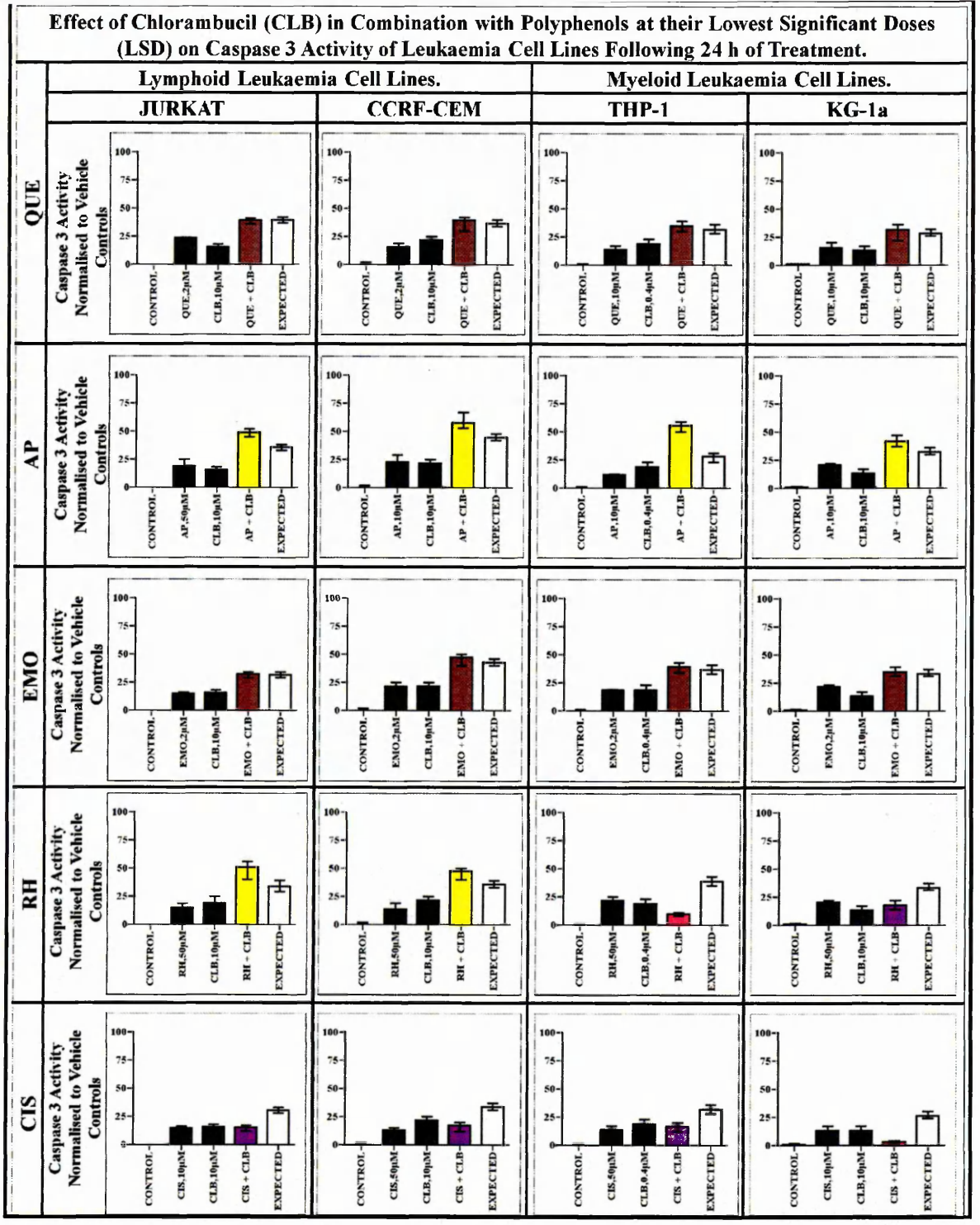


Figure 3.19: The effect of CLB when used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with CLB and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

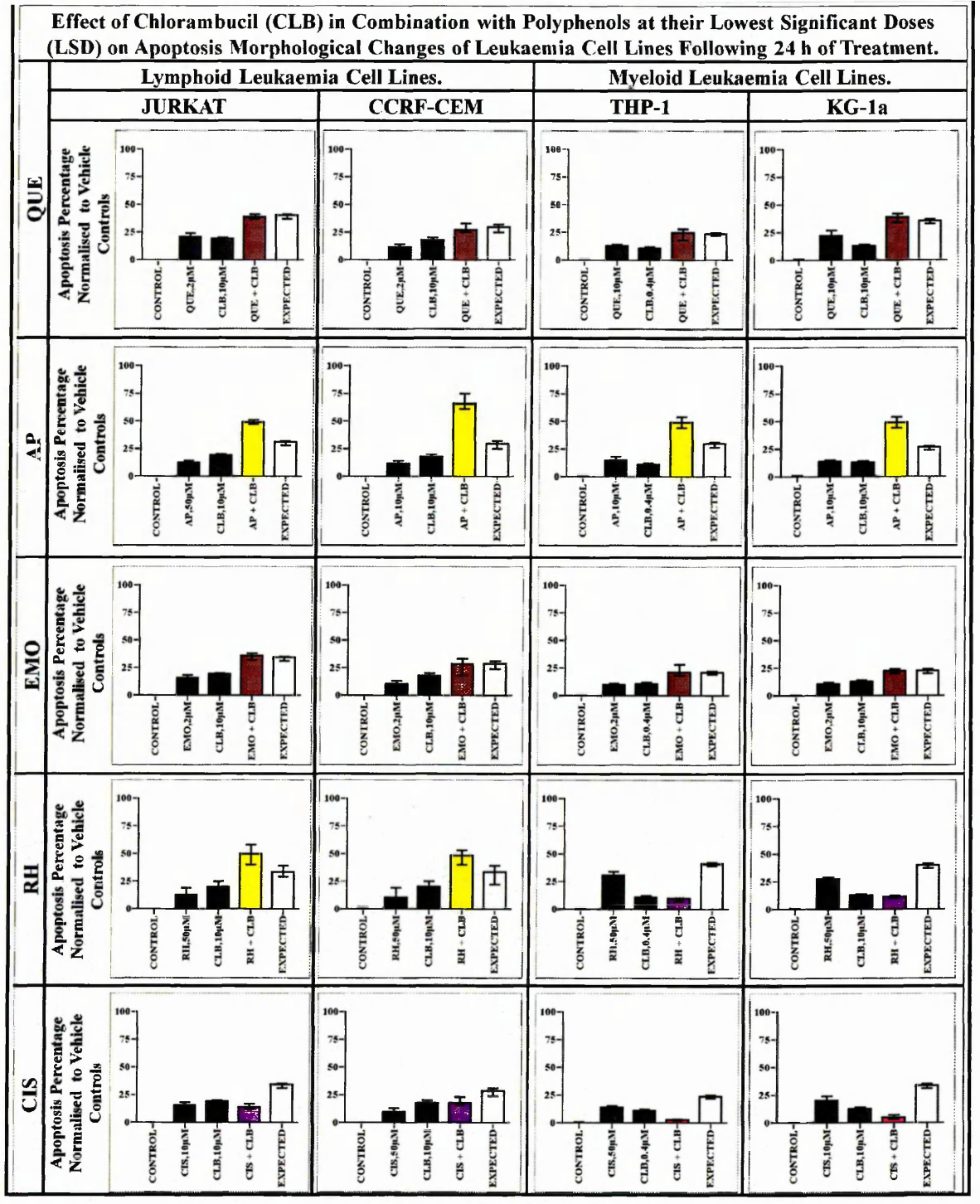


Figure 3.20: The effect of CLB when used in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with CLB and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

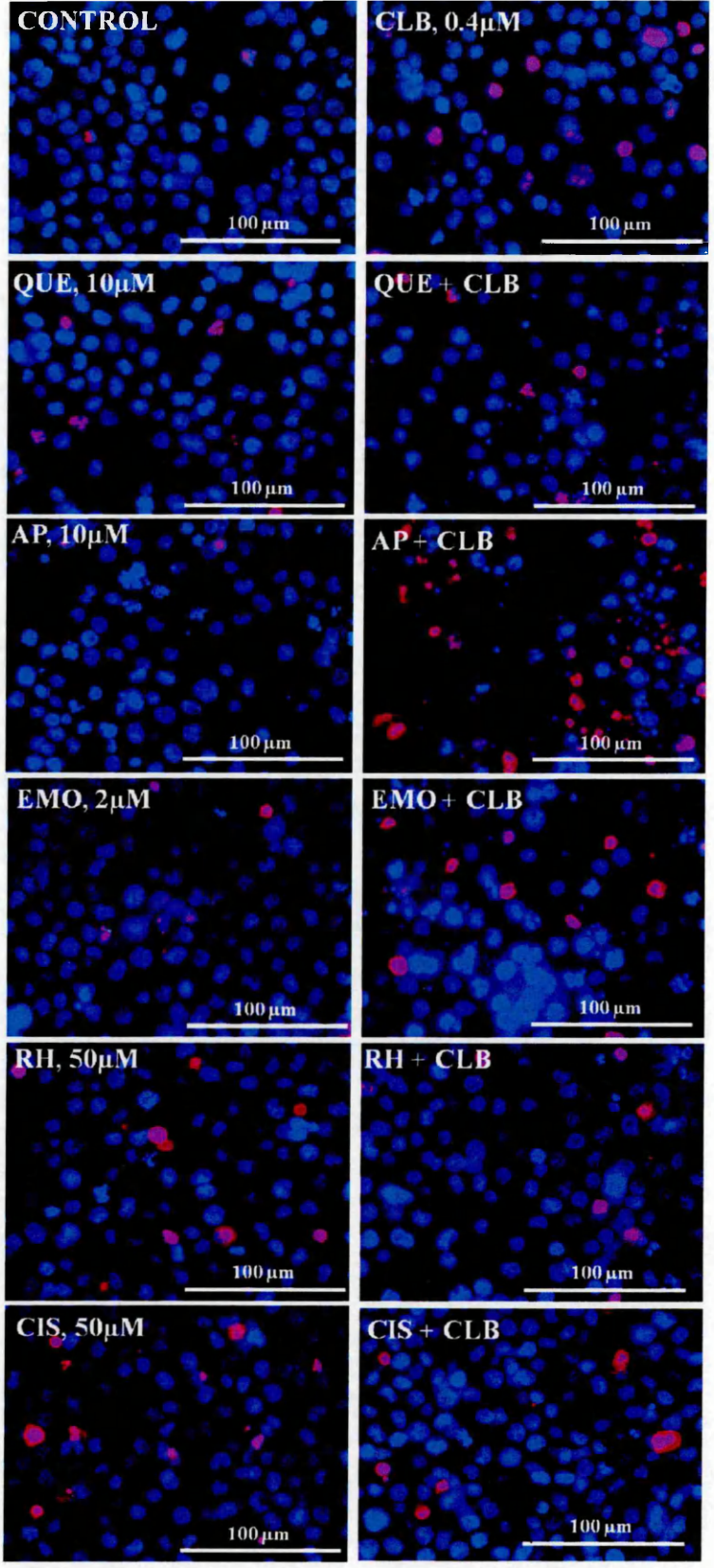
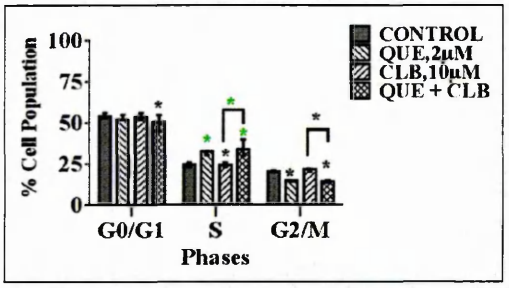
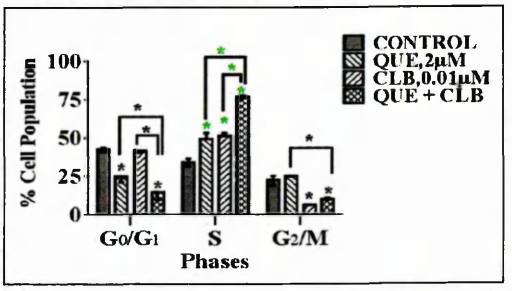
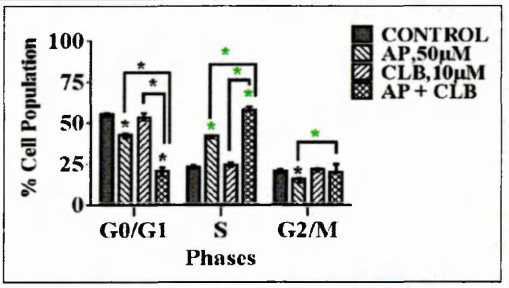
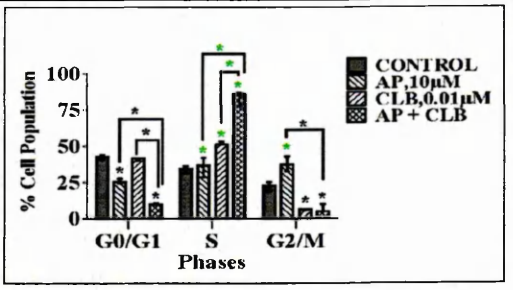
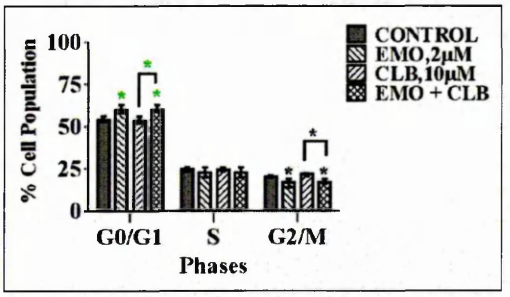
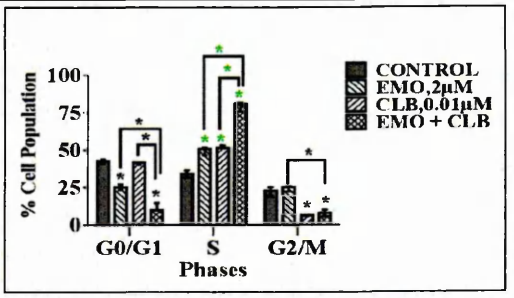
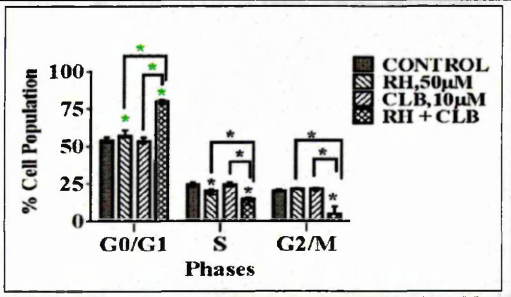
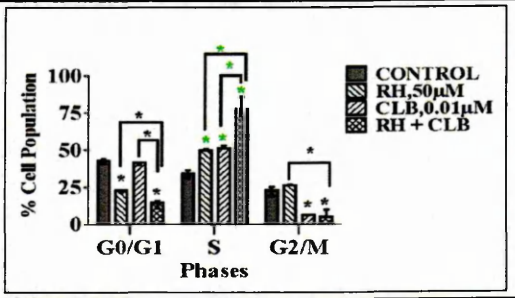
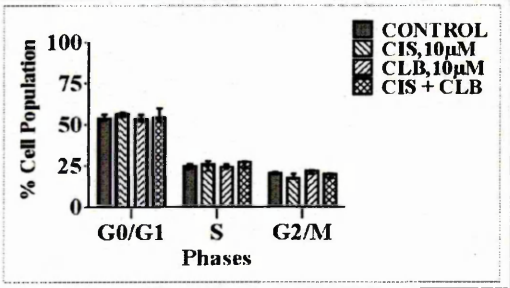
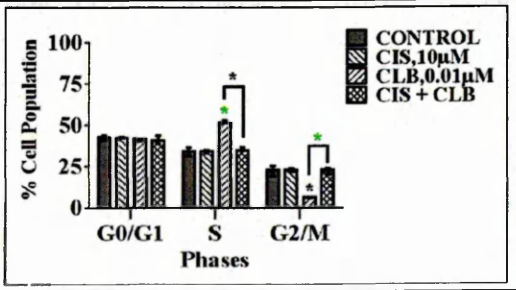


Figure 3.21: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of CLB and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A)Effect of Chlorambucil (CLB) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of Chlorambucil (CLB) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

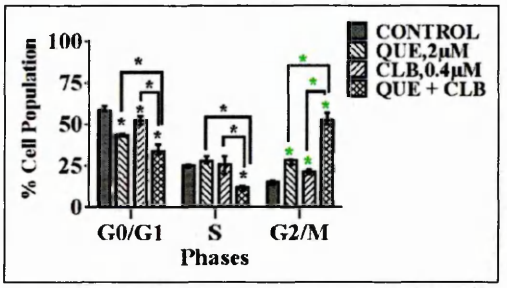
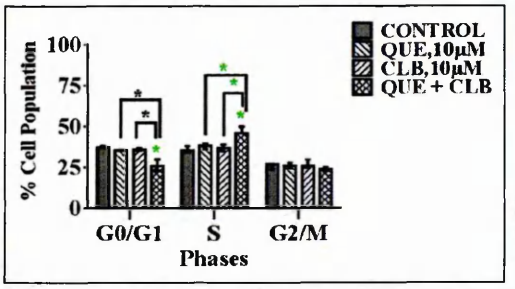
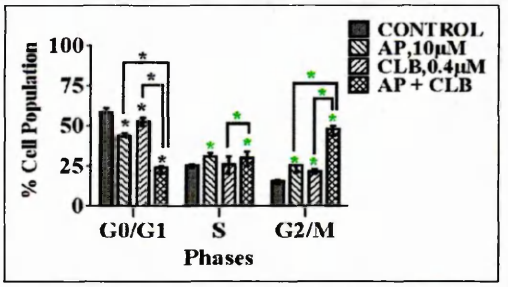
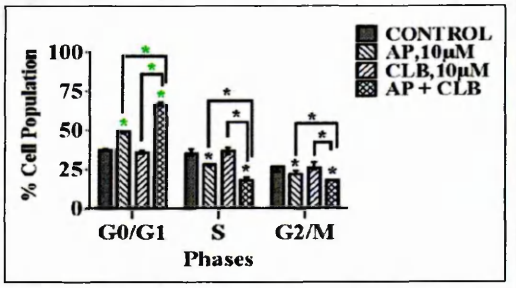
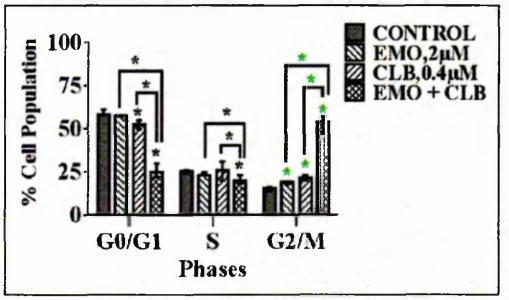
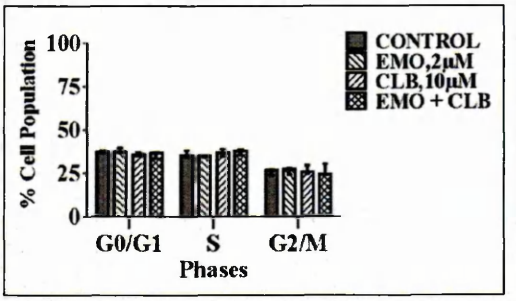
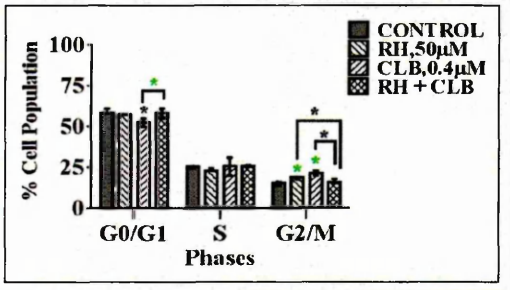
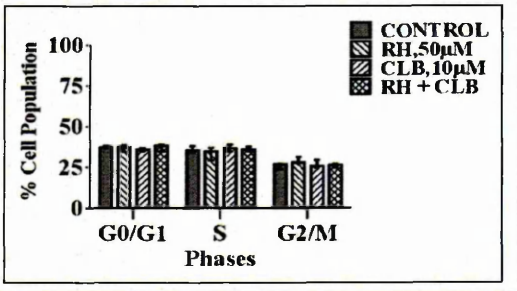
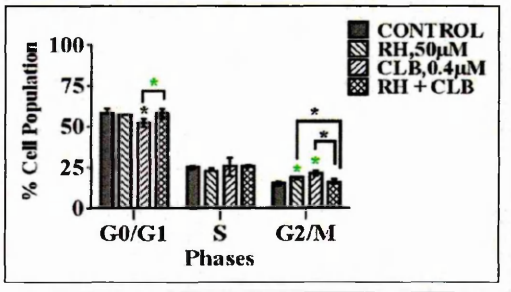
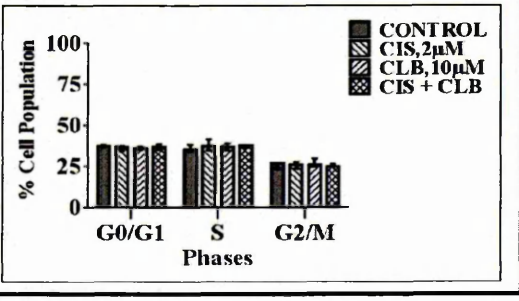
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.22: The effect of CLB when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with CLB and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (⋆) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (⋆) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.8.

Assays	Targets	Combination of Chlorambucil and Polyphenols	The Significant Effects of Chlorambucil (CLB) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	CLB + QUE (NSD)	ADD	ADD	ADD	ADD
		CLB + QUE (LSD)	ADD	ADD	ADD	ADD
Caspase 3 Activity	Apoptosis	CLB + QUE (NSD)	ADD	ADD	ADD	ADD
		CLB + QUE (LSD)	ADD	ADD	ADD	ADD
Hoechst/ PI	Apoptosis	CLB + QUE (LSD)	ADD	ADD	ADD	ADD
Cell Cycle/PI	Cell Cycle	CLB + QUE (LSD)	Non Interaction	Interaction	Interaction	Interaction
CellTiter-Glow	ATP Level	CLB + AP (NSD)	SYN	SYN	SYN	ADD
		CLB + AP (LSD)	SYN	SYN	SYN	SYN
Caspase 3 Activity	Apoptosis	CLB + AP (NSD)	SYN	SYN	SYN	ADD
		CLB + AP (LSD)	SYN	SYN	SYN	SYN
Hoechst/ PI	Apoptosis	CLB + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	CLB + AP (LSD)	Interaction	Interaction	Interaction	Interaction
CellTiter-Glow	ATP Level	CLB + EMO (NSD)	ADD	ADD	ADD	ADD
		CLB + EMO (LSD)	ADD	ADD	ADD	ADD
Caspase 3 Activity	Apoptosis	CLB + EMO (NSD)	ADD	ADD	ADD	ADD
		CLB + EMO (LSD)	ADD	ADD	ADD	ADD
Hoechst/ PI	Apoptosis	CLB + EMO (LSD)	ADD	ADD	ADD	ADD
Cell Cycle/PI	Cell Cycle	CLB + EMO (LSD)	Non Interaction	Interaction	Interaction	Non Interaction
CellTiter-Glow	ATP Level	CLB + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CLB + RH (LSD)	SYN	SYN	ANTG	ANTG
Caspase 3 Activity	Apoptosis	CLB + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CLB + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CLB + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	CLB + RH (LSD)	Interaction	Interaction	ANTG	Non Interaction
CellTiter-Glow	ATP Level	CLB + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	ANTG
		CLB + CIS (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Caspase 3 Activity	Apoptosis	CLB + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		CLB + CIS (LSD)	C-ANTG	C-ANTG	C-ANTG	ANTG
Hoechst/ PI	Apoptosis	CLB + CIS (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	CLB + CIS (LSD)	ANTG	ANTG	ANTG	Non Interaction

Table 3.8: A summary of the effects of chlorambucil (CLB) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.2.3 Cisplatin (CSP)

(A) Effect of Cisplatin (CSP) Combined with Polyphenols on ATP Levels

CSP effectively interacted with QUE, and caused a synergistic reduced in ATP levels in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 3.23 and Table 3.9). Furthermore, when CSP was used in combination with EMO, RH, or CIS, it caused a synergistic reduced in ATP levels in the lymphoid leukaemia cell lines only (JURKAT and CCRF-CEM) ($p<0.05$) (Figure 3.23 and Table 3.9). In contrast, when CSP was used in combination with EMO, RH, or CIS, it caused a competitive antagonistic or antagonistic effects and significantly increased ATP level in myeloid leukaemia cell lines (THP-1 and KG-1a) ($p<0.05$) (Figure 3.23 and Table 3.9). The only exception was shown when CSP was combined with AP, here there was an additive reduction of ATP level in both lymphoid and myeloid leukaemia cell lines, ($p<0.05$) (Figure 3.23 and Table 3.9).

(B) Effect of Cisplatin (CSP) Combined with Polyphenols on Apoptosis

CSP effectively interacted with QUE, which they caused a synergistic increased in caspase 3 activity (Figure 3.24) and percentage of apoptosis (Figure 3.25) in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 3.24, 3.25 and Table 3.9). In addition, when CSP was used in combination with EMO, RH, or CIS it caused a synergistic increased caspase 3 activity (Figure 3.24) and percentage of apoptosis (Figure 3.25) in the lymphoid leukaemia cell lines only (JURKAT, CCRF-CEM) ($p<0.05$) (Figure 3.24, 3.25 and Table 3.9). Once again in the myeloid cell lines (THP-1 and KG-1a), when CSP was combined with EMO, RH, or CIS it caused a competitive antagonistic or antagonistic effects; and significantly decreased caspase 3 activity ($p<0.05$) and the percentage of cells undergoing apoptosis ($p<0.05$) (Figure 3.24, 3.25 and Table 3.9). CSP when used in combination with AP this produced an additive effects on apoptosis in both lymphoid and myeloid leukaemia cell lines, ($p<0.05$) (Figure 3.24, 3.25 and Table 3.9). An example of the morphological assessment of apoptosis for CSP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.26.

(C) Effect of Cisplatin (CSP) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had a significant increase of cell accumulation in S phase caused by CSP, QUE and AP, in G_0/G_1 phase caused by EMO and RH; while they had no significant increase of cell accumulation in any phases of cell cycle caused by CIS when compared to the vehicle control ($p<0.05$) (Figure 3.27). Treatment of JURKAT cells with CSP in combination with QUE, AP, EMO, RH or CIS had an interactive effect causing a highly

significant increase of cell accumulation in S phase of the cell cycle ($p<0.05$) (Figure 3.27 and Table 3.9).

CCRF-CEM lymphoid leukaemia cells had a significant increase of cell accumulation in S phase caused by CSP, QUE, EMO, and RH, and in G_2/M phase caused by AP; while they had no significant increase of cell accumulation in any phases of cell cycle caused by CIS when compared to the vehicle control ($p<0.05$) (Figure 3.27). Treatment of CCRF-CEM cells with CSP in combination with QUE, AP, EMO, RH or CIS had an interactive effect causing a highly significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.27 and Table 3.9).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in G2/M phase caused by CSP, QUE, EMO, RH and CIS; and in S and G_2/M phases caused by AP when compared to the vehicle control ($p<0.05$) (Figure 3.27). Treatment of THP-1 cells with CSP in combination with QUE or AP had an interactive effect causing a significantly increase of cell accumulation in G_2/M phase ($p<0.05$) (Figure 3.27 and Table 3.9). In contrast, treatment of THP-1 cells with CSP in combination with EMO, RH or CIS had an antagonistic effect on cell cycle significantly decreasing cell accumulation; there was no significant different when compared to the vehicle control ($p<0.05$) and they were significantly reduced cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.27 and Table 3.9).

KG-1a myeloid leukaemia cells had a significant increase of cell accumulation in G2/M phase caused by CSP, in G_0/G_1 phase caused by AP; while they had no significant increase of cell accumulation in any phases of cell cycle caused by QUE, EMO, RH or CIS when compared to the vehicle control ($p<0.05$) (Figure 3.27). Treatment of KG-1a cells with CSP in combination with QUE or AP had an interactive effect on cell cycle which significantly increasing cell accumulation in G_2/M phase ($p<0.05$) (Figure 3.27 and Table 3.9). In contrast, treatment of KG-1a cells when treated with CSP in combination with EMO, RH or CIS had an antagonistic effect on cell cycle; there was no significant different when compared to the vehicle control ($p<0.05$) and they were significantly reduced when compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.27 and Table 3.9).

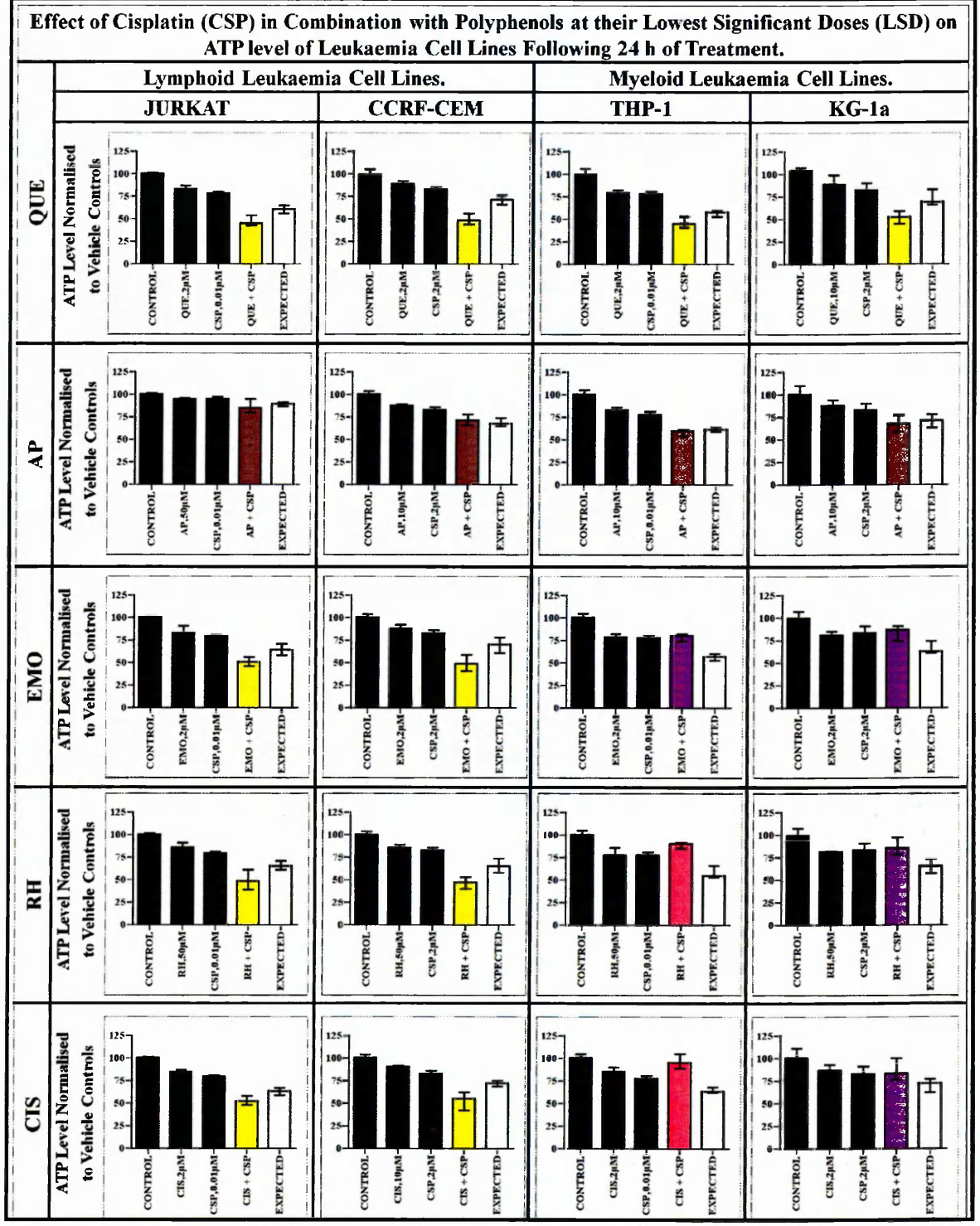


Figure 3.23: The effect of CSP when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

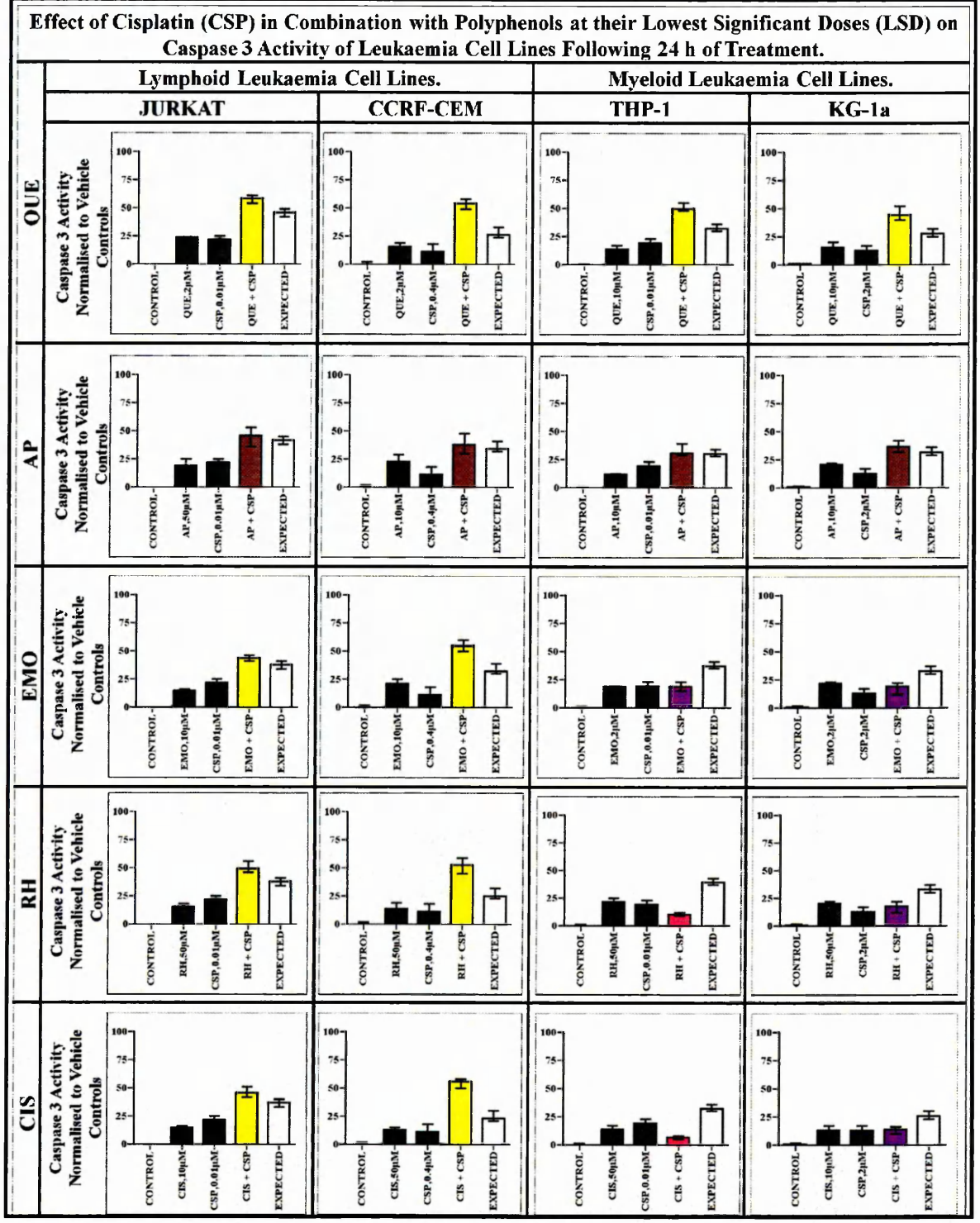


Figure 3.24: The effect of CSP when used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

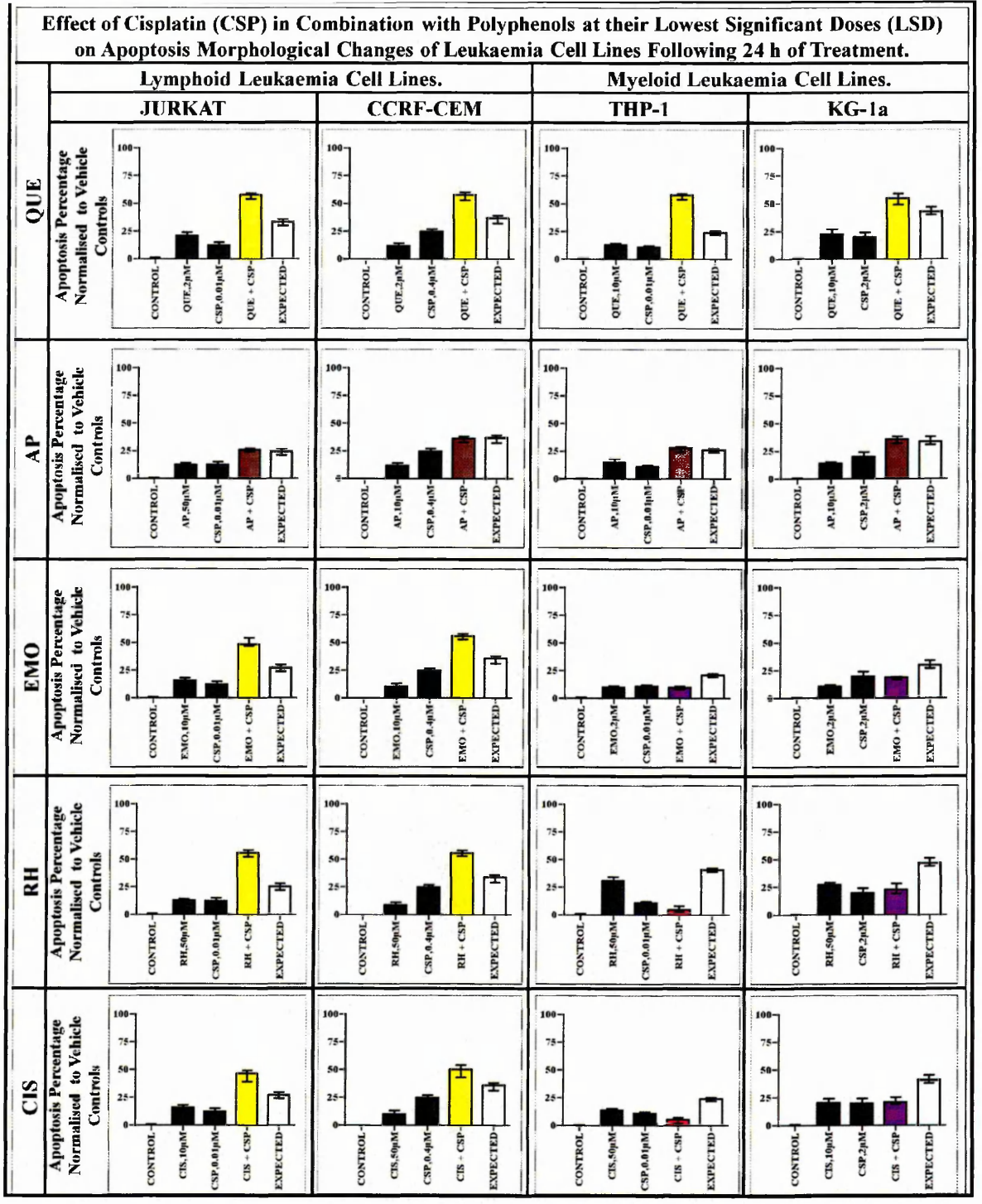


Figure 3.25: Effect of CSP when used in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

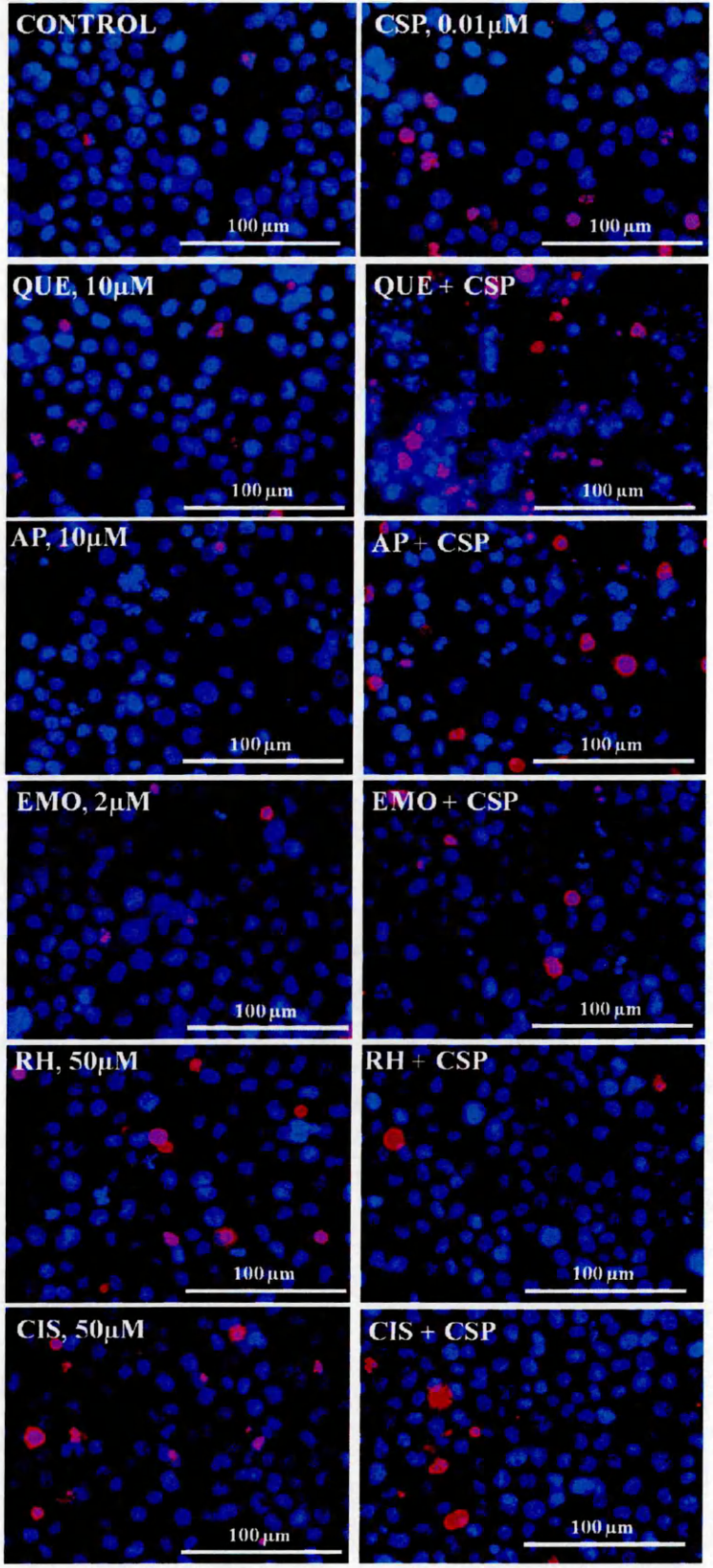
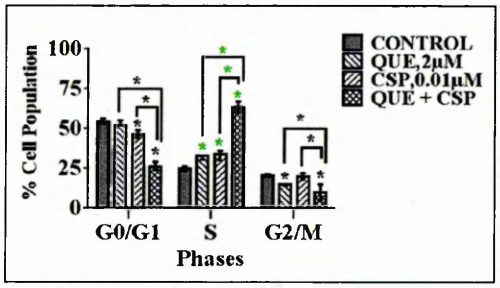
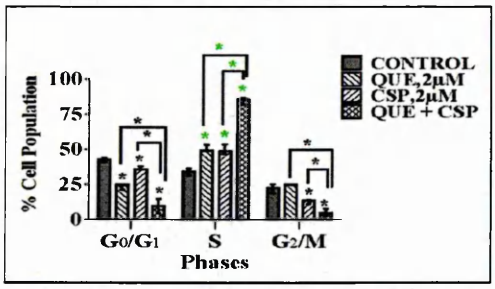
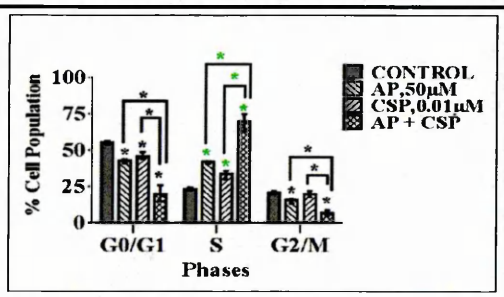
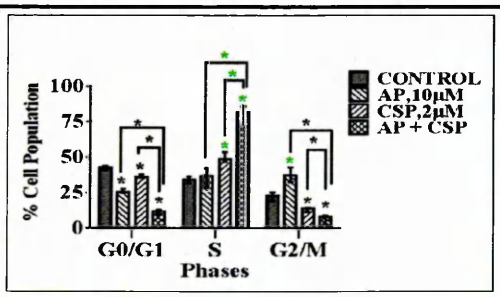
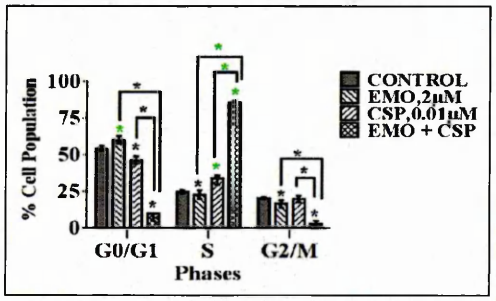
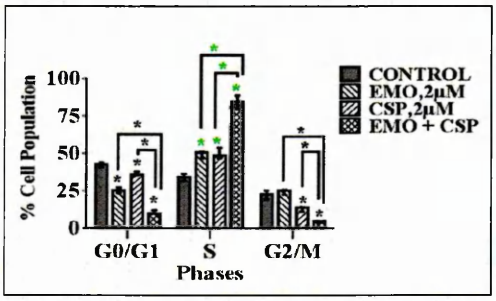
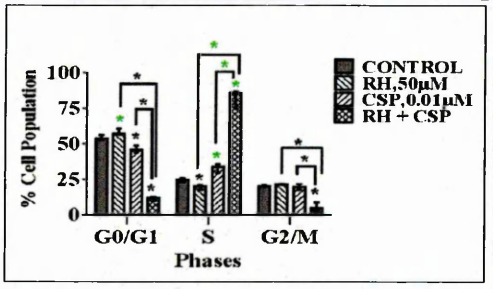
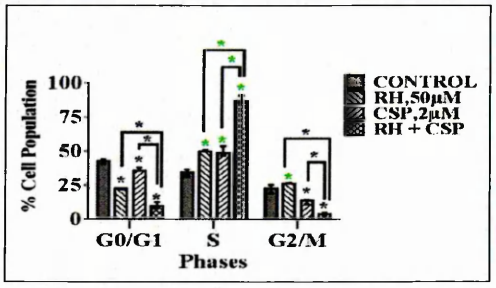
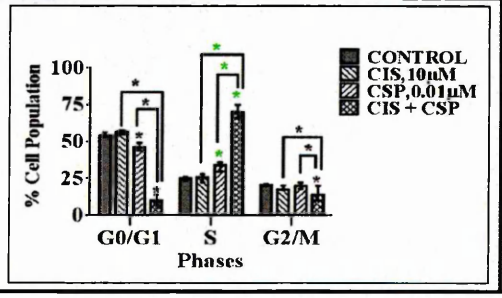
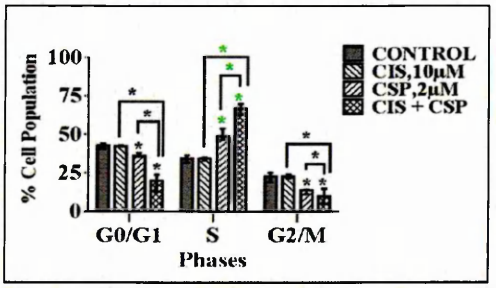


Figure 3.26: : An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of CSP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A) Effect of Cisplatin (CSP) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of Cisplatin (CSP) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

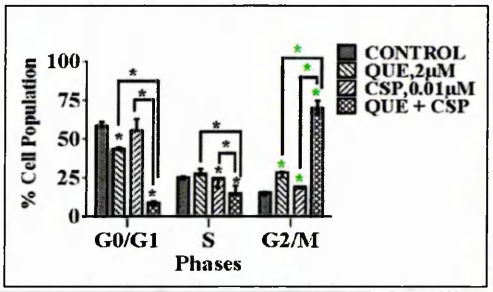
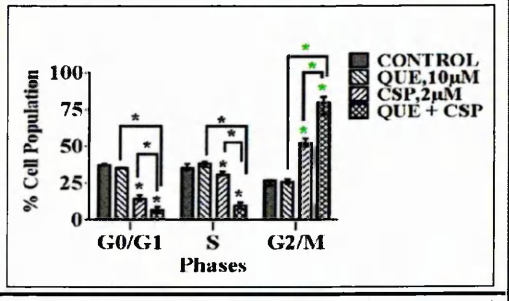
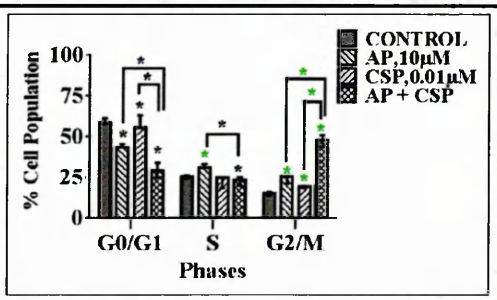
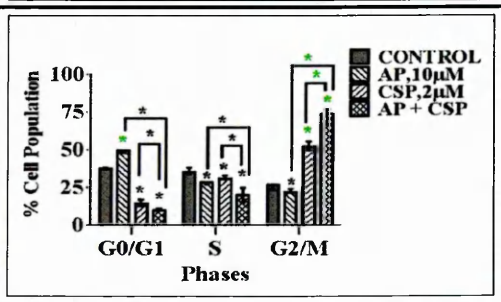
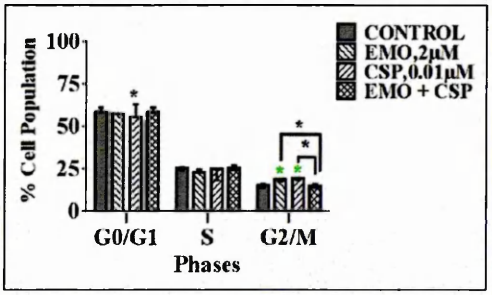
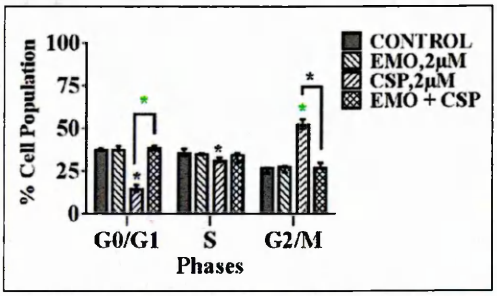
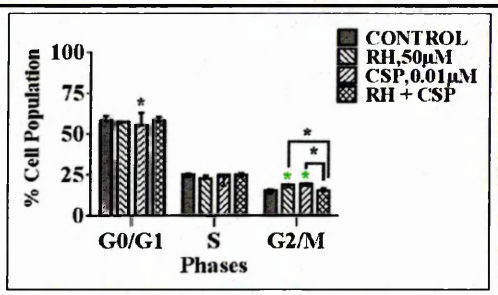
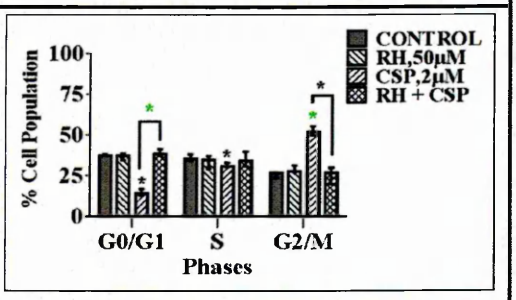
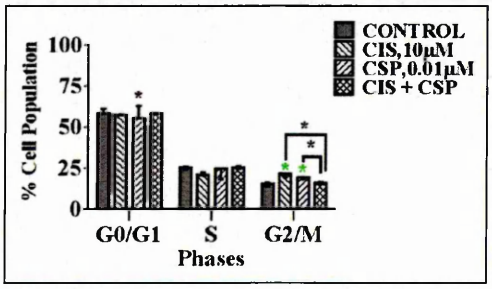
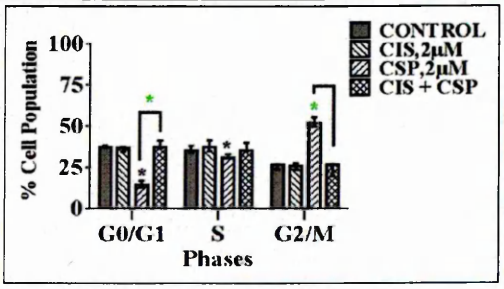
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.27: The effect of CSP when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with CSP and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.9.

Assays	Targets	Combination of Cisplatin and Polyphenols	The Significant Effects of Cisplatin (CSP) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	CSP + QUE (NSD)	SYN	SYN	SYN	ADD
		CSP + QUE (LSD)	SYN	SYN	SYN	SYN
Caspase 3 Activity	Apoptosis	CSP + QUE (NSD)	SYN	SYN	SYN	ADD
		CSP + QUE (LSD)	SYN	SYN	SYN	SYN
Hoechst/ PI	Apoptosis	CSP + QUE (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	CSP + QUE (LSD)	Interaction	Interaction	Interaction	Interaction
CellTiter-Glow	ATP Level	CSP + AP (NSD)	ADD	ADD	ADD	ADD
		CSP + AP (LSD)	ADD	ADD	ADD	ADD
Caspase 3 Activity	Apoptosis	CSP + AP (NSD)	ADD	ADD	ADD	ADD
		CSP + AP (LSD)	ADD	ADD	ADD	ADD
Hoechst/ PI	Apoptosis	CSP + AP (LSD)	ADD	ADD	ADD	ADD
Cell Cycle/PI	Cell Cycle	CSP + AP (LSD)	Interaction	Interaction	Interaction	Interaction
CellTiter-Glow	ATP Level	CSP + EMO (NSD)	ADD	SYN	C-ANTG	C-ANTG
		CSP + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	CSP + EMO (NSD)	ADD	SYN	C-ANTG	C-ANTG
		CSP + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CSP + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	CSP + EMO (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glow	ATP Level	CSP + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CSP + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	CSP + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CSP + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CSP + RH (LSD)	SYN	SYN	ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	CSP + RH (LSD)			ANTG	ANTG
CellTiter-Glow	ATP Level	CSP + CIS (NSD)	SYN	SYN	C-ANTG	C-ANTG
		CSP + CIS (LSD)	SYN	SYN	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	CSP + CIS (NS)	ADD	SYN	C-ANTG	C-ANTG
		CSP + CIS (LSD)	SYN	SYN	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	CSP + CIS (LSD)	SYN	SYN	ANTG	C-ANTG
Cell cycle/PI	Cell Cycle	CSP + CIS (LSD)	Interaction	Interaction	ANTG	ANTG

Table 3.9: A summary of the effects of cisplatin (CSP) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.3 Effects of Anti-Metabolites Chemotherapy Agents in Combined with Polyphenols

3.3.2.3.1 Methotrexate (MTX)

(A) Effect of Methotrexate (MTX) Combined with Polyphenols on ATP Levels

MTX when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) caused a competitively antagonistic and/or antagonistic effects significantly increasing the ATP levels in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 3.28 and Table 3.10).

(B) Effect of Methotrexate (MTX) Combined with Polyphenols on Apoptosis

Similarly, when MTX used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) caused a competitively antagonistic and/or antagonistic effects significantly decreasing caspase 3 activity (Figure 3.29) and the percentage of cells undergoing apoptosis (Figure 3.30) in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 3.29, 3.30 and Table 3.10). An example of the morphological assessment of apoptosis for MTX and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.31.

(C) Effect of Methotrexate (MTX) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had a significant increase of cell accumulation in S phase following treated with MTX, QUE or AP alone, and in G_0/G_1 phase with EMO or RH alone; while there was no significant increase of cell accumulation in any phases of cell cycle following treatment with CIS ($p<0.05$) (Figure 3.32). Treatment of JURKAT cells with MTX in combination with QUE, AP, EMO, RH or CIS, caused an antagonistic effect; there were no significant differences compared to the vehicle control ($p<0.05$), but there were significant decrease of cell accumulation compared to the phases arrested by the individual treatments alone ($p<0.05$) (Figure 3.32 and Table 3.10).

CCRF-CEM lymphoid leukaemia cells had a significant increase of cells in S phase following treatment with MTX, QUE, EMO or RH alone; and in G_2/M phase following treatment with AP alone; whilst they had no significant increase of cell accumulation in any phases of cell cycle following CIS treatment alone ($p<0.05$) (Figure 3.32). Treatment of CCRF-CEM cells with MTX in a combination QUE, AP, EMO, RH or CIS had an antagonistic effect on cell cycle accumulation; there were no significant differences compared to the vehicle control ($p<0.05$), but there was a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.32 and Table 3.10).

THP-1 myeloid leukaemia cells had a significant increase in accumulation of cells in the S and G_2/M phases of the cell cycle following treatment with MTX and AP alone ($p<0.05$); in G_2/M

phase following treatment of with QUE and CIS alone ($p<0.05$); whilst they had no significant increase of cell accumulation in any phases of the cell cycle following treatment with EMO or RH alone ($p<0.05$) (Figure 3.32). Treatment of THP-1 cells with MTX in combination with QUE, AP, EMO, RH or CIS had an antagonistic effect on the cell cycle; there were no significant differences compared to the vehicle control ($p<0.05$), but there was a significant decrease in cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.32 and Table 3.10).

KG-1a myeloid leukaemia cells had a significant increase of cells in G_0/G_1 and S phases when treated with MTX alone, and in G_0/G_1 phase only when treated with AP alone; while they had no significant increase of cell accumulation in any phases of cell cycle when treated with QUE, EMO, RH or CIS alone ($p<0.05$) (Figure 3.32). Treatment of KG-1a cells with MTX in combination with QUE, AP, EMO, RH or CIS had an antagonistic effect on cell cycle accumulation, there were no significant differences compared to the vehicle control ($p<0.05$) but there were significant decreases of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.32 and Table 3.10).

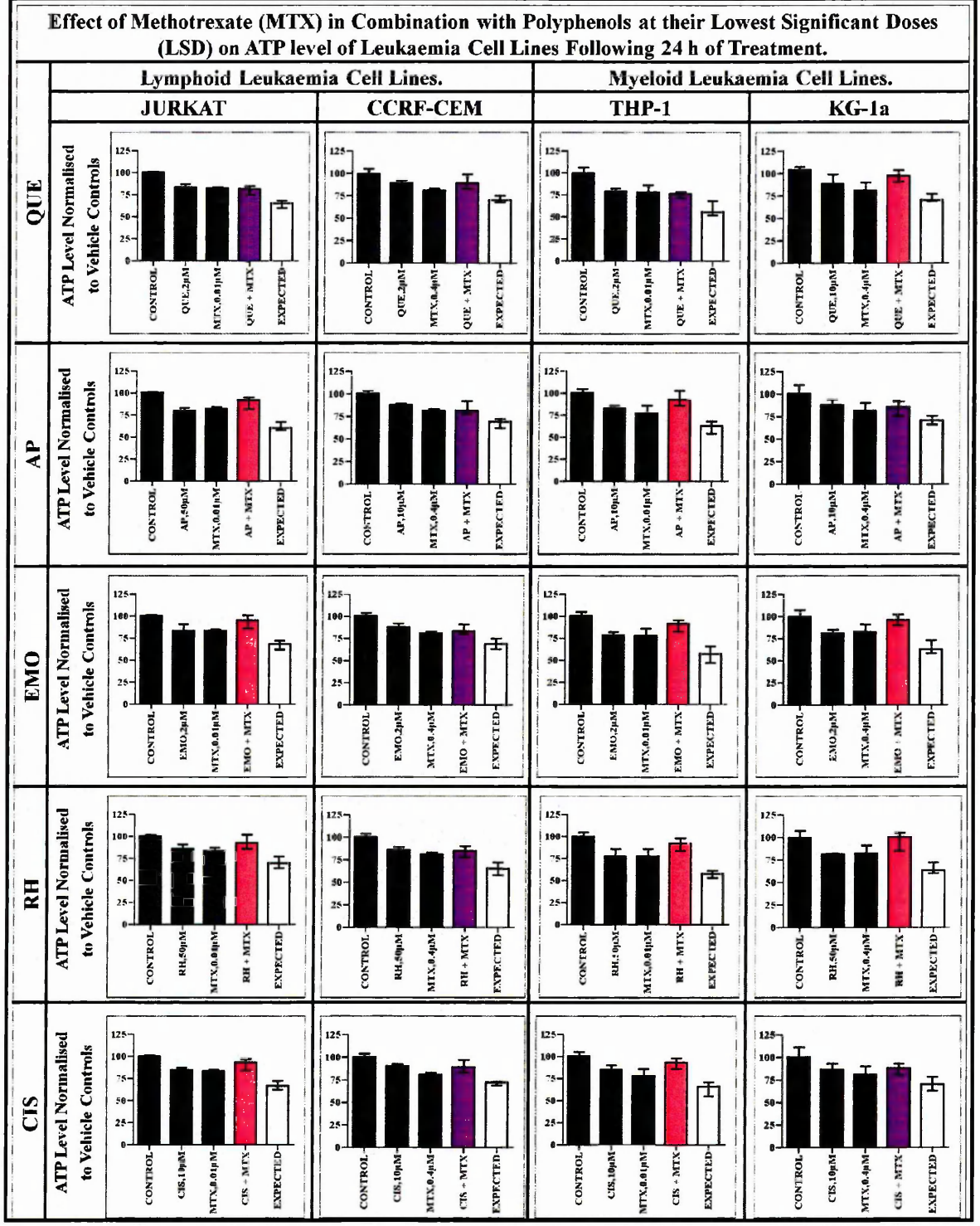


Figure 3.28: The effect of MTX when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with MTX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

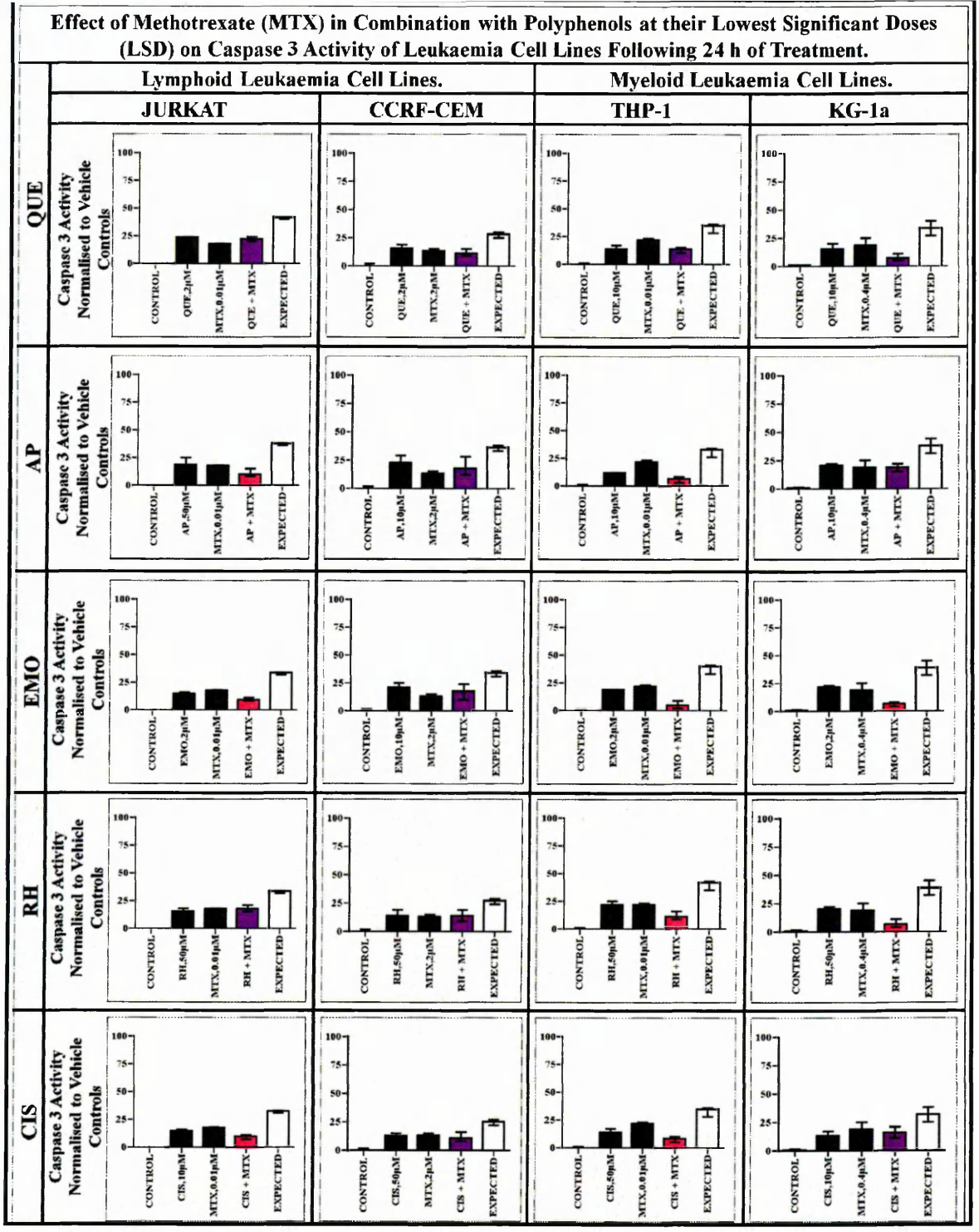


Figure 3.29: The effect of MTX when used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with MTX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

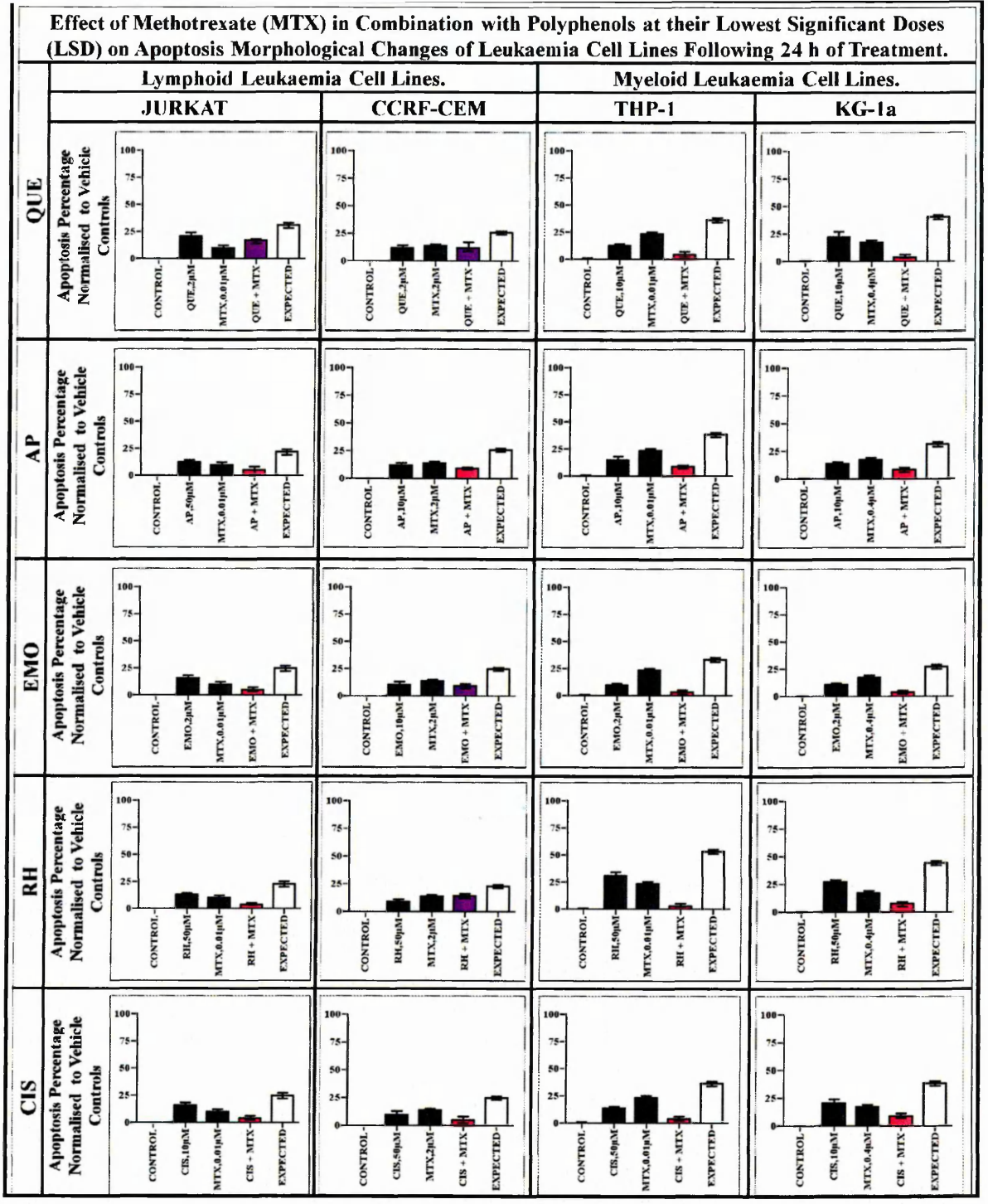


Figure 3.30: The effect of MTX in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with MTX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

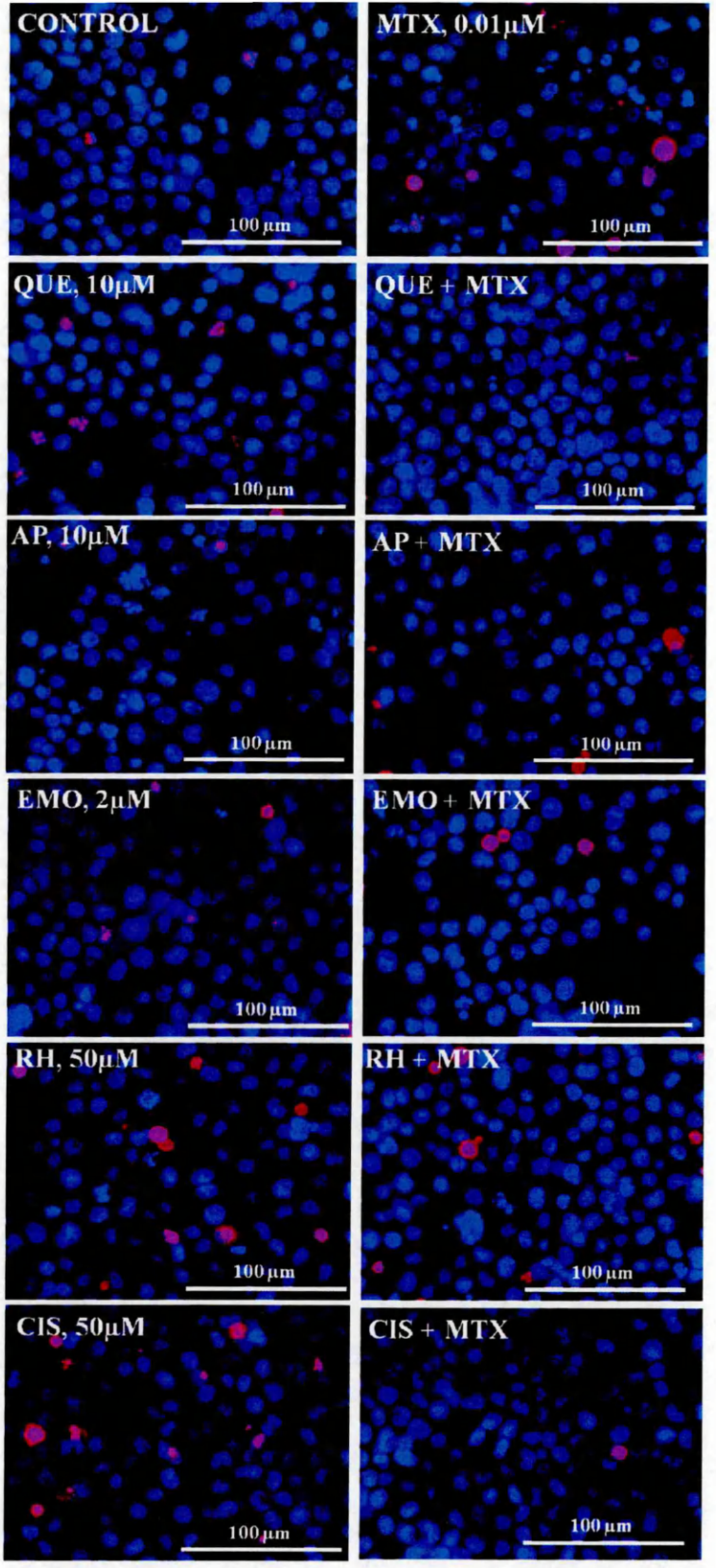
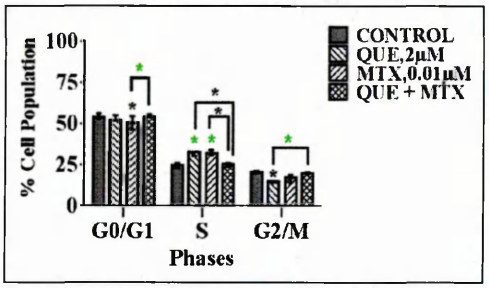
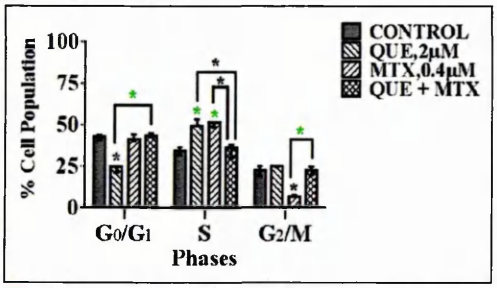
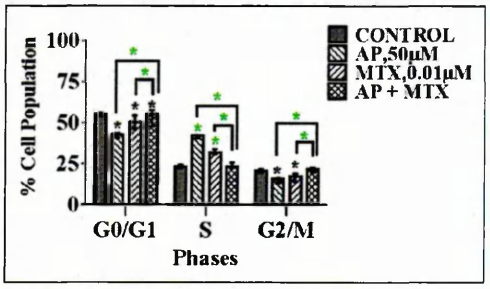
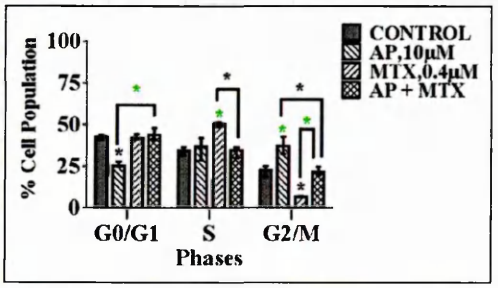
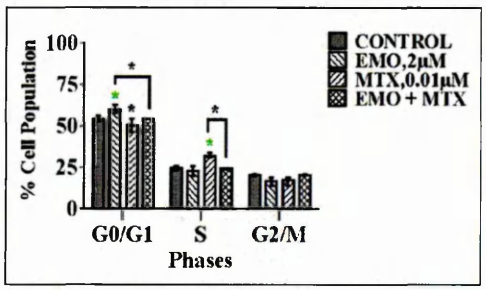
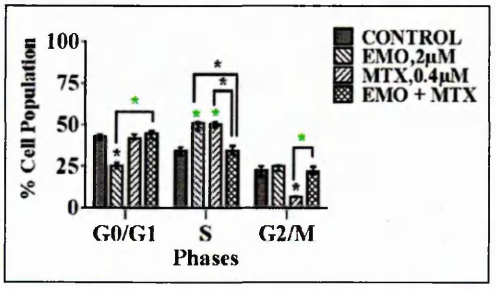
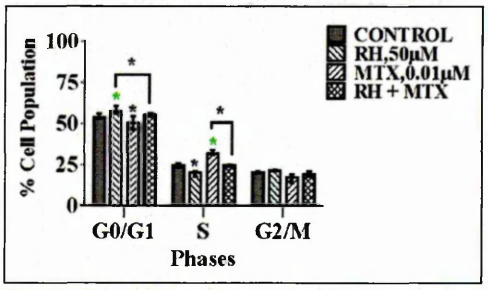
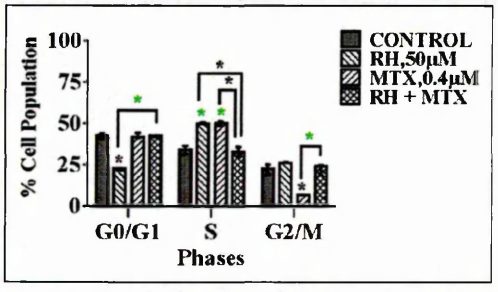
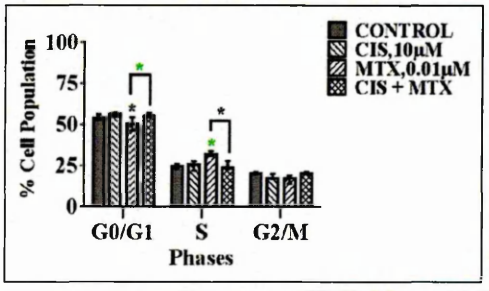
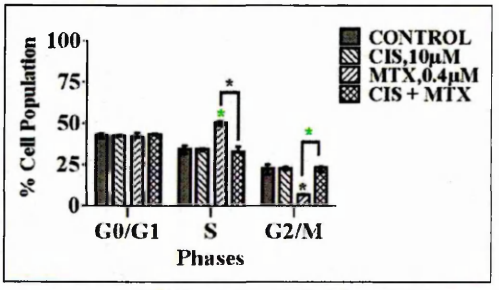


Figure 3.31: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of MTX and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A)Effect of Methotrexate (MTX) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of Methotrexate (MTX) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

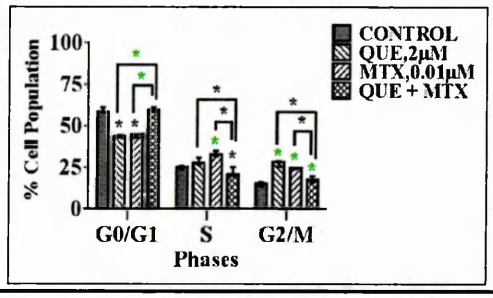
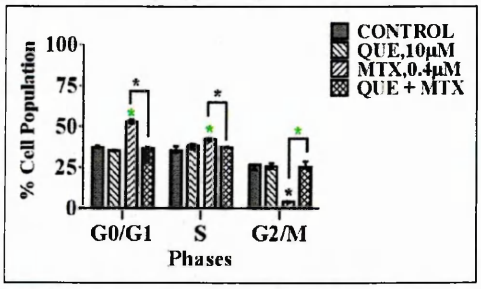
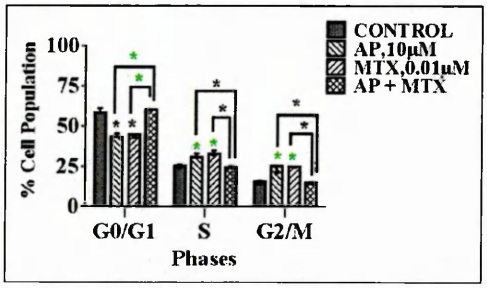
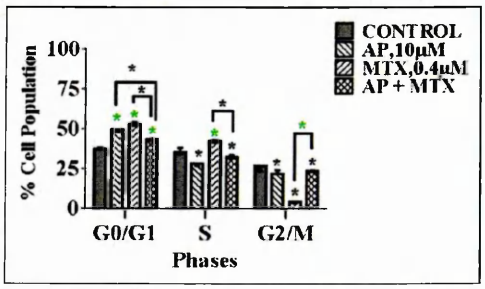
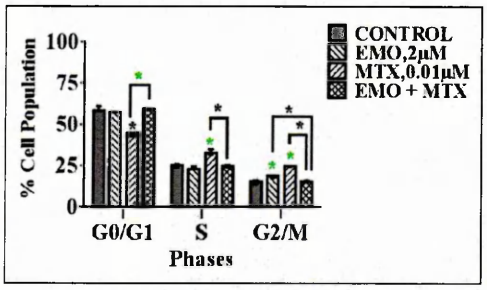
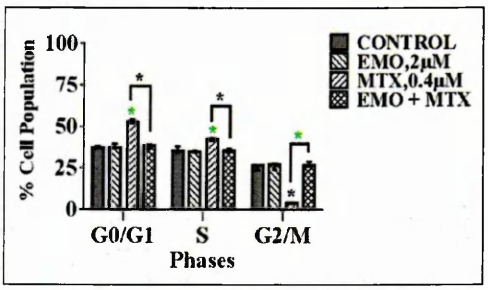
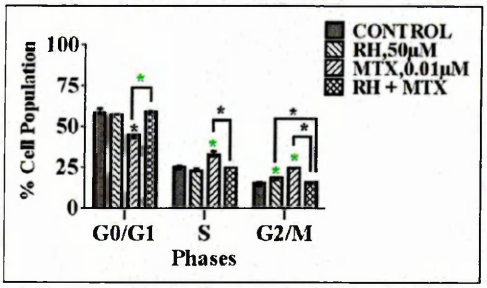
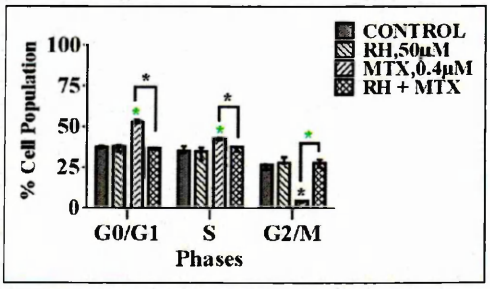
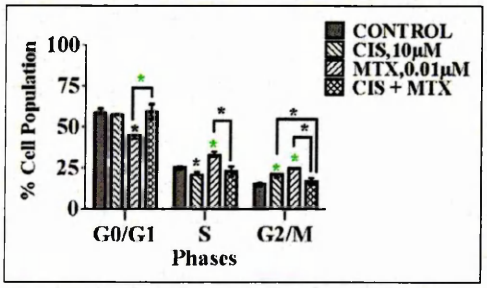
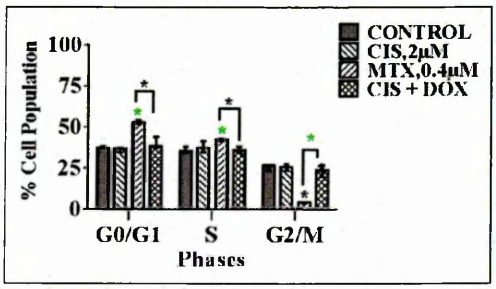
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.32: The effect of MTX when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with MTX and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.10.

Assays	Targets	Combination of Methotrexate and Polyphenols	The Significant Effects of Methotrexate (MTX) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	MTX + QUE (NSD)	C-ANTG	C-ANTG	C-ANTG	ANTG
		MTX + QUE (LSD)	C-ANTG	C-ANTG	C-ANTG	ANTG
Caspase 3 Activity	Apoptosis	MTX + QUE (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		MTX + QUE (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	MTX + QUE (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	MTX + QUE (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	MTX + AP (NSD)	C-ANTG	C-ANTG	ANTG	C-ANTG
		MTX + AP (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	MTX + AP (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		MTX + AP (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	MTX + AP (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	MTX + AP (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	MTX + EMO (NSD)	C-ANTG	C-ANTG	ANTG	ANTG
		MTX + EMO (LSD)	ANTG	C-ANTG	ANTG	ANTG
Caspase 3 Activity	Apoptosis	MTX + EMO (NS)	C-ANTG	C-ANTG	ANTG	C-ANTG
		MTX + EMO (LS)	ANTG	C-ANTG	ANTG	ANTG
Hoechst/ PI	Apoptosis	MTX + EMO (LS)	ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	MTX + EMO (LS)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	MTX + RH (NSD)	C-ANTG	C-ANTG	ANTG	C-ANTG
		MTX + RH (LSD)	ANTG	C-ANTG	ANTG	ANTG
Caspase 3 Activity	Apoptosis	MTX + RH (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		MTX + RH (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Hoechst/ PI	Apoptosis	MTX + RH (LSD)	ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	MTX + RH (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	MTX + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		MTX + CIS (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	MTX + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		MTX + CIS (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	MTX + CIS (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	MTX + CIS (LSD)	ANTG	ANTG	ANTG	ANTG

Table 3.10: A summary of the effects of methotrexate (MTX) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.3.2 6-Mercaptopurine (6-MP)

(A) Effect of **6-Mercaptopurine (6-MP)** ***Combined with Polyphenols on ATP Levels***

6-MP when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) produced a competitive antagonistic and/or antagonistic effects significantly increasing the ATP levels in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 3.33 and Table 3.11).

(B) Effect of **6-Mercaptopurine (6-MP)** ***Combined with Polyphenols on Apoptosis***

6-MP when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) produced a competitive antagonistic and/or antagonistic effects on apoptosis significantly decreasing the caspase 3 activity and the percentage of cells undergoing apoptosis in both lymphoid and myeloid leukaemia cell lines($p<0.05$) (Figure 3.34, 3.35 and Table 3.11). An example of the morphological assessment of apoptosis for 6-MP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.36.

(C) Effect of **6-Mercaptopurine (6-MP)** ***Combined with Polyphenols on Cell Cycle***

JURKAT lymphoid leukaemia cells had a significant increase in cell accumulation in S phase when treated with 6-MP, QUE or AP alone, and in G_0/G_1 phase when treated with EMO or RH alone; while there was no increase in cell accumulation in any phases of cell cycle when treated with CIS alone ($p<0.05$) (Figure 3.37). Treatment of JURKAT cells with 6-MP in combination with QUE, AP, EMO, RH or CIS had an antagonistic effect, there were no significant difference compared to the vehicle control ($p<0.05$), but there was a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.37 and Table 3.11).

CCRF-CEM lymphoid leukaemia cells had a significant increase in cell accumulation in S phase when treated with 6-MP, QUE, EMO or RH alone; and in G_2/M phase when treated with AP alone; while there was no significant increase of cell accumulation in any phases of cell cycle when treated with CIS alone ($p<0.05$) (Figure 3.37). Treatment of CCRF-CEM cells with 6-MP in combination with QUE, AP, EMO, RH or CIS had an antagonistic effect on cell cycle; there were no significant differences compared to the vehicle control ($p<0.05$) but there was a significant decrease in cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.37 and Table 3.11).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in S and G_2/M phases when treated with 6-MP or AP alone; in G_2/M phase when treated with QUE or CIS alone; however there was no significant increase of cell accumulation in any phases of cell

cycle when treated with EMO or RH alone ($p<0.05$) (Figure 3.37). Treatment of THP-1 cells with 6-MP in combination with QUE, AP, EMO, RH or CIS had an antagonistic effect on cell cycle; there were no significant differences between the combination treatment and the vehicle control ($p<0.05$), but there were significant decreases in the cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$(Figure 3.37 and Table 3.11).

KG-1a myeloid leukaemia cells had a significant increase of cell accumulation in G_0/G_1 and S phases when treated with 6-MP or AP alone; while there was no significant increase of cell accumulation in any phases of cell cycle when treated with QUE, EMO, RH or CIS alone ($p<0.05$) (Figure 3.37). Treatment of KG-1a cells with 6-MP in combination with QUE, AP, EMO, RH or CIS had an antagonistic effect on cell cycle; there were no significant differences compared to the vehicle control ($p<0.05$) but there were significantly decreased when compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.37 and Table 3.11).

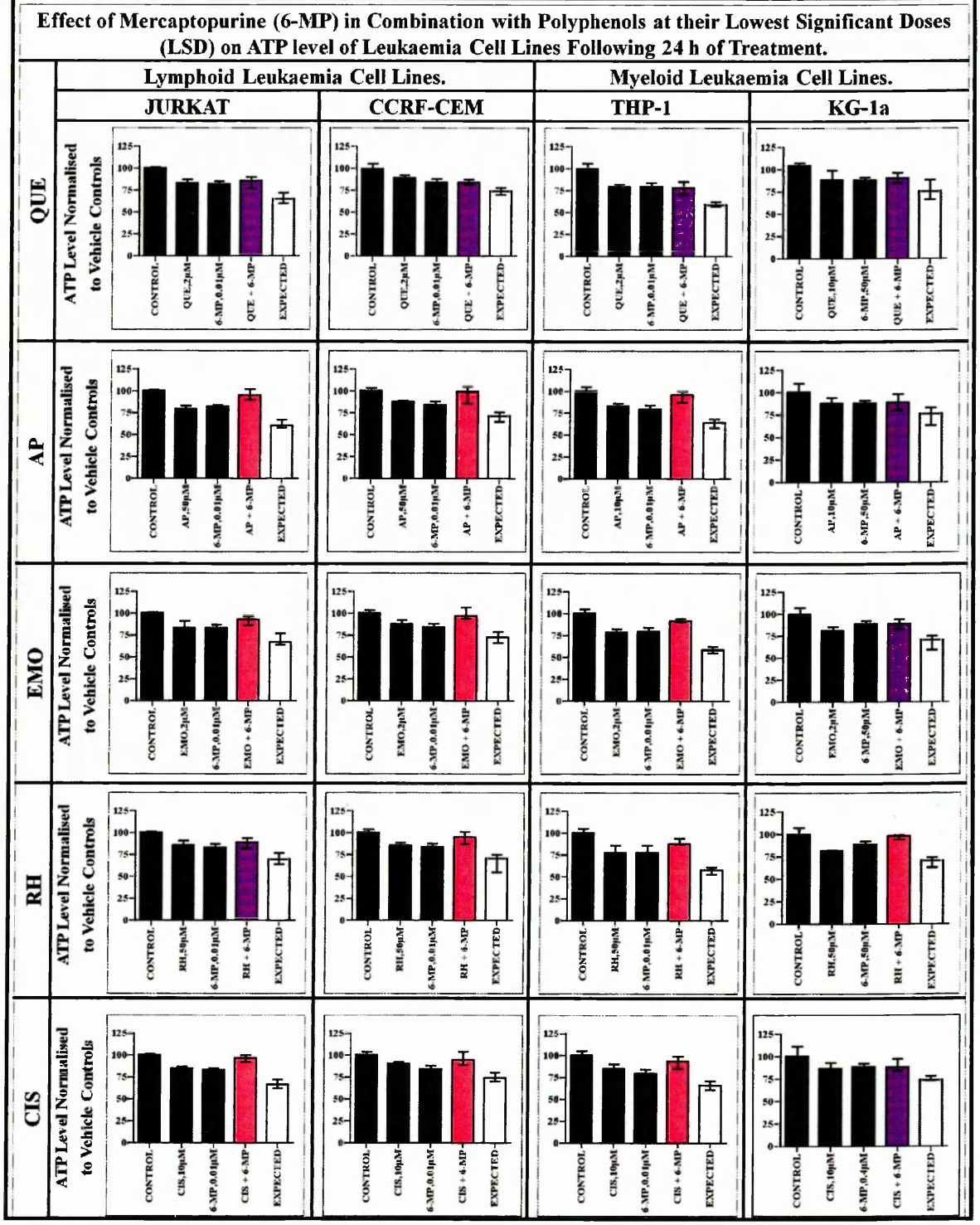


Figure 3.33: The effect of 6-MP used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

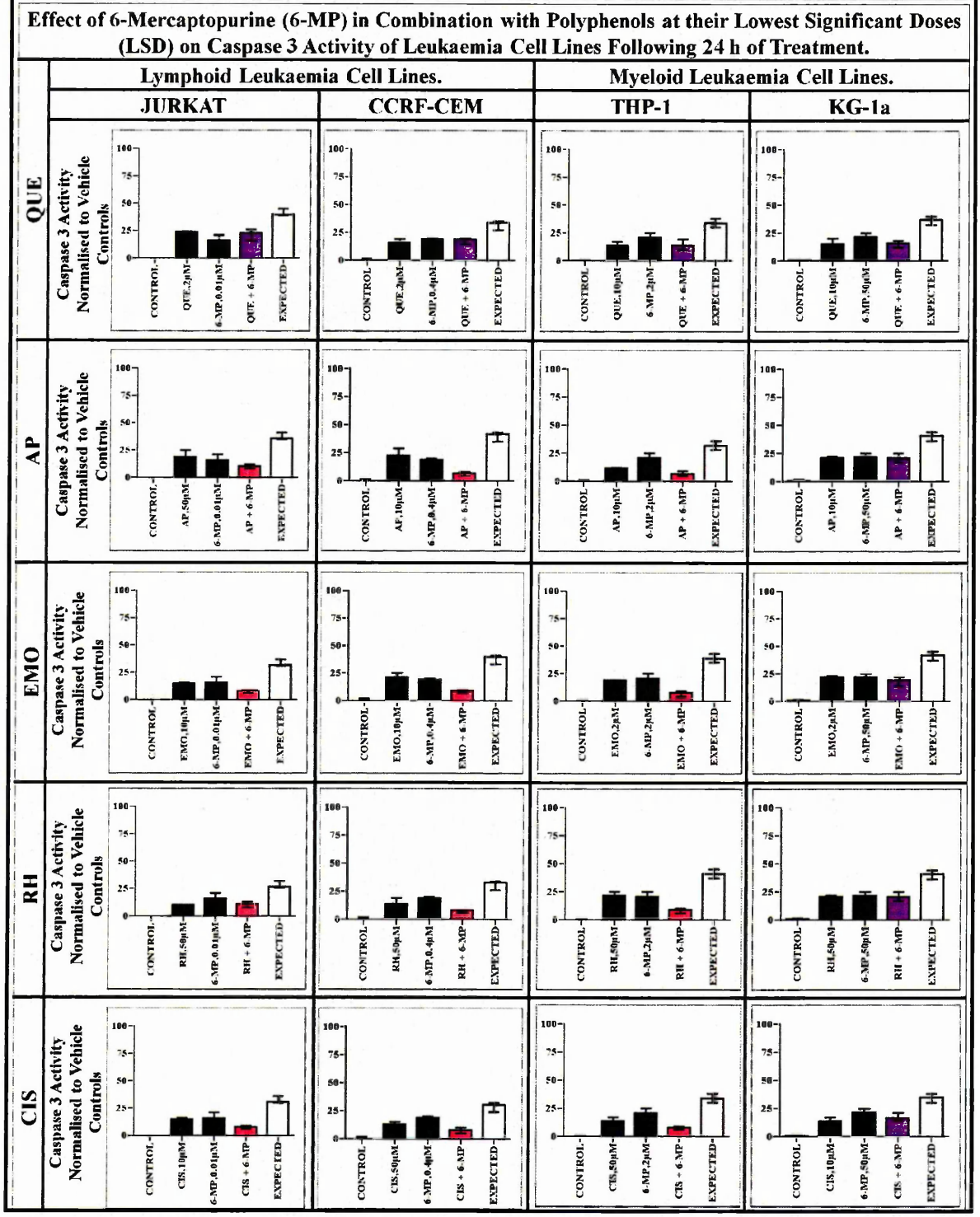


Figure 3.34: The effect of 6-MP used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

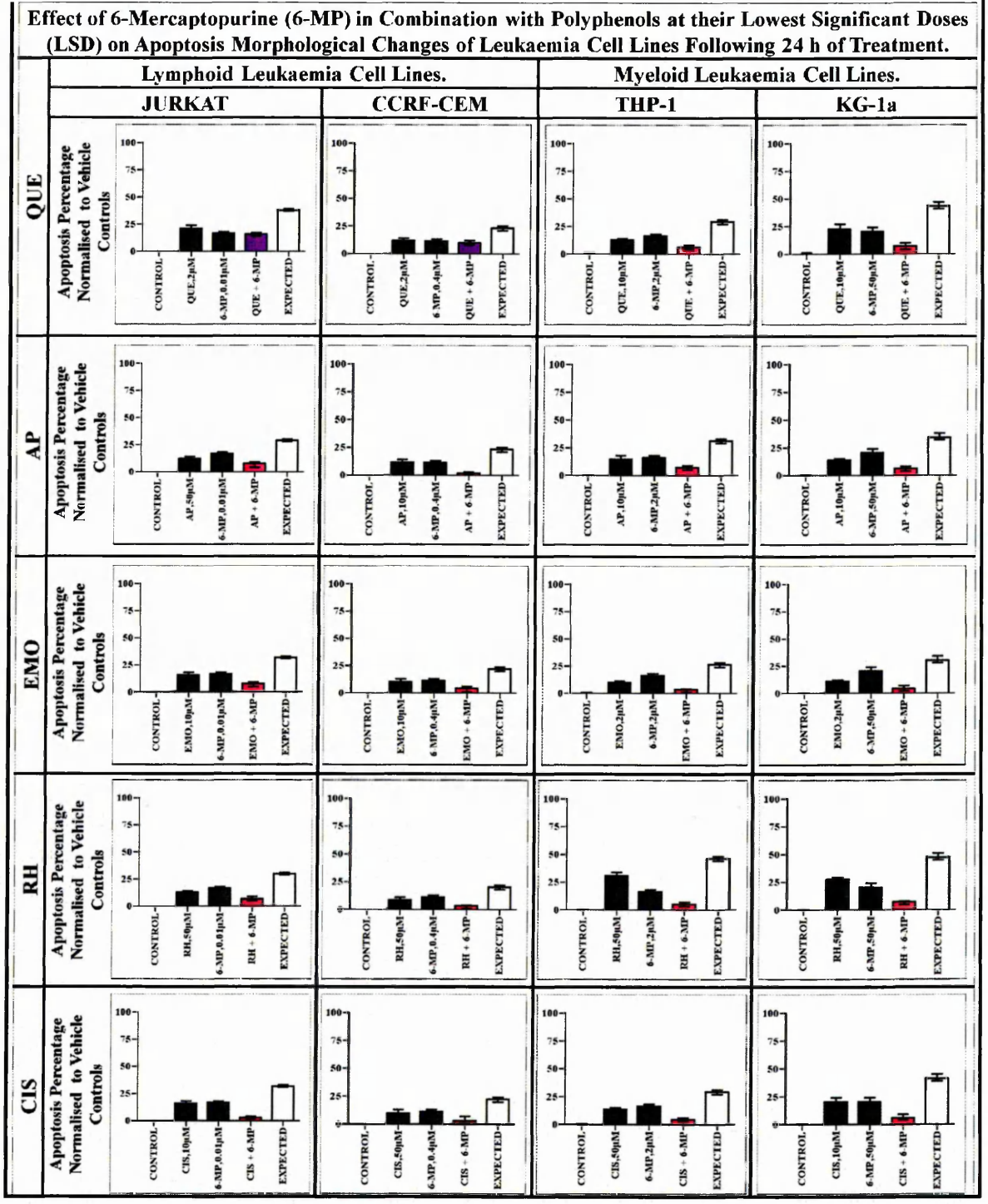


Figure 3.35: The effect of 6-MP in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

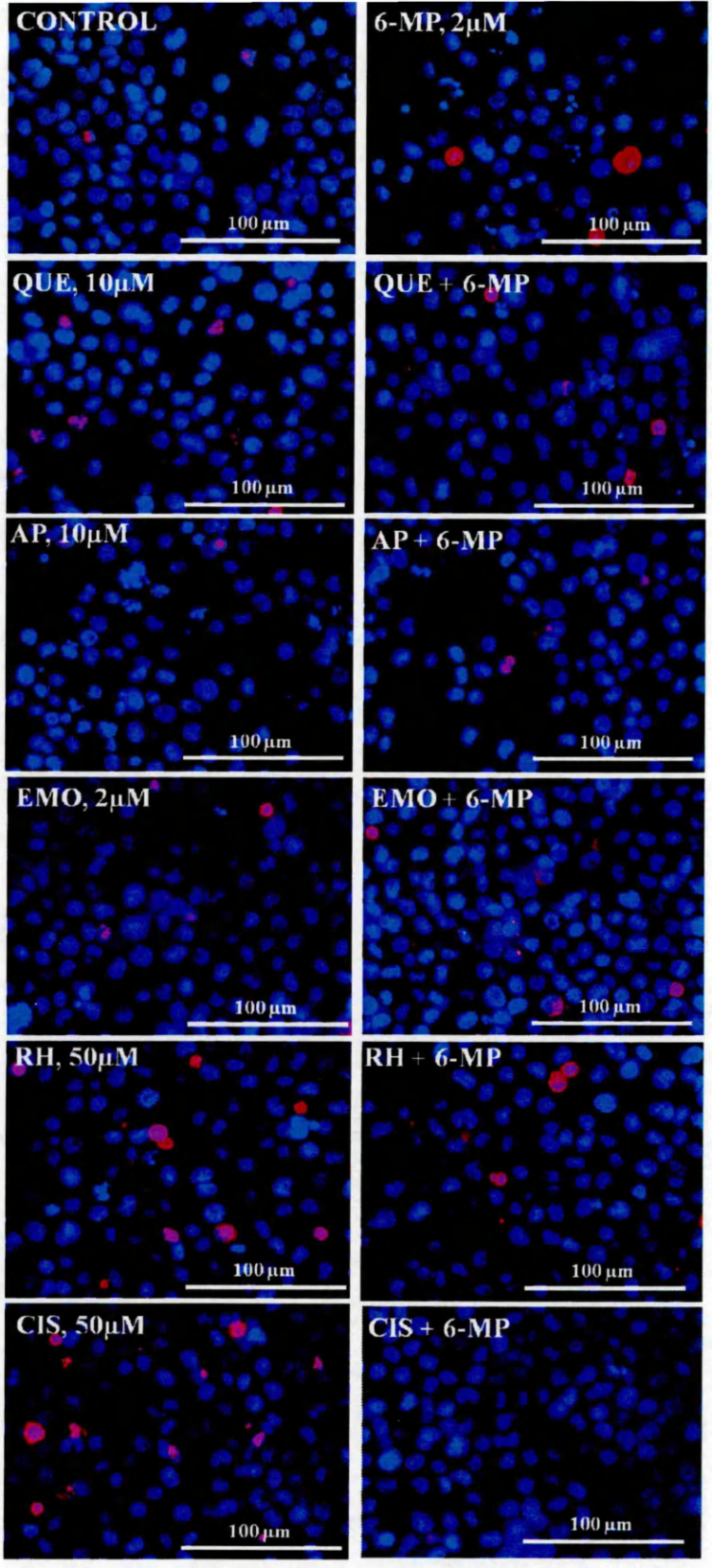
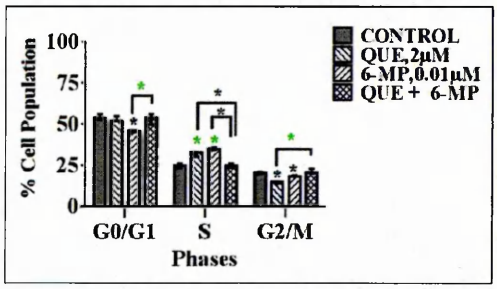
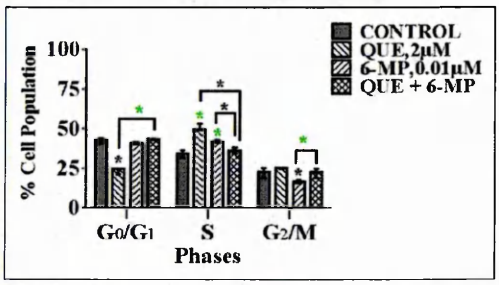
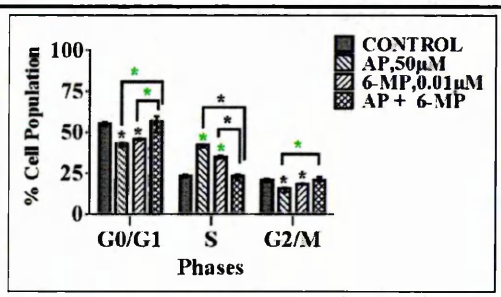
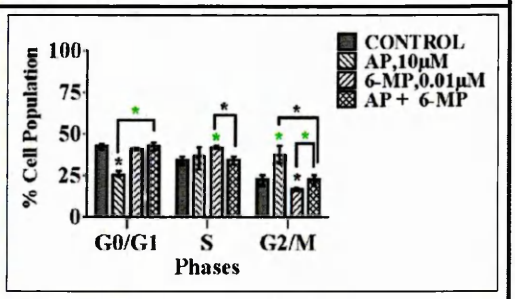
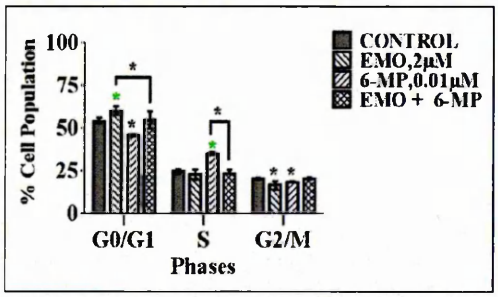
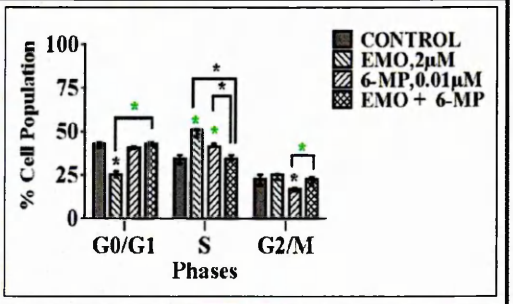
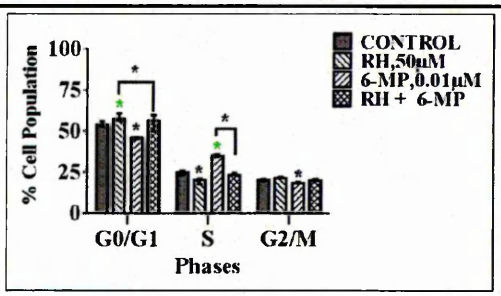
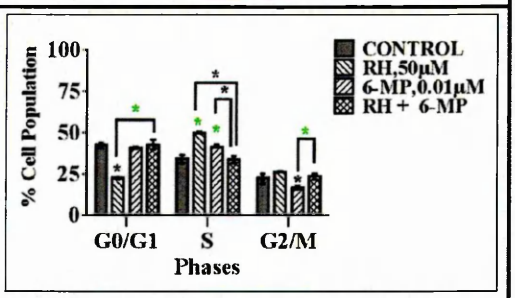
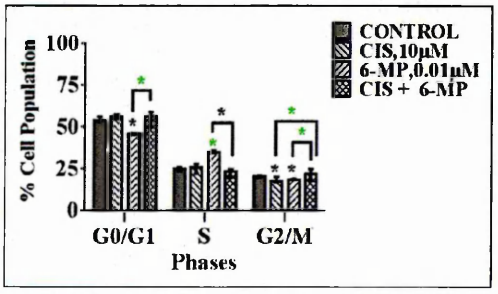
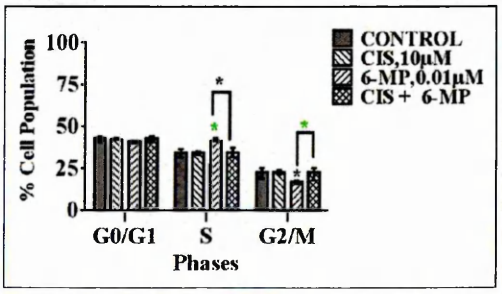


Figure 3.36: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of 6-MP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A) Effect of 6-Mercaptopurine (6-MP) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of 6-Mercaptopurine (6-MP) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

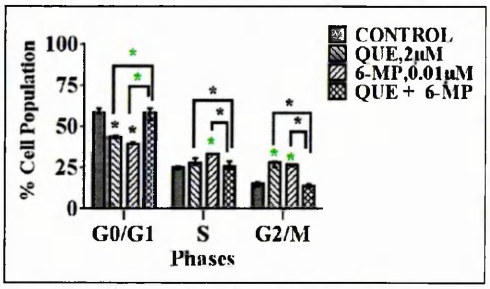
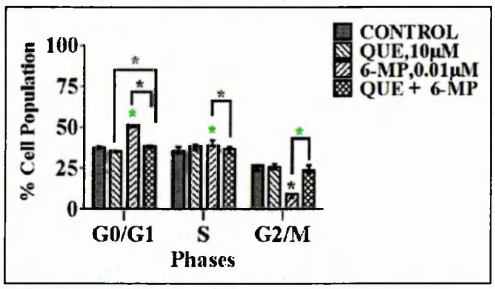
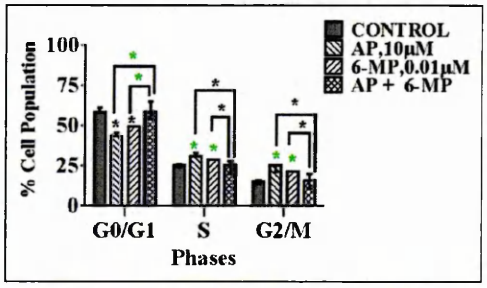
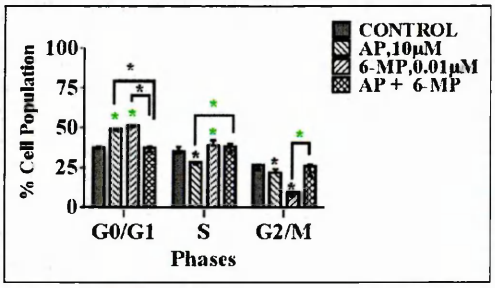
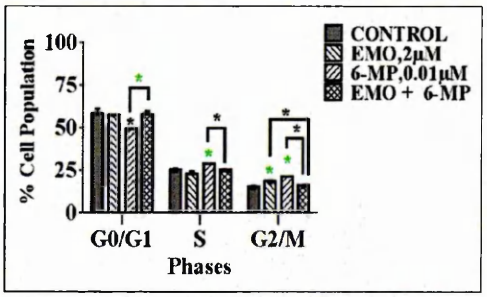
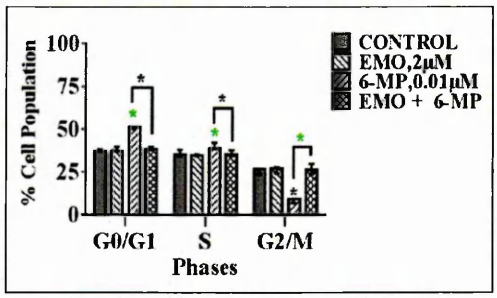
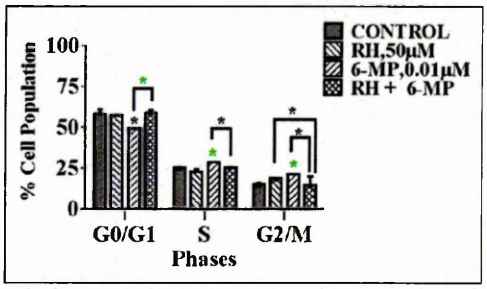
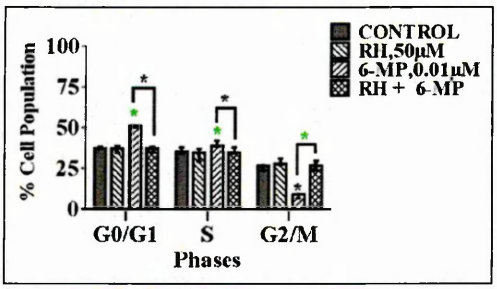
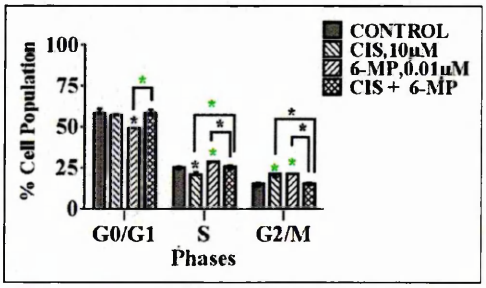
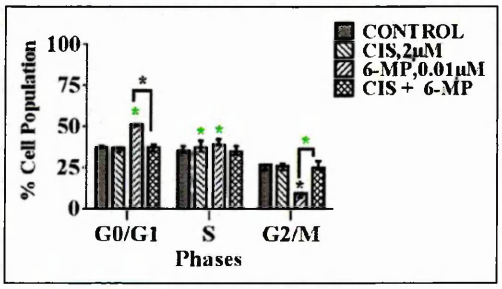
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.37: The effect of 6-MP when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.11.

Assays	Targets	Combination of Mercaptopurine and Polyphenols	The Significant Effects of Mercaptopurine (6-MP) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	6-MP + QUE (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + QUE (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	6-MP + QUE (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + QUE (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	6-MP + QUE (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	6-MP + QUE (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	6-MP + AP (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + AP (LSD)	ANTG	ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	6-MP + AP (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + AP (LSD)	ANTG	ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	6-MP + AP (LSD)	ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	6-MP + AP (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	6-MP + EMO (NSD)	C-ANTG	ANTG	C-ANTG	C-ANTG
		6-MP + EMO (LSD)	ANTG	ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	6-MP + EMO (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + EMO (LSD)	ANTG	ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	6-MP + EMO (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	6-MP + EMO (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	6-MP + RH (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + RH (LSD)	C-ANTG	ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	6-MP + RH (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + RH (LSD)	ANTG	ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	6-MP + RH (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	6-MP + RH (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	6-MP + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + CIS (LSD)	ANTG	ANTG	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	6-MP + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		6-MP + CIS (LSD)	ANTG	ANTG	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	6-MP + CIS (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	6-MP + CIS (LSD)	ANTG	ANTG	ANTG	ANTG

Table 3.11: A summary of the effects of mercaptopurine (6-MP) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.3.3 5-Fluorouracil (5-FLU)

(A) Effect of 5-Fluorouracil (5-FLU) Combined with Polyphenols on ATP Levels

5-FLU when used in combination with QUE and EMO produced an additive effect significantly decreasing the ATP levels in both lymphoid and myeloid leukaemia cell lines (Figure 3.38 and Table 3.12). The most potent effect was seen when 5-FLU was combined with AP, this combination caused a synergistic reduction in ATP levels in all the leukaemia cell lines ($p<0.05$) (Figure 3.38 and Table 3.12). Similarly, 5-FLU also had a synergistic interaction with RH in lymphoid leukaemia cell lines ($p<0.05$); whilst it was antagonistically interacted in myeloid leukaemia cell lines, ($p<0.05$) (Figure 3.38 and Table 3.12). However, 5-FLU when used in combination with CIS produced a competitive antagonistic or antagonistic effects significantly increased ATP level in both lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.45 and Table 3.12).

(B) Effect of 5-Fluorouracil (5-FLU) Combined with Polyphenols on Apoptosis

5-FLU when used in combination with QUE and EMO produced an additive effect on apoptosis with both the lymphoid and myeloid leukaemia cell lines (Figure 3.39, 3.40 and Table 3.12). The most potent affect was shown when 5-FLU was combined with AP, there was a synergistic increase in caspase 3 activity (Figure 3.39) and the percentage of cells undergoing apoptosis (Figure 3.40) in both lymphoid and myeloid leukaemia cell lines($p<0.05$) (Figure 3.39, 3.40 and Table 3.12). Similarly, 5-FLU acted synergistically when combined with RH increasing apoptosis in the lymphoid leukaemia cell lines, ($p<0.05$); whilst they were found to have an antagonistic effect on apoptosis in myeloid leukaemia cell lines ($p<0.05$) (Figure 3.39, 3.40 and Table 3.12). This competitive antagonistic or antagonistic effects was also seen when 5-FLU used in combination with CIS once again there was a significantly decreased in the caspase 3 activity (Figure 3.39) and the percentage of cell undergoing apoptosis (Figure 3.40) in both lymphoid and myeloid leukaemia cell lines($p<0.05$) (Figure 3.39, 3.40 and Table 3.12). An example of the morphological assessment of apoptosis for 5-FLU and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.41.

(C) Effect of 5-Fluorouracil (5-FLU) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had a significant increase of cell accumulation in S phase following treatment with 5-FLU, QUE or AP alone; and in G_0/G_1 phase following treatment with EMO or RH alone; while there were no significant increase of cell accumulation in any phases of cell cycle following treatment with CIS alone, ($p<0.05$) (Figure 3.42). Treatment of JURKAT cells with 5-FLU in combination with AP or RH had an interactive effect causing a significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.42 and Table 3.12). In

addition, treatment of JURKAT cells with 5-FLU in combination with QUE or EMO had a non-interactive effect, there were significant increases in cell accumulation in S phase when compared to the vehicle control; but this was not significant when compared to the phases of arrest caused by individual treatments alone ($p<0.05$) (Figure 3.42 and Table 3.12). In contrast, treatment of JURKAT cells with combination of 5-FLU and CIS had an antagonistic effect on cell cycle, although there were no significant differences compared to the vehicle control ($p<0.05$), but there were significant decreases in the cell accumulation compared to the phases arrested by individual treatment alone ($p<0.05$) (Figure 3.42 and Table 3.12).

CCRF-CEM lymphoid leukaemia cells had a significant increase of cell accumulation in S phase following treatment with 5-FLU, QUE, EMO or RH alone, and in G_2/M phase following treatment with AP alone; while they had no significant increase of cell accumulation in any phases of cell cycle following treatment with CIS alone, when compared to the vehicle control ($p<0.05$) (Figure 3.42). Treatment of CCRF-CEM cells with 5-FLU in combination with AP or RH had an interactive effect causing a significant increase of cell accumulation in S phase ($p<0.05$) (Figure 3.42 and Table 3.12). In addition, treatment of CCRF-CEM cells with 5-FLU in combination with QUE or EMO had a non-interactive effect, there were significant increases in cell accumulation in S phase when compared to the vehicle control; but these were not significant when compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.42 and Table 3.12). On the other hand, treatment of CCRF-CEM cells with combination of 5-FLU and CIS had an antagonistic effect on cell cycle, although there were no significant differences compared to the vehicle control ($p<0.05$) but there were significant decreases in the cell accumulation compared to the phases arrested by individual treatment alone ($p<0.05$) (Figure 3.42 and Table 3.12).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in G_0/G_1 phase following treatment with 5-FLU alone, in G_2/M phase following treatment with QUE or CIS alone, and in S and G_2/M phases following treatment with AP alone. There were no significant increases of cell accumulation in any phases of cell cycle following treatment with EMO or RH alone, when compared to the vehicle control ($p<0.05$) (Figure 3.42). Treatment of THP-1 cells with combination of 5-FLU and AP had an interactive effect causing a significant increase of cell accumulation in G_0/G_1 phase when compared to the vehicle control and individual treatments ($p<0.05$) (Figure 3.42 and Table 3.12). In addition, treatment of THP-1 cells with 5-FLU in combination with QUE or EMO had a non-interactive effect, there were significant increases in cell accumulation in G_0/G_1 when compared to the vehicle control; but these were not significant when compared to phases arrested by individual treatments alone effect ($p<0.05$) (Figure 3.42 and Table 3.12). In contrast, treatment of THP-1 cells with 5-FLU in combination with RH or CIS had an antagonistic effect, there were no significant differences

compared to the vehicle control ($p<0.05$), but there were significant decreases in the cell accumulation compared to the phases arrested by individual treatment alone ($p<0.05$) (Figure 3.42 and Table 3.12).

KG-1a myeloid leukaemia cells had a significant increase of cell accumulation in G_0/G_1 phase following treatment with 5-FLU and AP alone; while they had no significant increase of cell accumulation in any phases of cell cycle following treatment with QUE, EMO, RH or CIS alone when compared to the vehicle control ($p<0.05$) (Figure 3.42). Treatment of KG-1a cells with combination of 5-FLU and AP had an interactive effect causing a significant increase of cell accumulation in G_0/G_1 phase when compared to the vehicle control and individual drugs treatments ($p<0.05$) (Figure 3.42 and Table 3.12). In contrast, treatment of KG-1a cells with 5-FLU in combination with QUE or EMO had a non-interactive effect there were significant increases in cell accumulation in G_0/G_1 when compared to the vehicle control; but these were not significant when compared to phases arrested by individual treatments alone ($p<0.05$) (Figure 3.42 and Table 3.12). Alternatively, treatment of KG-1a cells with 5-FLU in combination with RH or CIS had an antagonistic effect, although there were no significant differences compared to the vehicle control ($p<0.05$), there were significant decreases in the cell accumulation compared to the phases arrested by individual treatment alone ($p<0.05$) (Figure 3.42 and Table 3.12).

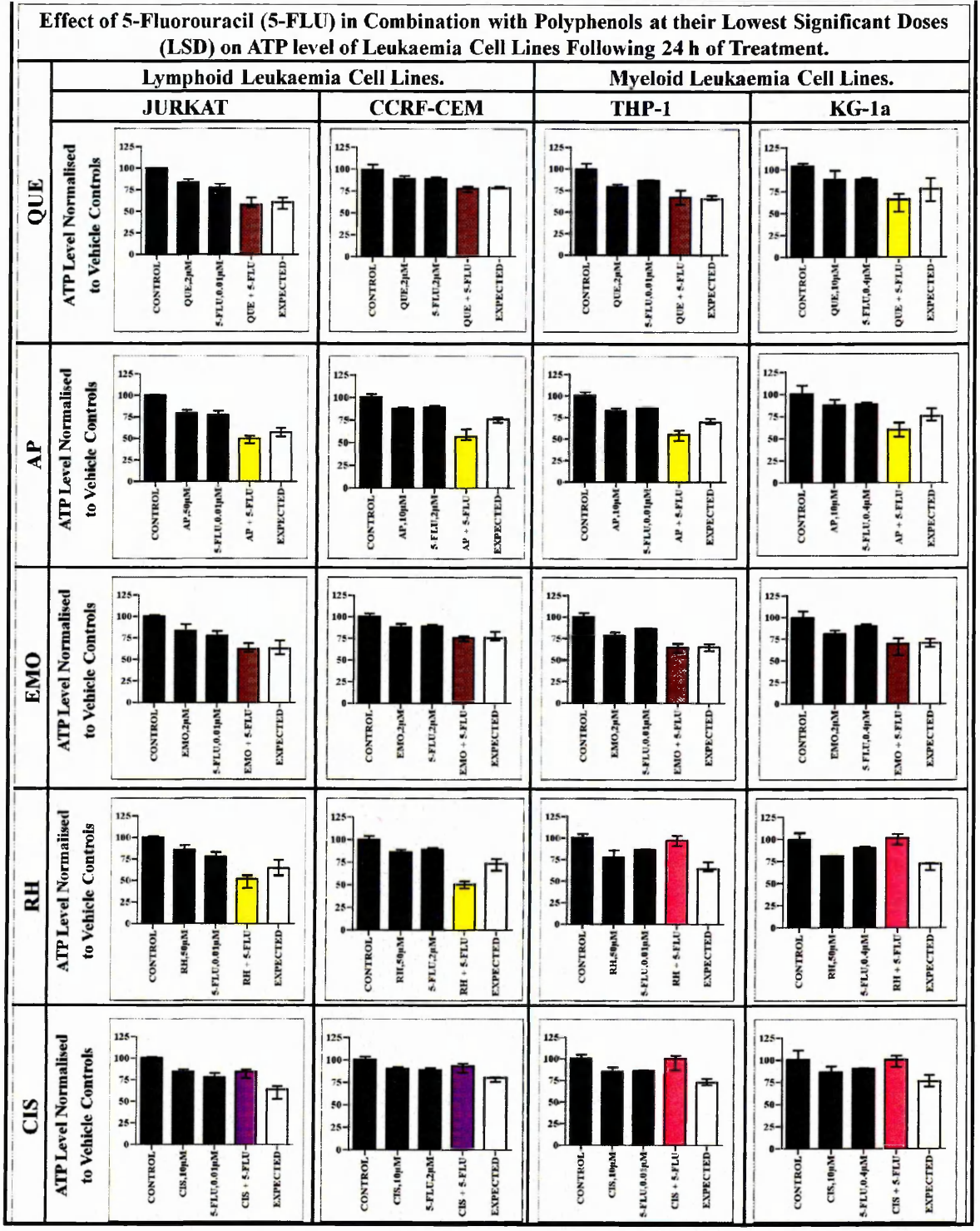


Figure 3.38: The effect of 5-FLU when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines. This was evaluated by CellTiter-Glo® assay. Cells were treated with 5-FLU and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

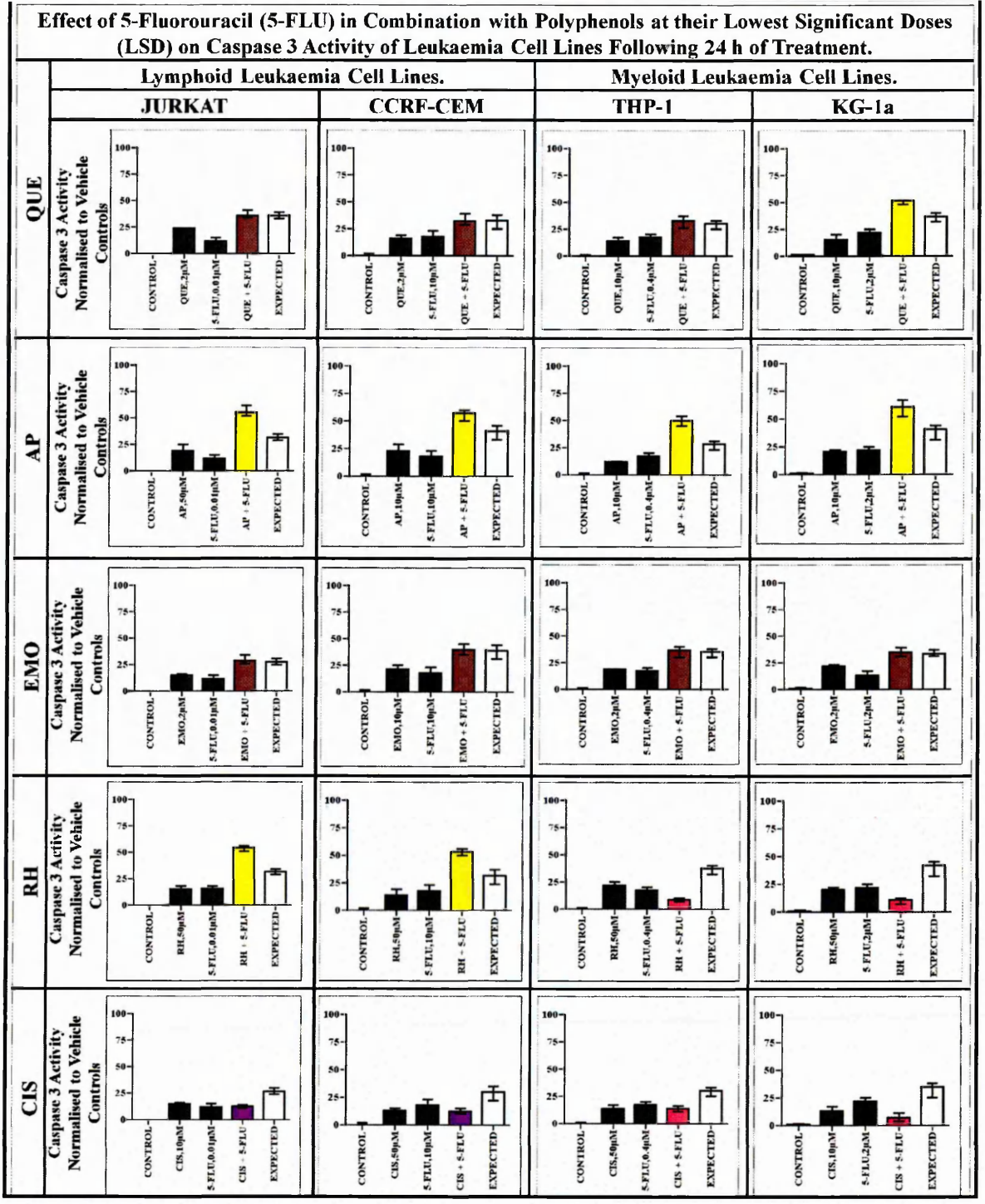


Figure 3.39: The effect of 5-FLU used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with 5-FLU and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

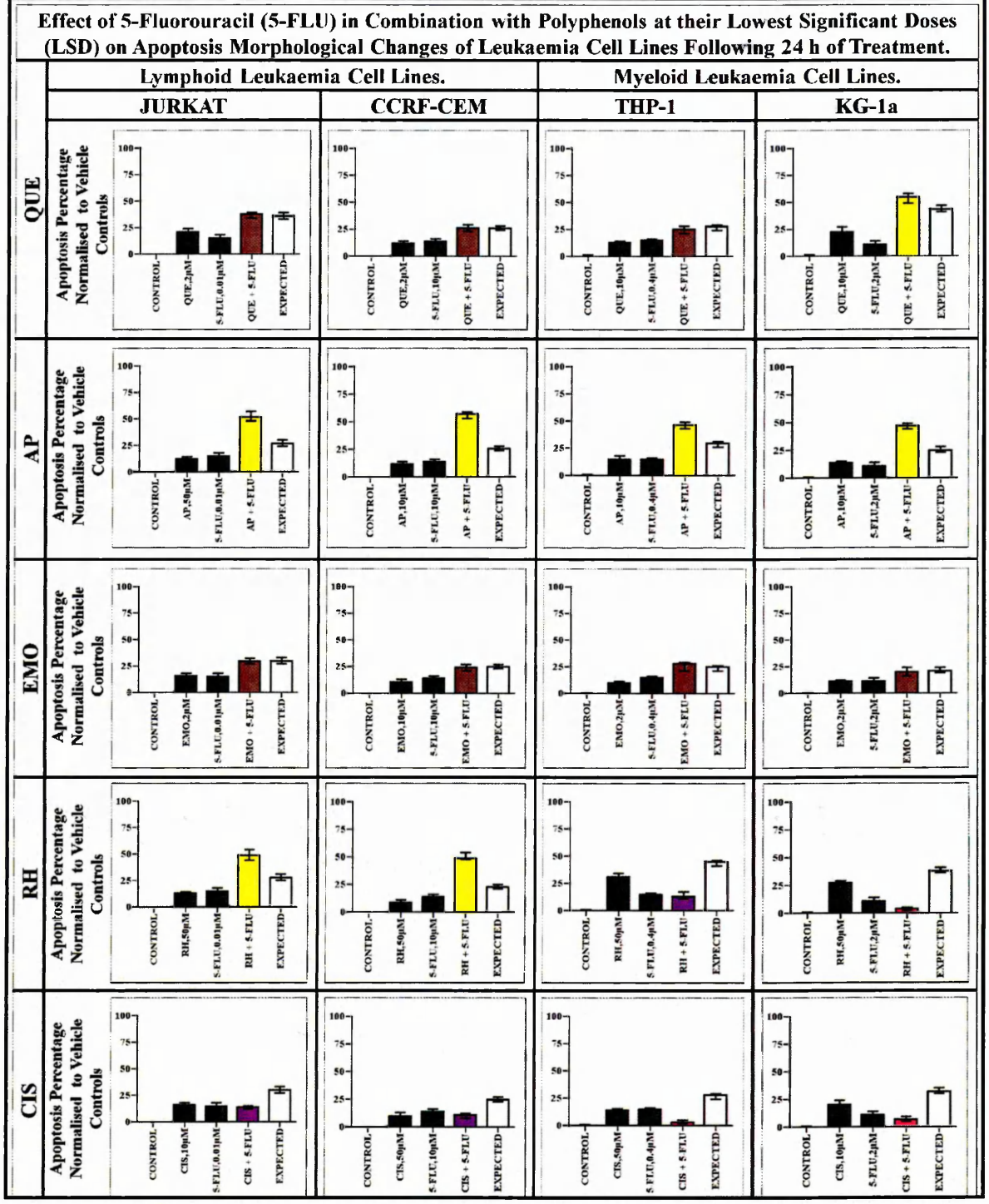


Figure 3.40: The Effect of 5-FLU when used in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with 5-FLU and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

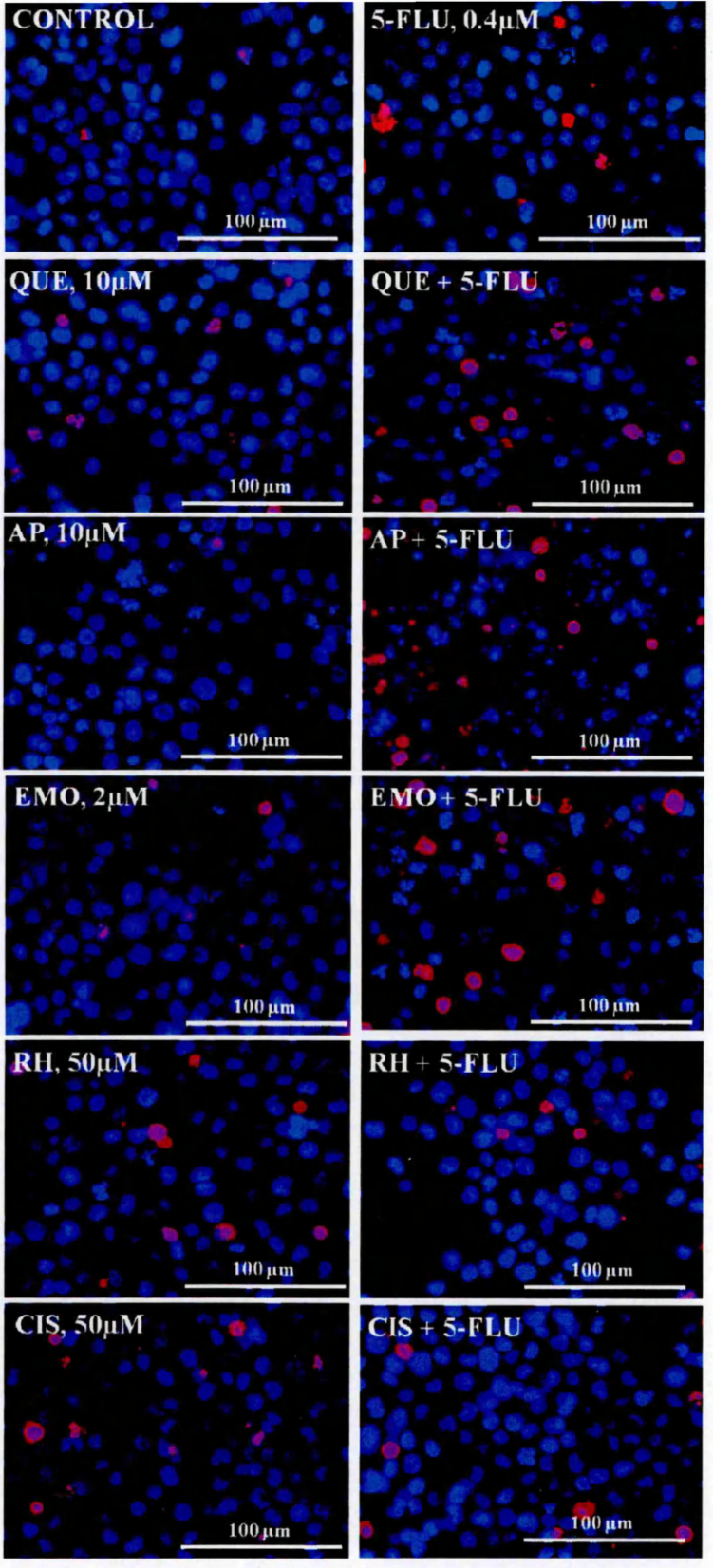
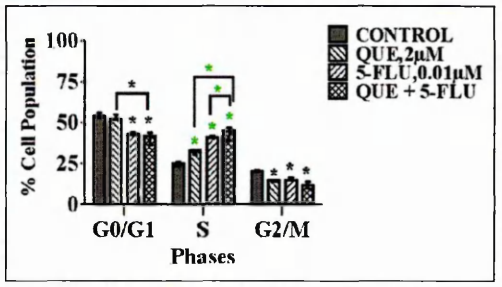
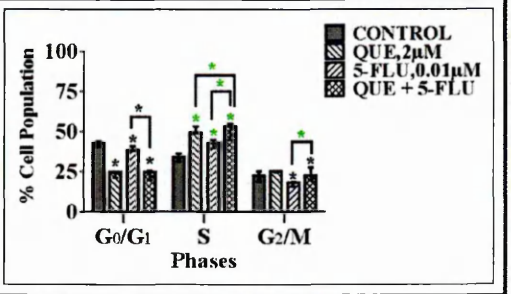
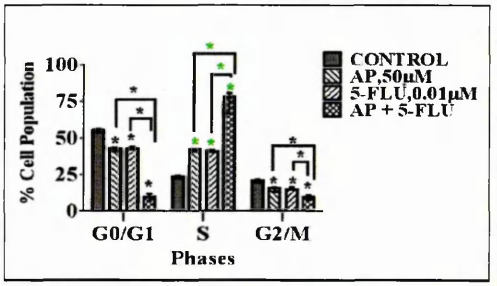
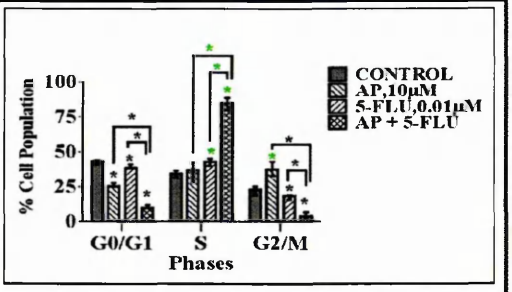
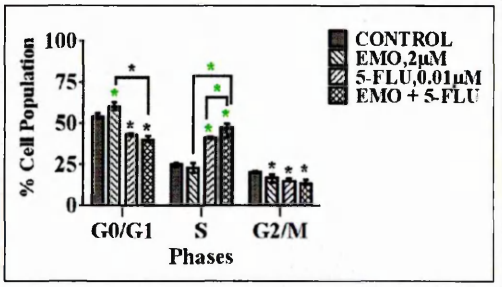
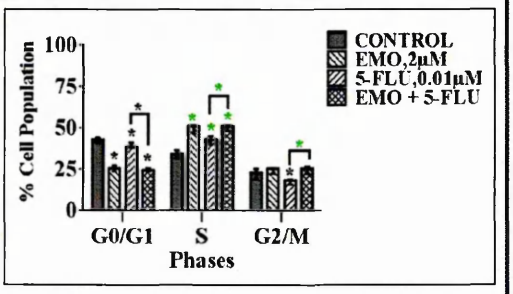
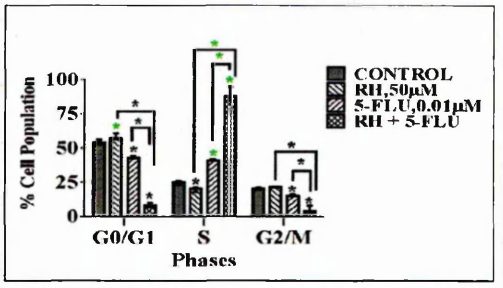
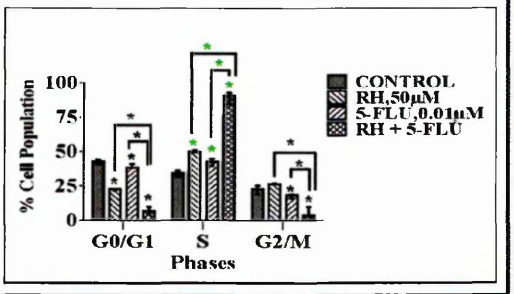
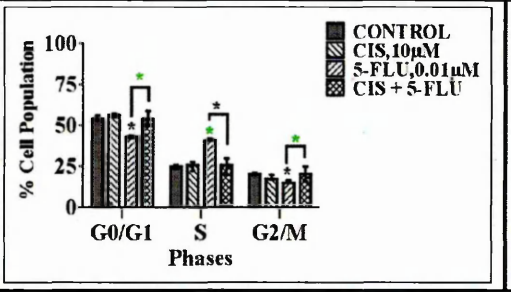
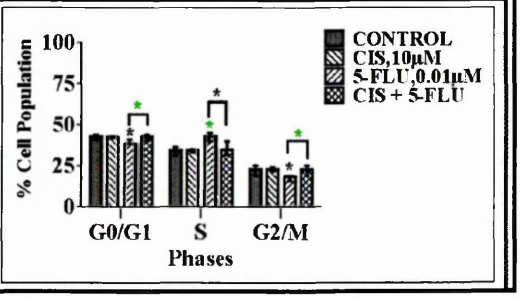


Figure 3.41: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of 5-FLU and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A) Effect of 5-Fluorouracil (5-FLU) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of 5-Fluorouracil (5-FLU) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

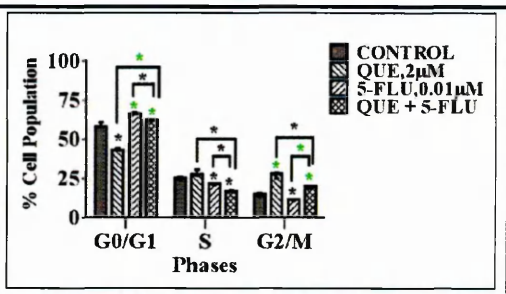
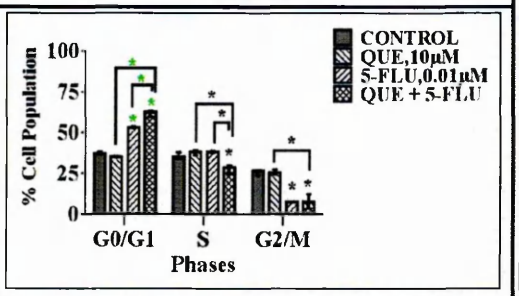
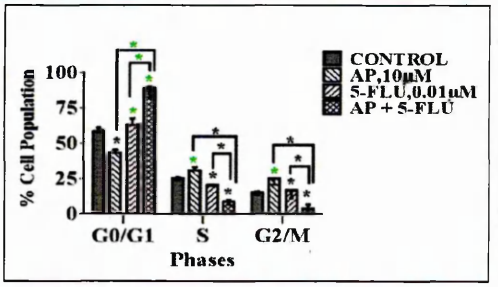
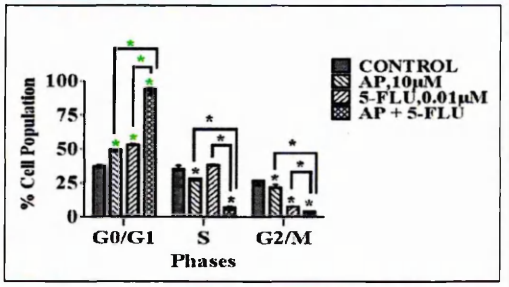
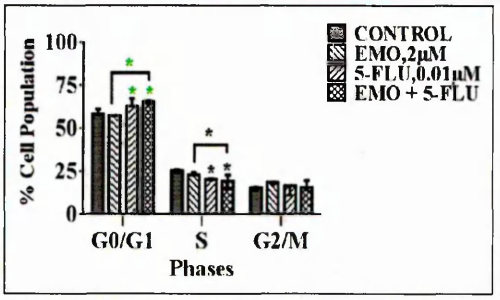
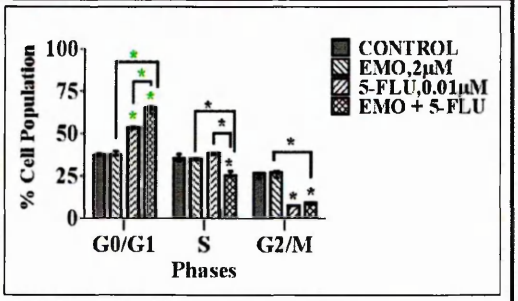
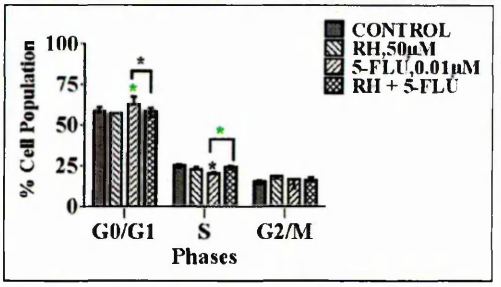
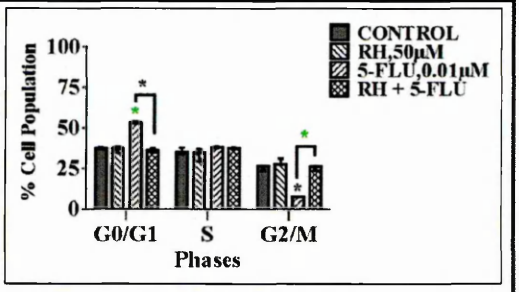
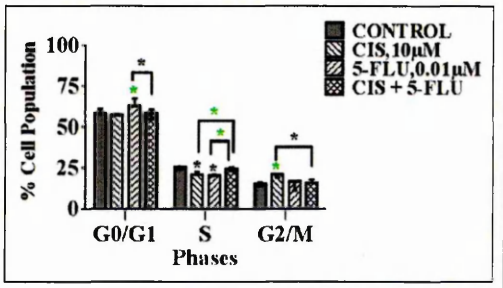
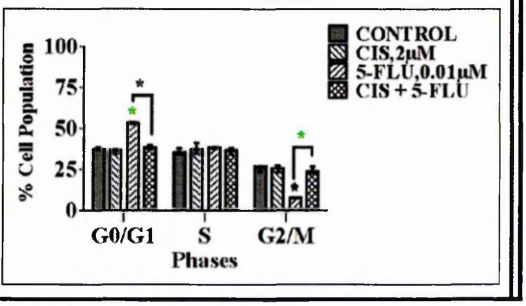
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.42: The effect of 5-FLU when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with 5-FLU and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.12.

Assays	Targets	Combination of Fluorouracil and Polyphenols	The Significant Effects of 5-Fluorouracil and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	5-FLU + QUE (NSD)	ADD	ADD	ADD	SYN
		5-FLU + QUE (LSD)	ADD	ADD	ADD	SYN
Caspase 3 Activity	Apoptosis	5-FLU + QUE (NSD)	ADD	ADD	ADD	SYN
		5-FLU + QUE (LSD)	ADD	ADD	ADD	SYN
Hoechst/ PI	Apoptosis	5-FLU + QUE (LSD)	ADD	ADD	ADD	SYN
Cell Cycle/PI	Cell Cycle	5-FLU + QUE (LSD)	Non Interaction	Non Interaction	Non Interaction	Non Interaction
CellTiter-Glow	ATP Level	5-FLU + AP (NSD)	SYN	SYN	SYN	SYN
		5-FLU + AP (LSD)	SYN	SYN	SYN	SYN
Caspase 3 Activity	Apoptosis	5-FLU + AP (NSD)	SYN	SYN	SYN	SYN
		5-FLU + AP (LSD)	SYN	SYN	SYN	SYN
Hoechst/ PI	Apoptosis	5-FLU + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Cell Cycle	5-FLU + AP (LSD)	Interaction	Interaction	Interaction	Interaction
CellTiter-Glow	ATP Level	5-FLU + EMO (NSD)	ADD	ADD	ADD	ADD
		5-FLU + EMO (LSD)	ADD	ADD	ADD	ADD
Caspase 3 Activity	Apoptosis	5-FLU + EMO (NSD)	ADD	ADD	ADD	ADD
		5-FLU + EMO (LSD)	ADD	ADD	ADD	ADD
Hoechst/ PI	Apoptosis	5-FLU + EMO (LSD)	ADD	ADD	ADD	ADD
Cell Cycle/PI	Cell Cycle	5-FLU + EMO (LSD)	Non Interaction	Non Interaction	Non Interaction	Non Interaction
CellTiter-Glow	ATP Level	5-FLU + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		5-FLU + RH (LSD)	SYN	SYN	ANTG	ANTG
Caspase 3 Activity	Apoptosis	5-FLU + RH (NSD)	SYN	SYN	C-ANTG	C-ANTG
		5-FLU + RH (LSD)	SYN	SYN	ANTG	ANTG
Hoechst/ PI	Apoptosis	5-FLU + RH (LSD)	SYN	SYN	C-ANTG	ANTG
Cell Cycle/PI	Cell Cycle	5-FLU + RH (LSD)	Interaction	Interaction	ANTG	ANTG
CellTiter-Glow	ATP Level	5-FLU + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		5-FLU + CIS (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Caspase 3 Activity	Apoptosis	5-FLU + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
		5-FLU + CIS (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Hoechst/ PI	Apoptosis	5-FLU + CIS (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Cell Cycle	5-FLU + CIS (LSD)	ANTG	ANTG	ANTG	ANTG

Table 3.12: A summary of the effects of 5-fluorouracil (5-FLU) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.3.2.4 Effects of Tyrosine Kinase Inhibitors Agent in Combined with Polyphenols

3.3.2.4.1 Imatinib (IM)

(A) Effect of Imatinib (IM) Combined with Polyphenols on ATP Levels

IM was caused a significant synergistic reduction of ATP levels when used in combination with AP in the myeloid cell lines (THP-1 and KG1a) ($p<0.05$). In contrast, in the lymphoid cell lines (JURKAT and CCRF-CEM), this combination caused a competitively antagonistic or antagonistic effects, significantly increased ATP level when compared to the expected values and individual drugs ($p<0.05$) (Figure 3.43 and Table 3.13). IM when combined with EMO produced an additive effects on reduction of ATP level in both the lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.43 and Table 3.13). In addition, IM when used in combination with RH produced additive effects on reduction of ATP level while only in the lymphoid cell lines (JURKAT, CCRF-CEM) ($p<0.05$); however, they produced competitively antagonistic or antagonistic effects causing significantly increased in ATP level in the myeloid leukaemia cell lines (THP-1 and KG1a) ($p<0.05$) (Figure 3.43 and Table 3.13). Similarly, IM in combination with QUE and CIS produced competitively antagonistic or antagonistic effects causing significantly increased ATP level when compared to the expected values and individual drugs, but this time in both lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.43 and Table 3.13).

(B) Effect of Imatinib (IM) Combined with Polyphenols on Apoptosis

IM when used in combination with AP produced a synergistic effects on apoptosis in myeloid cell lines (THP-1 and KG1a), significantly increasing caspase 3 activity and the percentage of cell undergoing apoptosis ($p<0.05$) (Figure 3.44, 3.45 and Table 3.13). However, in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) the combination of IM and AP produced competitively antagonistic or antagonistic effects, significantly decreasing the caspase 3 activity and the percentage of cells undergoing apoptosis, when compared to the individual treatments alone and the expected value ($p<0.05$) (Figure 3.44, 3.45 and Table 3.13). IM when used in combination with EMO produced an additive effect on caspase 3 activity and the percentage of cell undergoing apoptosis in both lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 3.44, 3.45 and Table 3.13). Furthermore, IM in combination with RH also produced additive effects on apoptosis, however only in the lymphoid cell lines (JURKAT and CCRF-CEM). In the myeloid leukaemia cell lines (THP-1 and KG1a) this combination produced a competitively antagonistic or antagonistic effects on apoptosis ($p<0.05$) (Figure 3.44, 3.45 and Table 3.13). Similarly, IM when used combination with QUE or CIS in both lymphoid and myeloid leukaemia cell lines, produced competitively antagonistic or antagonistic effects, significantly decreasing caspase 3 activity and the percentage of a cell undergoing apoptosis when compared to the individual treatments alone and the expected

values ($p<0.05$) (Figure 3.44, 3.45 and Table 3.13). An example of the morphological assessment of apoptosis for IM and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination at their LSD for 24 h on THP-1 myeloid leukaemia cell lines is showed in Figure 3.46.

(C) Effect of Imatinib (IM) Combined with Polyphenols on Cell Cycle

JURKAT lymphoid leukaemia cells had a significant increase of cell accumulation in G2/M phase when treated with IM alone, in S phase when treated with QUE or AP alone, and in G_0/G_1 phase when treated with EMO or RH alone; while they had no significant increase of cell accumulation in any phases of cell cycle when treated with CIS alone, when compared to the vehicle control ($p<0.05$) (Figure 3.47). Treatment of JURKAT cells with IM in combination with EMO or RH had an interactive effect that significantly increased the cell accumulation in G_0/G_1 phase when compared to the vehicle control; but this was not significant when compared to phases arrested by EMO or RH alone effect ($p<0.05$) (Figure 3.47 and Table 3.13). In contrast, treatment of JURKAT cells with IM in combination with QUE, AP or CIS had an antagonistic effect, there were no significant differences compared to the vehicle control ($p<0.05$), but there was a significant decrease in cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.47 and Table 3.13).

CCRF-CEM lymphoid leukaemia cells had a significant increase of cell accumulation in S phase when treated with IM, QUE, EMO or RH alone, in G_2/M phase when treated with AP alone; but there was no significant increase in cell accumulation in any phases of cell cycle when treated with CIS alone, when compared to the vehicle control ($p<0.05$) (Figure 3.47). Treatment of CCRF-CEM cells with IM in combination with EMO or RH had an interactive effect significantly increasing cell accumulation in S phase when compared to the vehicle control; but not when compared to phases arrested by EMO or RH alone ($p<0.05$) (Figure 3.47 and Table 3.13). In contrast when CCRF-CEM cells were treated with IM in combination with QUE, AP or CIS there was an antagonistic effect, there were no significant differences compared to the vehicle control ($p<0.05$) but there was a significant decrease of cell accumulation compared to the phases arrested by individual treatments alone ($p<0.05$) (Figure 3.47 and Table 3.13).

THP-1 myeloid leukaemia cells had a significant increase of cell accumulation in S phase when treated with IM alone, in G_2/M phase when treated with QUE or CIS alone, and in S and G_2/M phases when treated with AP alone; while they had no significant increase of cell accumulation in any phases of cell cycle when treated with EMO or RH alone when compared to the vehicle control ($p<0.05$) (Figure 3.47). Treatment of THP-1 cells with IM in combination with AP or EMO had an interactive effect which significantly increased the cell

accumulation in S phase when compared to the vehicle control; but this was not significant when compared to phases arrested by individual treatments alone ($p<0.05$) (Figure 3.47 and Table 3.13). In contrast when THP-1 cells were treated with IM in combination with QUE, RH or CIS there was an antagonistic effect, there were no significant differences compared to the vehicle control ($p<0.05$) but there was a significant decrease of cell accumulation compared to the phases arrested by the individual treatments alone ($p<0.05$) (Figure 3.47 and Table 3.13).

KG-1a myeloid leukaemia cells had a significant increase of cell accumulation in G_0/G_1 phase when treated with IM or AP alone; while they had no significant increase of cell accumulation in any phases of cell cycle when treated with QUE, EMO, RH or CIS alone when compared to the vehicle control ($p<0.05$) (Figure 3.47). Treatment of KG-1a cells with IM in combination with AP or EMO had an interactive effect which significantly increased the cell accumulation in G_0/G_1 phase when compared to the vehicle control; but this was not significant when compared to phases arrested by the individual treatments alone ($p<0.05$) (Figure 3.47 and Table 3.13). On other hand, the treatment of KG-1a cells with IM in combination with QUE, RH or CIS had an antagonistic effect, there were no significant differences compared to the vehicle control ($p<0.05$), but there were significant decreases in cell accumulation compared to the phases arrested by the individual treatments alone ($p<0.05$) (Figure 3.47 and Table 3.13).

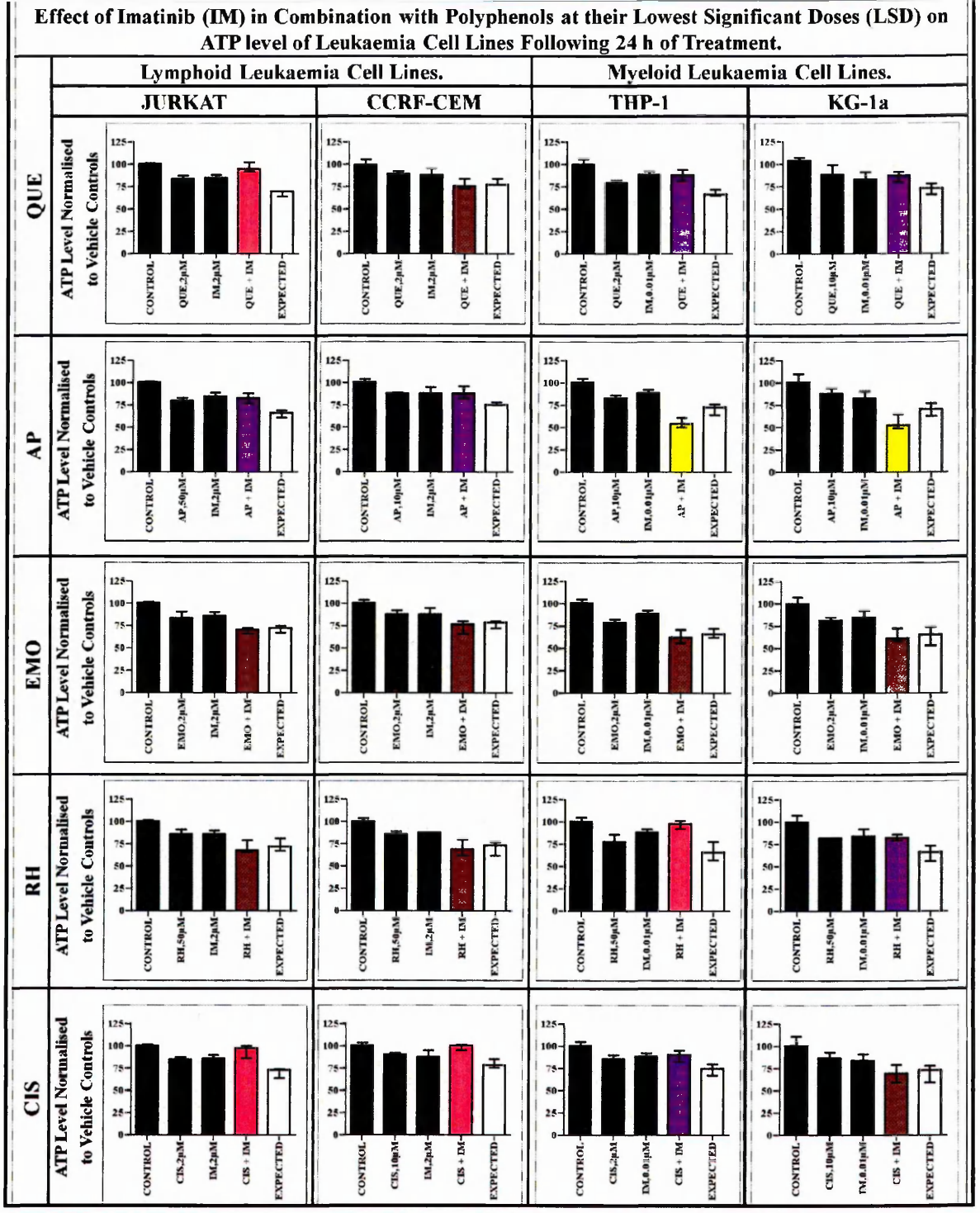


Figure 3.43: **The effect of IM when used in combination with QUE, AP, EMO, RH or CIS on ATP levels of leukaemia cell lines.** This was evaluated by CellTiter-Glo® assay. Cells were treated with IM and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% cell viability. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on ATP levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

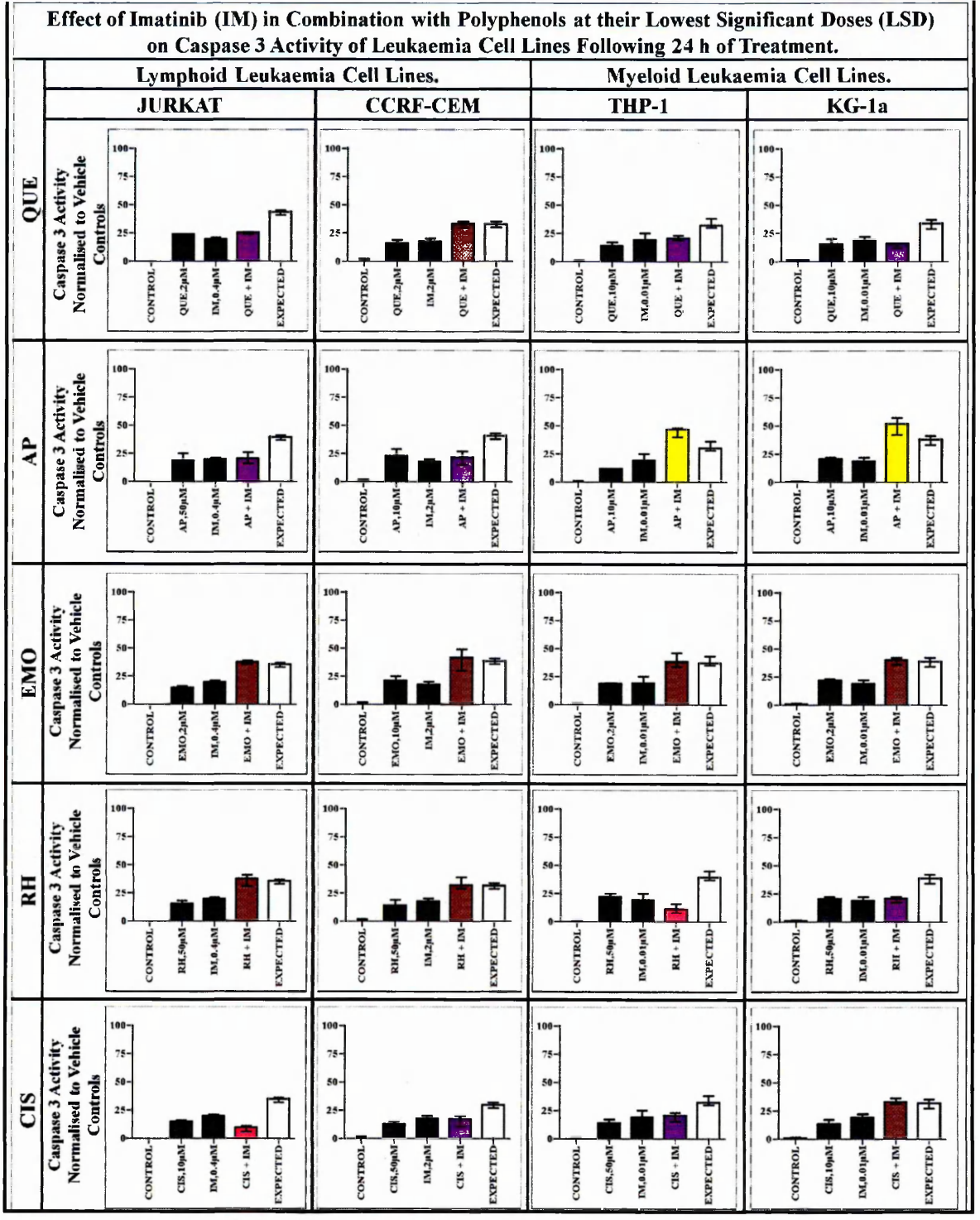


Figure 3.44: The effect of IM used in combination with QUE, AP, EMO, RH or CIS on caspase 3 activity in leukaemia cell lines. This was evaluated by NucView caspase 3 activity assay. Cells were treated with IM and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 3 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

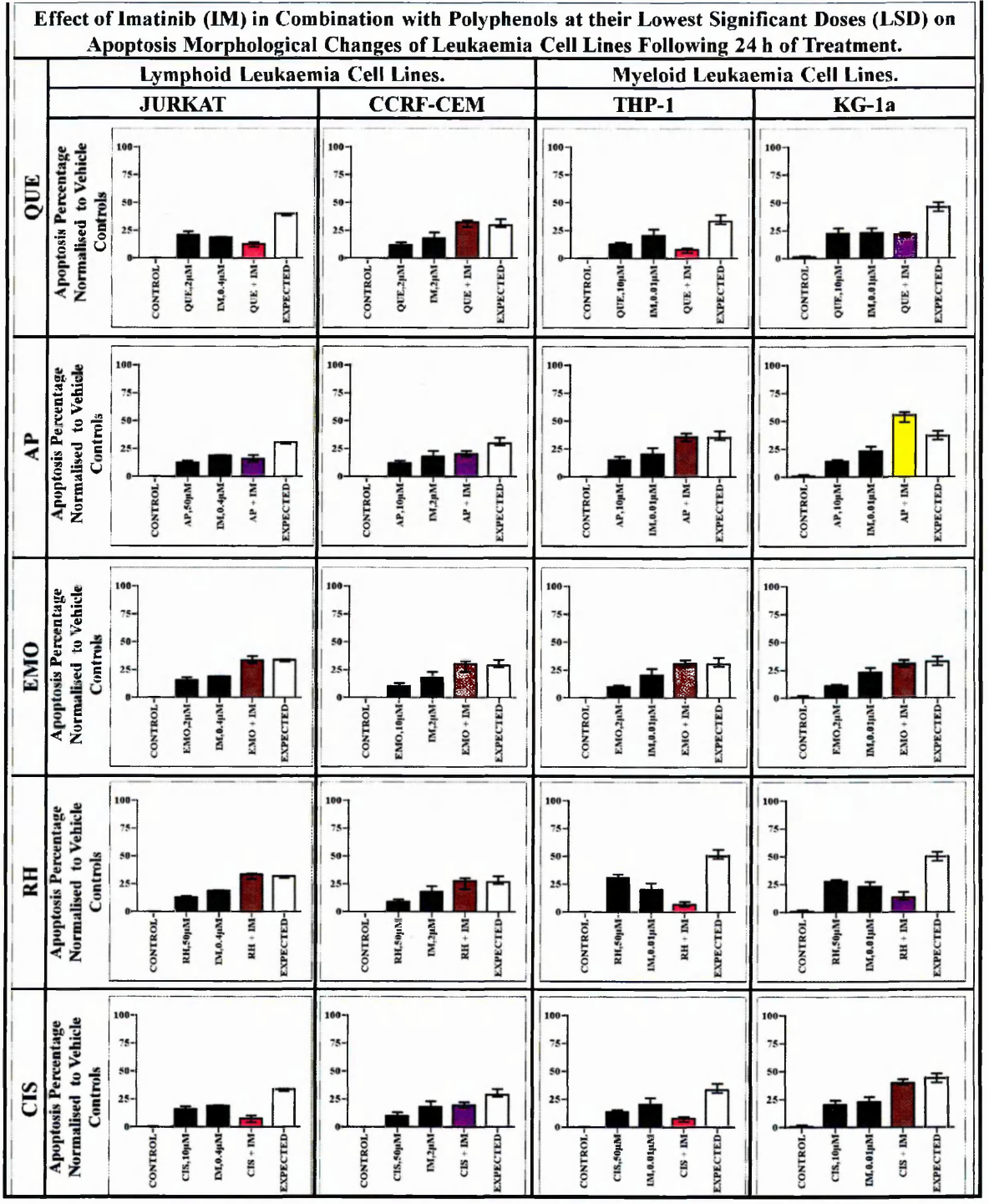


Figure 3.45: The effect of IM in combination with QUE, AP, EMO, RH or CIS on apoptosis morphological changes of leukaemia cell lines. This was evaluated by double staining with Hoechst 33342/PI using fluorescence microscopy. Cells were treated with IM and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-only control, which was assigned a 0% apoptotic level. The data was expressed as medians with ranges (n=4). The black bars shows the vehicle controls and treatments alone; the white bar shows the expected level of percentage apoptosis; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

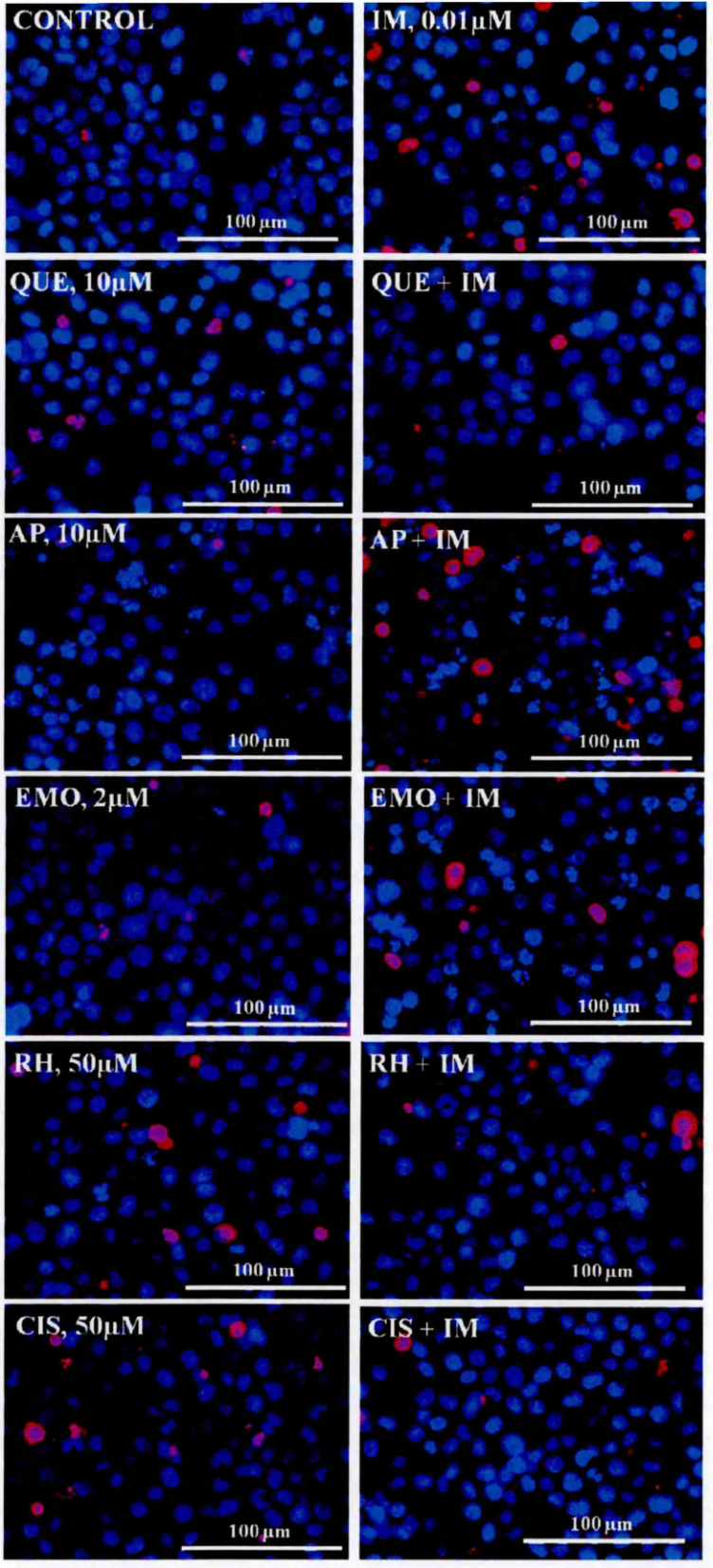
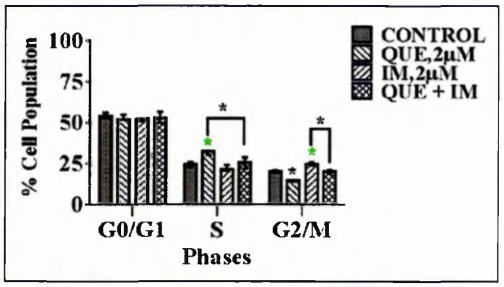
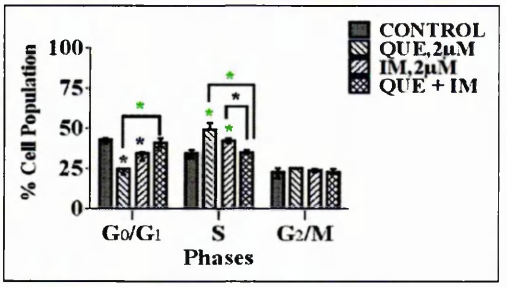
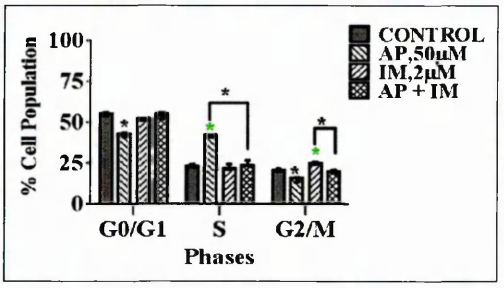
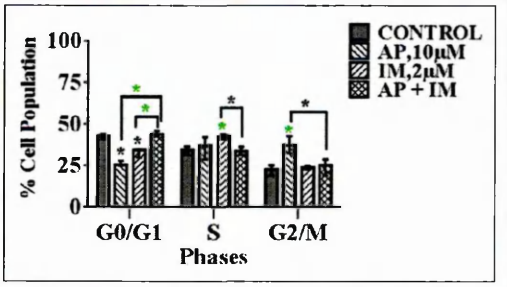
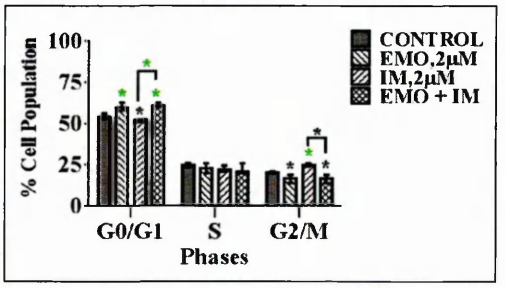
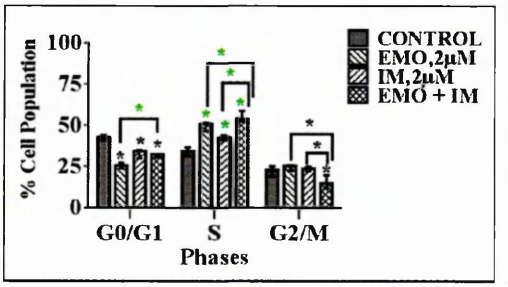
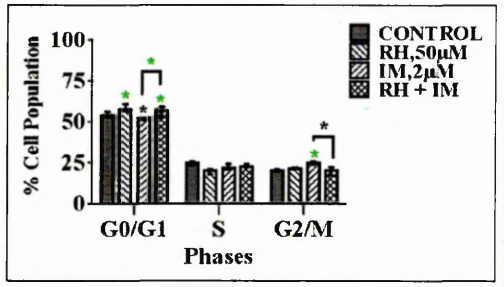
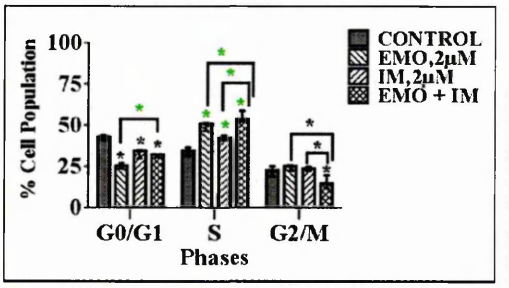
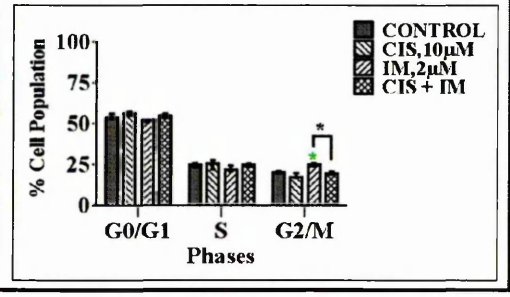
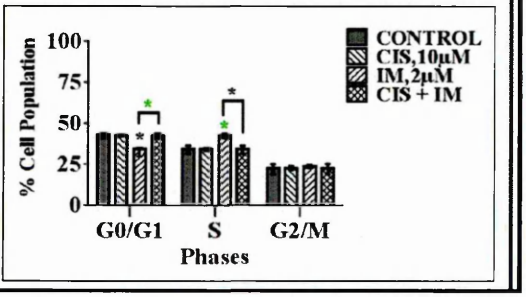


Figure 3.46: An example of morphological assessment of apoptosis using Hoechst 33342/PI for THP-1 myeloid leukaemia cells when treated with LSDs of IM and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combined for 24 h. Apoptotic cells were identified by their irregular shape, intensely bright blue stained nuclei, chromatin condensation, and the formation of apoptotic bodies. Normal cells were round and pale blue stained, and late apoptotic cells and dead cells appear pink. Scale bar = 100 µm.

(A) Effect of Imatinib (IM) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.

Drugs	JURKAT	CCRF-CEM
QUE		
AP		
EMO		
RH		
CIS		

(B) Effect of Imatinib (IM) in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Cell Cycle of Myeloid Leukaemia Cell Lines Following 24 h of Treatment.

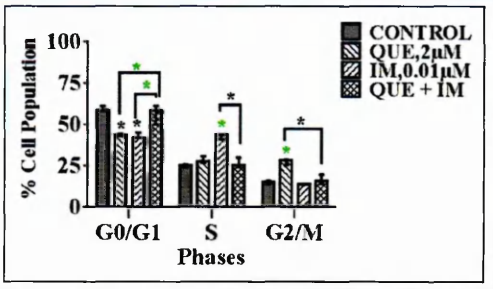
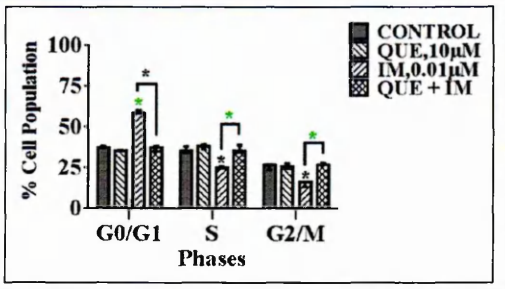
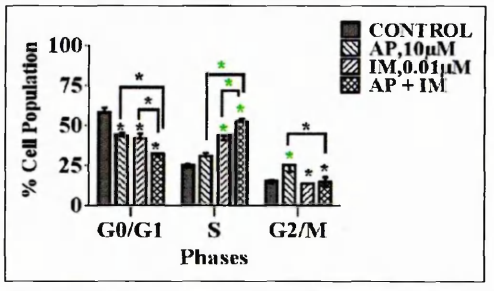
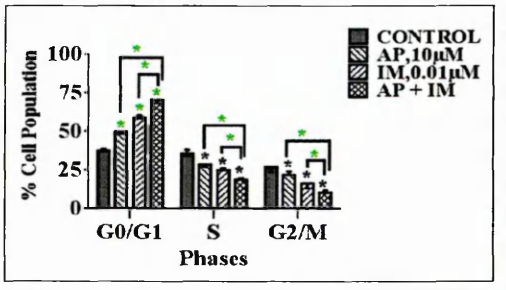
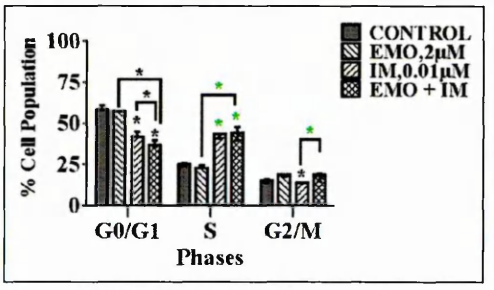
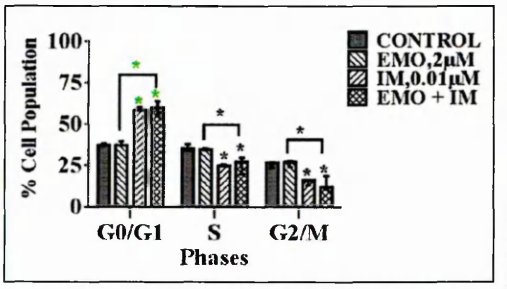
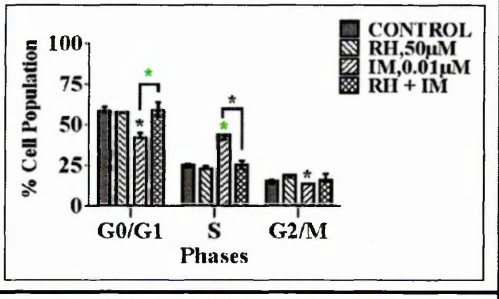
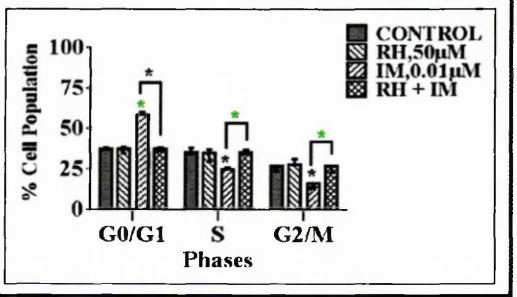
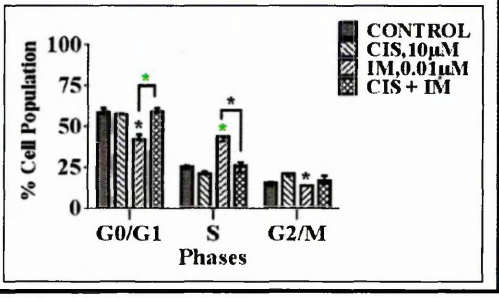
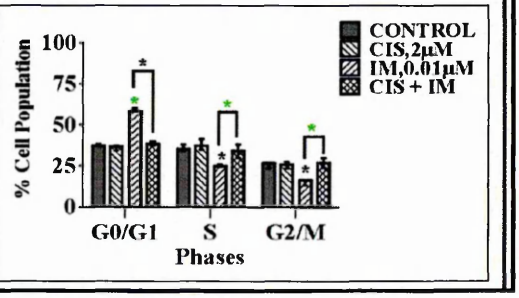
Drugs	THP-1	KG-1a
QUE		
AP		
EMO		
RH		
CIS		

Figure 3.47: The effect of IM when used in combination with QUE, AP, EMO, RH or CIS on cell cycle progression in: (A) lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and (B) Myeloid leukaemia cell lines (THP-1 and KG-1a). Cell cycle progression was analysed following propidium iodide staining using flow cytometry. Cells were treated with IM and polyphenols alone and in combined for 24 h using the lowest-significant doses (LSD) as determined by CellTiter-Glo assay. The percentage of cells in each phase was analysed with FlowJo software using Waston pragmatic model. The data was expressed as medians with ranges (n=4). The statistical significance of individual drugs and combined drugs was determined by comparison with the vehicle control. Statistical significance of the combined treatments was determined by comparison with the effect of the individual treatments alone. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone. The Green star (*) is used to indicate a significant increase in cell accumulation in a phase of the cell cycle; whilst the Black star (*) is used to indicate a significant decrease in cell accumulation in a phase of the cell cycle. The effects of combination treatments on cell cycle were classified as interactive or non-interactive as described in Section 3.2.5.2; and are summarised in Table 3.13.

Assays	Targets	Combination of Imatinib and Polyphenols	The Significant Effects of matinib (IM) and Polyphenols Combination Treatment on ATP Levels, Apoptosis and Cell Cycle Progression at 24 h.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
CellTiter-Glow	ATP Level	IM + QUE (NSD)	C-ANTG	ADD	C-ANTG	C-ANTG
		IM + QUE (LSD)	ANTG	ADD	C-ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	IM + QUE (NSD)	C-ANTG	ADD	C-ANTG	C-ANTG
		IM + QUE (LSD)	C-ANTG	ADD	C-ANTG	C-ANTG
Hoechst/ PI	Apoptosis	IM + QUE (LSD)	ANTG	ADD	ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	IM + QUE (LSD)	ANTG	ANTG	ANTG	ANTG
CellTiter-Glow	ATP Level	IM + AP (NSD)	C-ANTG	C-ANTG	SYN	SYN
		IM + AP (LSD)	C-ANTG	C-ANTG	SYN	SYN
Caspase 3 Activity	Apoptosis	IM + AP (NSD)	C-ANTG	C-ANTG	SYN	SYN
		IM + AP (LSD)	C-ANTG	C-ANTG	SYN	SYN
Hoechst/ PI	Apoptosis	IM + AP (LSD)	C-ANTG	C-ANTG	ADD	SYN
Cell Cycle/PI	Cell Cycle	IM + AP (LSD)	ANTG	ANTG	ADD	ADD
CellTiter-Glow	ATP Level	IM + EMO (NSD)	ADD	ADD	ADD	ADD
		IM + EMO (LSD)	ADD	ADD	ADD	ADD
Caspase 3 Activity	Apoptosis	IM + EMO (NSD)	ADD	ADD	ADD	ADD
		IM + EMO (LSD)	ADD	ADD	ADD	ADD
Hoechst/ PI	Apoptosis	IM + EMO (LSD)	ADD	ADD	ADD	ADD
Cell Cycle/PI	Cell Cycle	IM + EMO (LSD)	Non Interaction	Non Interaction	Non Interaction	Non Interaction
CellTiter-Glow	ATP Level	IM + RH (NSD)	ADD	ADD	C-ANTG	C-ANTG
		IM + RH (LSD)	ADD	ADD	ANTG	C-ANTG
Caspase 3 Activity	Apoptosis	IM + RH (NSD)	ADD	ADD	C-ANTG	C-ANTG
		IM + RH (LSD)	ADD	ADD	ANTG	C-ANTG
Hoechst/ PI	Apoptosis	IM + RH (LSD)	ADD	ADD	ANTG	C-ANTG
Cell Cycle/PI	Cell Cycle	IM + RH (LSD)	Non Interaction	Non Interaction	ANTG	ANTG
CellTiter-Glow	ATP Level	IM + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	ADD
		IM + CIS (LSD)	ANTG	ANTG	C-ANTG	ADD
Caspase 3 Activity	Apoptosis	IM + CIS (NSD)	C-ANTG	C-ANTG	C-ANTG	ADD
		IM + CIS (LSD)	ANTG	C-ANTG	C-ANTG	ADD
Hoechst/ PI	Apoptosis	IM + CIS (LSD)	ANTG	C-ANTG	ANTG	ADD
Cell Cycle/PI	Cell Cycle	IM + CIS (LSD)	ANTG	ANTG	ANTG	ANTG

Table 3.13: A summary of the effects of imatinib (IM) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on ATP level, apoptosis and cell cycle progression in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two myeloid leukaemia cell lines (THP-1and KG1a) at 24 h. Combination treatments were investigated at their non-significant dose (NSD) and lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 2.5.2. SYN = Synergistic, ADD = Additive, C-ANTG= Competitive Antagonistic, ANTG= Antagonistic.

3.4 Discussion

Clinically, polyphenols have been shown to enhance the efficacy of chemotherapy agents. These polyphenols have also been shown to decrease the occurrence of drug resistance, toxicity and treatment related side effects in cancer patients (Wu, 2006; Florea and Büsselberg, 2011; Sandeep *et al,* 2012; Mohan *et al*, 2013). This study has investigated the combination effects of nine standard chemotherapy agents and five polyphenols (identified in Chapter 2). Here we have demonstrated how each of these chemotherapy agents and polyphenols can be used in combination to inhibit cell proliferation, induce apoptosis and arrest the cell cycle in leukaemia cell lines. In order to study the molecular interaction of these polyphenols and chemotherapy agent; they need to be combined in micromolar concentrations.

Here these concentrations were defined as the non-significant dose (NSD) and lowest significant dose (LSD) that inhbited cell proliferation and induced apoptosis following 24 h treatment. Only at these low doses it is possible to measure how drug interactions mechanistically, and enable the evaluation of the effects of treatment combinations as synergistic, additive, etc. The use of IC_{50} doses would obscure these effects. This study demonstrated that the efficacy chemotherapeutic agents were differential modulated by polyphenols within the leukaemia cell lines. These differential combination effects could be dependent on the type of chemotherapy agents, the polyphenol used, and/or cell lineage. This section will discuss our combination results according to the chemotherapy classifications as described previously.

3.4.1 Toposiomerase Inhibitor Agents in Combination with Polyphenols

The topoisomerase inhibitor agents: DOX and ETP displayed synergistic effects on reduction of ATP levels, induction of apoptosis and arresting the cell cycle when combined with each of the polyphenols investigated (QUE, AP, EMO, RH or CIS) in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) (Table 3.5 and 3.6). However, DOX and ETP displayed differential effects either synergistic or additive or antagonistic effects when combined with the polyphenols (QUE, AP, EMO, RH or CIS) in myeloid leukaemia cell lines (THP-1 and KG-1a) (Table 3.5 and 3.6). Most importantly, DOX and ETP when combined with QUE; and DOX when combined with AP produced synergistic effects in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) (Table 3.5 and 3.6).

Previously DOX combined with QUE was shown to be highly effective against breast cancer cell lines (Du *et al,* 2010; Staedler *et al,* 2011; Li *et al,* 2013). Du *et al,* 2010 demonstrated using a xenograft murine breast cancer cell (4T1) model that intra-tumoural injection of quercetin and doxorubicin synergistically suppressed tumour growth and metastasis, and induced persistent T-cell tumour-specific responses through an increase in IFN-γ and IL-2

levels, a reduced IL-4 and IL-10 and an induction of apoptosis of CD4+ or CD8+ T cells (Du *et al,* 2010). This study reported that that tumour-specific T cells play a vital role in tumour immunosurveillance and immunorejection, the characteristics of immune responses are mainly directed by the activity of T-helper (Th) cell sub-populations and their cytokine product (Du *et al,* 2010). The type 1 cytokines such as IL-2 and IFN-γ are immunostimulants and are able to limit tumour growth (Du *et al,* 2010). Alternatively, the type 2 cytokines such as IL-4 and IL-10 are immunoinhibitory and thus able of drive tumour growth (Du *et al,* 2010). It was found that DOX-induced tumour cell death and quercetin was an effective immunoregulator which increased IFN-γ and IL-2 and decreased IL-4 and IL-10 with the host serum and tumour (Du *et al,* 2010). In addition, this study found quercetin was capable of modulation of the immune system and sensitize the tumour to chemotherapy, by the inducing immunogenic cancer cell death, and synergistic induction of long lasting tumour-specific immunity (Du *et al,* 2010). It was concluded that the combination of quercetin with intratumoral doxorubicin may represent a novel and highly effective strategy for inducing immune responses against tumours, plus help to reduce chemotherapy treatment doses an hence the toxic side effects seen in breast cancer patients treated with doxorubicin (Du *et al,* 2010). In addition, in human breast cancer cell lines (MCF-7, MDA-MB-231, and MCF-10A) the combination of DOX and QUE inhibited cells proliferation and induced apoptosis through decreased the cellular thiol level and blockage of the PKCδ signalling pathway (Staedler *et al,* 2011). The treatment of MCF-7 cells with combination of DOX and QUE caused inhibition of cell proliferation and invasion via the suppression of hypoxia-inducible factor-1 alpha (HIF-1α) and P-glycoprotein (P-gp) (Li *et al,* 2013). Similarly, the treatment of human hepatoma cell lines (SMMC7721 and QGY7701) with a combination of DOX and QUE induced apoptosis via accumulation of p53, followed by the activation of mitochondrial apoptotic pathway, resulting in activation of caspase 9 and caspase 3 (Wang *et al,* 2012). These studies suggest that the effects of DOX and ETP could be enhanced by polyphenols for solid tumours treatment; however studies in vivo may be identifying interactions that are different to cell culture models.

Cipak *et al*, 2003 reported that the antagonistic effects could be related to the anti-oxidant activity of the polyphenols, which could act as protective against reactive oxygen species (ROS) that are generated by DOX and thus inhibited the apoptosis, through suppressed ROS production and the activation of the c-Jun NH2-terminal kinase (JNK) pathway (Cipak *et al*, 2003; Somasundaram *et al,* 2002). This could be a possible explanation the effect caused by EMO and RH within the current study, which antagonised the activity of DOX in myeloid cell lines (THP-1 and KG-1a). EMO and RH may to act as anti-oxidants and mop up ROS generated by DOX (Cipak *et al*, 2003; Somasundaram *et al,* 2002).

Finally, it can be summarised that topoisomerase inhibitor agents when combined with flavonoid compounds QUE and AP synergistically interacted in both lymphoid and myeloid cell lines which could hold promise for leukaemia treatments. However DOX and ETP when combined with remaining polyphenols EMO, RH or CIS synergistically interacted in lymphoid leukaemia cell lines only; whilst in myeloid leukaemia cell lines no synergistic responses were observed. This suggests that combination of DOX and ETP with the polyphenols investigated here (QUE, AP, EMO, RH and CIS) could be promising for the treatment of lymphoid leukaemias. However, more importantly, patients with myeloid leukaemia treated with DOX should avoid anthraquinones such as EMO and RH; whilst patients given ETP therapy should avoid anthraquinones such as RH and stilbenoide such as CIS; or indeed any foods rich in these polyphenols that could potentially have an antagonistic effect on their chemotherapy treatment. These findings suggest that additional in vivo experiments require to be performed to determine whether diet-based EMO, RH or CIS could potentially affect DOX or ETP activity.

3.4.2 Alkylating Agents in Combination with Polyphenols

In this study, the alkylating agents CSP and CYCLO, followed by CLB were the next most effective chemotherapeutics to interact with the polyphenols. CSP and CYCLO demonstrated a potent synergistic effect when combined with 4 out of 5 polyphenols in the lymphoid cell lines (Table 3.7 and 3.9). Similarly CLB had a synergistic effect when combined with 2 out 5 polyphenols, but only in the lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) (Table 3.8). These synergistic effects were through the inhibition of ATP levels and induction of early apoptosis via increase the caspase 3 activation, plus morphological evidence of the late apoptosis and arresting the cell cycle mainly at S phase, and some combination at G_0/G_1 phase when compared to control and drugs alone. While, they had differential effects either synergistic or additive or antagonistic effects when combined with the 5 polyphenols (QUE, AP, EMO, RH, or CIS) in myeloid leukaemia cell lines (THP-1 and KG-1a) (Table 3.7, 3.8 and 3.8).

In summary, the significant observations on the combination of alkylating agents (CSP, CYCLO and CLB) with polyphenols were as follows: (1) the combination of CSP with QUE, CYCLO with AP and CLB with AP were interacted synergistically in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) and could be very promising for leukaemia treatment; however, (2) the combination of CYCLO and CLB with CIS had an antagonistic interaction in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CE, THP-1 and KG-1a). So, leukaemia patients given CYCLO or CLB therapy should avoid CIS supplementation or foods containing this type of polyphenol. Results

suggest that further in vivo experiments need to be performed to determine whether diet-based CIS could potentially affect CYCLO or CLB activity.

3.4.2.1 CSP in Combination with Polyphenols

The synergistic interaction between CSP and QUE in leukaemia cell lines occurred through the inhibition of ATP levels, induction of early apoptosis via upregulation the actvity of caspase 3 activity, plus morphological evidence of late apoptosis and arresting the cell cycle at S phase for lymphoid cell lines and at G_2/M phase for myeloid cell lines. This was confirmed by previous studies. CSP has been one of the most investigated chemotherapy drugs in combination with QUE in different cancer cell lines although mainly in solid tumour cells. Earlier work has shown that CSP and QUE when used in combination caused a synergistic inhibition of cell proliferation and induction of apoptosis in a number of cell lines: murine leukaemia cells (L1210) (Cipak *et al*, 2003), small cell lung cancer cells (SW1271), pulmonary adenocarcinoma cells (A549) (Borska *et al*, 2004), human non-small cell lung carcinoma cells (NCL-H-520) (Kuhar *et* al, 2006), human laryngeal carcinoma cells (Hep-2) (Kuhar *et* al, 2007), human malignant mesothelioma cells (SPC212 and SPC111) (Zergeroglu *et al*, 2010), ovarian cancer cells (A2780) (Meher *et al,* 2011), nasopharyngeal carcinoma cells (HK1 and C666-1) (Darker *et al,* 2012) and human hepatoculallular carcinoma cells (HepG2) (Zhao *et al*, 2014). Mechanistically, studies by Kuhar *et* al, in 2006 and 2007 and by Zergeroglu *et al*, in 2010 suggested that the synergistic effect induced by the combination of CSP and QUE was caused by the activation of the intrinsic apoptotic pathway by the down regulation of Bcl-xl and Bcl-2; and an increase in cytochrome c and caspase 3 activity Furthermore, it was found that CSP when used in combination with QUE synergistically arrested the cell cycle at S and G_2/M phases in murine leukaemia cell lines (L1210) (Cipak *et al*, 2003), at S phase only human malignant mesothelioma cell lines (SPC212 and SPC111) (Zergeroglu *et al*, 2010), and at G_0/G_1 phase by elevation of p16 expression in human hepatocellular carcinoma cell lines (HepG2) (Zhao *et al*, 2014). These earlier studies along with the evidence shown here support the theory that the combination of CSP and QUE could have promise in the treatment of a number of cancers including leukaemia.

However, there is a recent study by Li *et al,* 2014 who showed that when CSP was combined with low concentrations (5 μM–30 μM) of QUE, it could induce an antagonistic effect via inhibition the apoptosis. However, when CSP was combined with higher concentrations of QUE (40 μM–100 μM) this resulted in synergistic effects in human epithelial ovarian cancer cell lines (C13* and SKOV3) (Li *et al,* 2014). It was suggested that the therapeutic effects of CSP in combination with QUE, can be influenced by dose, with the lower concentrations of QUE causing suppression of ROS-induced injury, reduce intracellular ROS level and

increasing the expression of endogenous antioxidant enzymes such as glutathione enzymes, leading to a reduced therapeutic efficacy of CSP in human epithelial ovarian cancer cell lines (Li *et al,* 2014). Thus it is essential that if CSP and QUE are to be used together, the dose need to be sufficient to drive their pro-apoptotic effect, and may require ex vivo testing on a patient-by-patient basis. Consideration of QUE in diet is particularly important given the fact that diet-derived QUE can accumulate at levels that inhibit bortezomib via a direct antagonistic mechanisms-dependent on a direct chemical interaction between the vicinal diol of quercetin and the boronic acid of bortezomib, which converting the active triangular boronic acid of bortezomib to an inactive tetrahedral in human multiple myeloma cell lines (MC/CAR, RPMI8226 and U266) (Kim et al, 2009).

In this study, CSP was found to interact with AP in both lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1, and KG-1a). In previous studies CSP when used in combination with AP has caused a synergistic inhibition of cells proliferation and induction of apoptosis in head and neck squamous cell carcinoma cell lines (SCC25) through the increase in intracellular ROS levels, reduce the levels of glutathione (GSH), and increased Bcl-2-mediated caspase production (Chan *et al*, 2012); in human nasopharyngeal carcinoma cell lines (CNE-2Z) through up-regulation of Bax expression and down-regulation of Bcl-2 expression (Yong *et al,* 2012); and finally in human laryngeal carcinoma cell lines (Hep-2) through inhibition of glucose transporter-1 (GLUT-1) and PI3K/Akt expression (Xu et *al,* 2014).

Furthermore, here CSP has also been shown to act synergistically with all the remaining polyphenols (EMO, RH and CIS), however only in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) as showed by reduced ATP levels, induction of caspase 3 activity and apoptosis; plus arrest of the cell cycle at S phase. However, in the myeloid cell lines (THP-1 and KG-1a) CSP had an antagonistic effect when combined with EMO, RH or CIS. There was an elevation of ATP levels, a reduction in capase 3 and an inhibition the apoptosis; with no effect been seen on the cell cycle profile. Thus it would seem that the therapeutic effects of CSP in combination EMO, RH or CIS appears to be dependent on the cell lineage; which lead to the suggestion that the use of CSP in combination with polyphenols is only useful in the treatment of lymphoid leukaemia, but not myeloid leukaemia.

Many studies have been reported that CSP can be potentiated by EMO, producing synergistic effects on inhibition of cells proliferation and induction of apoptosis in non-small cell lung cancer (NSCLC) with HER-2/neu-overexpressing (Zhang and Hung, 1996; Ko *et al,* 2010), in merkel cell carcinoma (MCC) (Fenig *et al,* 2004), human ovarian tumour cell lines (A2780) (Kurokawa *et al,* 2010), and finally in gallbladder carcinoma cell lines (SGC996) through the depletion of glutathione (GSH) that related to ROS–mechanisms; and down-regulation the

expression of survivin protein and multidrug resistance-related protein 1 (MRP1) (Wang *et al*, 2010; Wang *et al,* 2011). To date there are no studies of the effect of CSP combination treatments with RH or CIS.

In general, the results show that CSP was differently modulated by polyphenols within the leukaemia cell lines. This is in agreement with the finding of C̆ipak *et al,* 2003, who demonstrated in murine leukaemia cell lines (L1210) that CSP can be differently modulated by polyphenols: having a synergistic effect with quercetin or luteolin, an additive effect with apigenin and an antagonistic effect with galangin and chrysin on cells proliferation and apoptosis (C̆ipak *et al*, 2003).

3.4.2.2 CYCLO in Combination with Polyphenols

CYCLO differently interacted with each polyphenols (Table 3.7): CYCLO when combined with AP only produced a synergistic effect in both lymphoid and myeloid leukaemia. Likewise when CYCLO was combined QUE, EMO or RH it also produced synergistic effects and but only in the lymphoid leukaemia cells. In the myeloid leukaemia treatment these combinations produced antagonistic effects. Finally, when CYCLO was used in combined with CIS this caused an antagonistic effect in both lymphoid and myeloid leukaemia cells. This demonstrated that CYCLO is influenced by the polyphenol type and the lineage of the cells. Thus the most effective polyphenol to work in combination with CYCLO is AP for all leukaemia types; whilst CYCLO activity is inhibited when combined with QUE, EMO, RH and CIS in myeloid leukaemia type. Findings suggest that further in vivo investigations require to be performed to determine whether diet-based QUE, EMO, RH and CIS could potentially affect CYCLO activity.

There are two previous studies that show that the efficacy of CYCLO can be differentially modulated by natural agents such as curcumin and resveratrol in human breast cancer cell lines (Somasundaram *et al,* 2002; Singh *et al*, 2011). It was reported that the CYCLO activity was blocked by curcumin in human breast cancer cell lines (MCF-7, MDA-MB-231, and BT-474). It caused an antagonistic inhibition of apoptosis via the reduction of reactive oxygen species (ROS) production and the inhibition of the c-Jun NH2-terminal kinase (JNK) pathway (Somasundaram *et al,* 2002). Alternatively, when CYCLO was combined with resveratrol in human breast cancer cell lines (MCF-7) it caused a synergistic inhibition of cells proliferation and induced apoptosis (Singh *et al*,2012). This supported the findings of this study that the efficacy of CYCLO is differently modulated by polyphenols, dependent on polyphenol type and/or cell lineage.

3.4.2.3 CLB in Combination with Polyphenols

In comparison with the alkylating agents (CSP and CYCLO), the efficacy of CLB had the least synergistic effects. Synergy was only observed when CLB was combined with 2 out 5 polyphenols: with AP in both lymphoid and myeloid leukaemia cell lines, and with RH in lymphoid leukaemia cell lines only. Similar to CYCLO combination work, CLB synergistically interacted with AP, and an antagonistic effect with CIS in all the leukaemia cell lines (JURKAT, CCRF-CEM, THP-1, and KG-1a). According to CLB combination results, CLB had differential effects either synergistic or additive or antagonistic effects when combined with polyphenols within the leukaemia cell lines. The differential combination effects of CLB with polyphenols could be dependent on polyphenol type. In particular CLB when combined with flavonoides (QUE and AP) and anthraquinones (EMO and RH) there was either additive or synergistic interaction. Alternatively when CLB was combined with stilbenoide (CIS) there was an antagonistic interacted within all the leukaemia cell lines. To date there is only one study investigating the interaction of CLB with polyphenols. CLB when combined with QUE was shown to synergistically reduced glutathione (GSH) levels and induced apoptosis in human colon adenocarcinoma cell lines (SW480) (Zhang *et al,* 2003). A similar effect is shown here, the combination of CLB with QUE had an additive effect reduction ATP levels and inducing apoptosis in leukaemia cell lines.

3.4.3 Anti-Metabolites Agents in Combination with Polyphenols

3.4.3.1 MTX and 6-MP in Combination with Polyphenols

In this study, the anti-metabolite chemotherapy agents such as MTX and 6-MP were the least effective chemotherapy agents when combined with polyphenols within the leukaemia cell lines. Particularly, MTX and 6-MP when combined with all polyphenols (QUE, AP, EMO, RH, or CIS) had an antagonistic effect increasing ATP levels and inhibiting caspase 3 activity and apoptosis; as well as having no effect on cell cycle profile (Table 3.10 and 3.11). This study is the first study to investigate MTX and 6-MP in combined with polyphenols; suggesting a further in vivo experiment is urgently needed to determine whether diet-based polyphenols (QUE, AP, EMO, RH and CIS) could potentially affect MTX or 6-MP activity.

3.4.3.2 5-FLU in Combination with Polyphenols

5-FLU had a varied effects when combined with polyphenols, producing either synergistic or additive or antagonistic effects depending on the polyphenol type and the leukaemia lineage (Table 3.12). The most promising combination for 5-FLU was with AP in which they were effectively interacted in both lymphoid and myeloid leukaemia cell lines and a synergistic

reduced of ATP levels, an increase in capase 3 activity and apoptosis; plus arrest of the cell cycle at S phase in the lymphoid cell lines and in G_0/G_1 for the myeloid cell line.

These results are consistent with previous finding in which 5-FLU was shown to synergistically interacted with three polyphenols: (1) AP in head and neck squamous cell carcinoma cell lines (SCC25) through increase in intracellular ROS levels, reduction of GSH and increase caspase 3 activation (Chan *et al*, 2012); (2) QUE in colorectal cancer cell lines (HCT116), prostate cancer cell lines (PPC1) (Samuel *et al*, 2012) and human epithelial ovarian cancer cell lines (C13* and SKOV3) (Li *et al,* 2014) through the inhibition of cell proliferation and the induction of apoptosis through the up-regulation of p53, p21, BAX and down regulation of cyclin B1 and survivin , and arrested of the cell cycle at S and G_2/M phases (Samuel *et al*, 2012; Li *et al,* 2014); (3) EMO in non-small cell lung cancer cell lines (NSCLC) via decreased tyrosine phosphorylation of HER-2/neu which then preferentially suppressed proliferation of HER-2/neu-overexpressing NSCLC cells (Zhang and Hung, 1996). Most of these earlier studies suggested 5-FLU in combination with polyphenols could be promising for cancer treatment. However, in our study 5-FLU acted antagonistically with CIS in all leukaemia cell lines; and with RH in the myeloid leukaemia cell lines (Table 3.12). This suggesting that 5-FLU can be differential modulated by polyphenols according to polyphenol type or cell lineage.

3.4.4 Tyrosine Kinase Inhibitor Agent – Imatinib (IM)

In this study, tyrosine kinase inhibitor agent such IM, were found to be one of the least effective therapy when used in combination with the selected polyphenols; most likely reason as only active against BCR-ABL + ve or fairly specific with limited activity against other RTKs. The IM polyphenol combination treatment produced manly additive and antagonistic responses within the leukaemia cell lines. The only exception was seen when IM used in combination with AP, were it produced a synergistic effects; although this was only seem in the myeloid leukaemia cell lines (THP-1 and KG1a). This is the first time a study has been made of the interaction between IM and polyphenols, but these results do not identify any potential IM/polyphenol combination therapies that could be useful in the treatments of leukaemia.

3.5 Conclusions

The majority of prior studies suggest that the activity of chemotherapeutic agents can be enhanced by polyphenols, however, there are a few studies which have demonstrated inhibitory actions of polyphenols on chemotherapeutic agents, responses have been shown to be dependent on the polyphenols chemical structure (Golden *et al,* 2009; Kim *et al,* 2009;

Perrone *et al,* 2009), anti-oxidant functions (Somasundaram *et al,* 2002; Cipak *et al,* 2003; Kim *et al,* 2009; Davalli *et al,* 2012) or administration dose (Li *et al,* 2014). Furthermore, this highlights that urgent investigations are now required to determine whether cancer patients undergoing chemotherapy could successfully respond to polyphenols; or should avoid polyphenols supplementation, and possibly even limit their exposure to polyphenols-containing foods.

Our study has shown that the efficacy of standard chemotherapeutic drugs were differentially modulated by polyphenols, producing either synergistic, additive or competitive antagonistic/antagonistic effects within the lymphoid and myeloid leukaemia cell lines, which was dependent on type of polyphenol, chemotherapy agent and cell line. The combined effect of polyphenols on reduction of ATP levels, induction of early and late apoptosis, and cell cycle arrest indicate some of these agents could be useful in improving leukaemia therapies; however combination therapies could also prevent the actions of chemotherapeutic agents. Identification of interaction between polyphenolic compounds and chemotherapy drugs may provide new pathways to target cancerous cells. As a result, since some polyphenols enhance the activity of chemotherapy, and some inhibit the activity of chemotherapy, it is essential to consider these interactions for dietary advice during therapy of cancer patients.

The exact mechanism, by which these differential effects of polyphenols have on the activity of chemotherapeutics are still unknown. Some studies have proposed that polyphenols can enhance the chemotherapeutic agents through their ability to inhibit kinases such as PKC that are involved in phosphorylation of topo II enzyme which can lead to inhibition of cell proliferation, induce apoptosis and arrest the cell cycle in cancer cells (C̆ipak *et al*, 2003). Furthermore polyphenols which block chemotherapeutic agents have been proposed to act via their anti-oxidative activity which is proposed to be cytoprotective for the cancer cells against the reactive oxygen species (ROS) which are generated from chemotherapeutic agents (Somasundaram *et al,* 2002; Cipak *et al,* 2003; Kim *et al,* 2009; Davalli *et al,* 2012), or through direct chemical interaction between the polyphenols and anti-cancer agents (Golden *et al,* 2009; Kim *et al,* 2009; Perrone *et al,* 2009). In addition, it is known that polyphenols are powerful antioxidants and free radical scavengers and may modulate the activity of phase I and II enzymes, in particular glutathione (GSH)-related enzymes, which are the major enzymes responsible for antioxidant functions (Luceri *et al,* 2002). Moreover, usually cancer cells express high levels of glutathione *S*-transferase (GST), which are commonly associated with the failure of cancer chemotherapy and poor patient survival (Luceri *et al,* 2002). Thus, the resulting synergistic or antagonistic effects from combination of polyphenols with chemotherapy drugs could be dependent on a modulation in glutathione (GSH) level within the leukaemia cells, which requires further study.

Chapter 4

Mechanism of Polyphenol Induced Modulation of Chemotherapy Agent Activity.

4.1 Introduction

The polyphenols studied here have been shown to induce apoptosis in leukaemia cell lines (Chapter 2). Furthermore they can be ranked from the most to the least to effectiveness at inducing apoptosis (emodin> quercetin > *cis*-stilbene = apigenin > rhein = aloe-emodin ≥ *trans*-stilbene). These polyphenols have been shown to act synergistically with some chemotherapeutic agents such as toposiomerase inhibitor agents DOX or ETP in lymphoid cell lines. However these same polyphenols have been shown to have an antagonistic when used in combination with anti-metabolites agents MTX and 6-MP in both the lymphoid and myeloid leukaemia cell lines (Chapter 3). This leads to the suggestion that the effects of polyphenols alone and in combination with chemotherapeutic agents in leukaemia cell lines is clearly dependent of the polyphenol, the chemotherapy type, the cell lineage and/or the doses used (Chapter 3).

Here, the potential mechanisms of apoptosis were investigated. Firstly, it was important to determine whether polyphenols alone and in combination with chemotherapeutic agents induced apoptosis via the extrinsic or intrinsic pathways, as this could impact on the potential apoptosis regulatory molecules that polyphenols may modulate. Secondly, it was important to identify whether the sensitivity and resistance of leukaemia cell lines to polyphenols alone and in combined with chemotherapeutics occurred based on the changing basal glutathione (GSH) content in cells. Thirdly, it was important also to demonstrate whether the combination of polyphenols and chemotherapeutic agents caused differential effects (synergetic, additive or antagonistic effects) on apoptosis induction through the modulation in glutathione (GSH) levels. Finally, it was necessary also to confirm whether the combination of polyphenols and chemotherapeutic agents induced apoptosis, as well as, arrested cell cycle at S phase and/or G_2/M phase by high expression of γ-H2AX foci (a hallmark of DNA damage). The rationale being that polyphenols may deplete GSH, resulting in enhanced chemotherapy-induced DNA damage, as determined by γ-H2AX. These results may give clues to how polyphenols may overcome drug resistance.

4.1.1 Extrinsic Verse Intrinsic Apoptotic Pathways

Apoptotic cell death is a key regulator of cell growth; aberrant apoptosis is crucially involved in formation of leukaemia thus its control and regulation is crucial it determining treatments (Belloc *et al,* 2000; Lavrik *et al*, 2005; Elmore, 2007; Wong, 2011). Currently, most anti-cancer agents work by driving apoptosis either through the activation of the extrinsic and/or intrinsic pathways (Belloc *et al,* 2000; Lavrik *et al*, 2005; Elmore, 2007; Wong, 2011). The extrinsic pathway is initiated from outside the cell by pro-apoptotic ligands that interact with specialised cell surface death receptors (DRs) (Chapter 1; Section 1.6.1.1) (Elmore, 2007;

Wong, 2011). In contrast, the intrinsic pathway is activated from inside the cells by members of Bcl-2 protein family which increase the permeability of the outer mitochondrial membrane leading to caspases activation (Chapter 1; Section 1.6.1.2) (Elmore, 2007; Wong, 2011). Both pathways activate the enzymatic caspase cascade, which carries out numerous proteolytic events that mediate the apoptotic cell death programme (Elmore, 2007; Wong, 2011).

These caspases are a family of cysteine proteases that play crucial roles in regulating apoptosis and synthesised as inactive zymogens in cytoplasm (Elmore, 2007; Wong, 2011). They are both the initiators and executioners that are responsible for destruction of the cell (Belloc *et al,* 2000; Lavrik *et al*, 2005; Elmore, 2007; Wong, 2011). Caspase 8 and 9 are believed to be the initiator caspases which are the first to be activated the executioner caspase 3 (Belloc *et al,* 2000; Lavrik *et al*, 2005; Elmore, 2007; Wong, 2011). Caspase 8 is activated during the extrinsic apoptotic pathway and responsible for elimination of unwanted cells during the development and other stimulation signals (Elmore, 2007; Wong, 2011). On the other hand, caspase 9 is activated during the intrinsic/mitochondrial apoptotic pathway in response to a variety of stimuli such as γ- and UV-radiation, DNA damage and cytotoxic drugs (Elmore, 2007; Wong, 2011). Cell death receptor (extrinsic) and mitochondrial (intrinsic) apoptotic pathways are initiated by recruitment of caspase 8 into the DISC or caspase 9 into apoptosome (Elmore, 2007; Wong, 2011). These initiator caspases undergo then self-activation to start the caspase signalling cascade. Subsequently, these two pathways converge and trigger the execution pathway through the activation of caspase 3 (Elmore, 2007; Wong, 2011). However it is important to note that the exact mechanism for the activation of initiator caspases is still not clear.

Activation of apoptosis is considered as the key molecular mechanism responsible for the anti-cancer action of most of the currently studied potential anti-cancer agents, including polyphenols (Patel *et al,* 2007; Sharif *et al*, 2010; Shukla *et al*, 2010). However, polyphenols initiate apoptosis and its mechanism of action remains poorly understood in leukaemia. Some earlier works have demonstrated the pro-apoptotic activity of polyphenols in solid tumours cell lines of the breast, lung and cervix (Dai *et al*, 2013; Han *et al*, 2007; Jaganathan *et al*, 2009; Patel *et al,* 2007; Sharif *et al*, 2010; Shukla *et al*, 2010). Here, they demonstrated that polyphenols could induce apoptosis by the direct activation of the caspase cascade (Dai *et al*, 2013; Han *et al*, 2007; Jaganathan *et al*, 2009; Patel *et al,* 2007; Sharif *et al*, 2010; Shukla *et al*, 2010).

4.1.2 Glutathione (GSH)

Glutathione (GSH) is the most abundant non-protein thiol intracellular antioxidant molecules, in the cell Wilson *et al*, 2006; Franco *et al*, 2007; Franco and Cidlowski, 2009; Abdalla, 2011;

Ortega *et al*, 2011; Traverso *et al*, 2013). It has an important role in cellular defence against oxidant aggression, redox regulation of proteins thiols and maintaining redox homeostasis. GSH is critical to regulate several metabolic processes including enzyme activity, transport activity, signal transduction and gene expression through redox-sensitive nuclear transcription factors such as NFkB and p53 (Traverso *et al*, 2013). GSH is required for proper cellular function, including DNA and protein synthesis, cell differentiation, cell proliferation and apoptosis (Wilson *et al*, 2006; Franco *et al*, 2007; Franco and Cidlowski, 2009; Abdalla, 2011; Ortega *et al*, 2011; Traverso *et al*, 2013).

GSH accounts for greater than 90% of the cellular thiol content (Wilson *et al*, 2006; Murphy *et al*, 2008; Abdalla, 2011; Ortega *et al*, 2011; Traverso *et al*, 2013); of this 90% is found in the cytosol, while nearly 10 % is found in the mitochondria and a tiny amount is found in endoplasmic reticulum (Abdalla, 2011; Traverso *et al*, 2013). GSH is known as a ubiquitous tripeptide glutathione, which is formed by glutamic acid, cysteine, and glycine (Abdalla, 2011; Traverso *et al*, 2013). These peptides are assembled into active GSH in the presence of γ-glutamylcysteine synthetase (GCS), glutathione synthetase (GS), GSH reductase (GR), GSH synthetase (GSS), gamma glutamyltransferase (GTT) and glutathione S-transferases (GSTs) (Abdalla, 2011; Brechbuhl *et al*, 2012; Traverso *et al*, 2013; Irwin *et al*, 2013). These enzymes play an important role in synthesis, turnover, homeostasis and maintenance of intracellular GSH concentration (Brechbuhl *et al*, 2012; Irwin et al, 2013). Due to nucleophilic thiol group of tripeptide glutathione it can detoxify substances within the cell in one of three ways: (1) conjugation of glutathione to substrates catalyzed by glutathione S-transferases (GSTs); (2) chemical reaction with a reactive metabolite to form a conjugate; and (3) donation protons or hydrogen atoms to reactive metabolites or free radicals (Durgo *et al*, 2007). The action GSH and its enzymes are regulated by the specific transcription factor called nuclear factor E2-related factor 2 (Nrf2) (Abdalla, 2011; Traverso *et al*, 2013).

GSH is usually present in two forms: the reduced form GSH and the oxidized form GSSG (Abdalla, 2011; Traverso *et al*, 2013). The reduced form (GSH) represents the majority of GSH range between 1 and 10 mM and this form can be converted to the oxidized form (GSSG) during oxidative stress; which is estimated to be less than 1% of the total GSH (Abdalla, 2011; Traverso *et al*, 2013). The majority of GSH within the cell is found in a reduced form (Traverso *et al*, 2013; Irwin *et al*, 2013). Normally, maintaining optimal GSH: GSSG ratios in the cell are critical for cell survival, and a deficiency of GSH can result in oxidative damage (Abdalla, 2011; Traverso *et al*, 2013; Irwin *et al*, 2013). Their disruptions are contributed to development of many human diseases including neurodegenerative disorders, cystic fibrosis and aging and cancers including leukaemia, melanoma, hepatoma and ovarian cancer (Abdalla, 2011; Traverso *et al*, 2013).

In both normal and cancer cells, increased GSH level is associated with a proliferative response and is essential for cell cycle progression (Traverso *et al*, 2013). Particularly in normal healthy cells, a higher level of GSH is important for: (1) protecting cells from reactive oxygen species (ROS), toxins, mutagens, carcinogens and drugs, (2) normal cellular functions including signal transduction, and (3) regulation of gene expression, cell survival, apoptosis, and membrane transportation (Meurette *et al*, 2005; Murphy *et al*, 2008; Abdalla, 2011; Ortega *et al*, 2011).

However, in cancerous cells, GSH plays an important role in regulating mutagenic and carcinogenic mechanisms including: continual DNA synthesis, cell growth, prevention of cell death, cell sensitivity or resistance against cytotoxic drugs; and multidrug and radiation resistance (Meurette *et al*, 2005; Murphy *et al*, 2008; Abdalla, 2011; Ortega *et al*, 2011; Traverso *et al*, 2013). Many cancers, including lung, ovarian breast, colon, larynx and haematological malignancies have high levels of GSH (Abdalla, 2011; Traverso *et al*, 2013). This high GSH level increases the antioxidant capacity of the cancerous cell preventing oxidative stress, repaired the DNA damage and inhibited cell death process; and as a result this can lead to resistance to cancer treatments (Abdalla, 2011; Traverso *et al*, 2013). Irwin *et al*, 2013 reported that GSH and its associated enzymes, such as GST are commonly highly present in AML, CML and CLL patients compared with healthy individuals (Irwin *et al*, 2013). It is believed that this high GSH level protecting these leukaemia cells from oxidative stress and DNA damage chemotherapeutics (Irwin *et al*, 2013; Traverso *et al*, 2013). It is reported that the GSH system is capable of removing ROS from leukaemia cells (Traverso *et al*, 2013). It is also reported that an elevated GSH in these types of leukaemia patients is correlated with increased risk of disease relapse and resistance to the chemotherapeutics (Irwin *et al*, 2013). In particular, it has been found that AML, CML and CLL patients with high GSH levels is associated with resistance to anti-cancer agents such as cisplatin, doxorubicin, daunorubicin and melphalan (Irwin *et al*, 2013). In contrast, some types of cancers such as melanoma have low intracellular GSH levels, which decreases the cellular antioxidant capacity and increased oxidative stress via excessive production of ROS, which can cause DNA damage and ultimately leads to cell death; this event consequently increased the sensitivity of cancer cells to the treatment (Abdalla, 2011; Traverso *et al*, 2013). In this regard, measurment of the basal levels of GSH in cancer cells and detection of GSH is relevant to understanding the mechanisms of action of many cancer treatments, since many earlier reports show that depletion of GSH level sensitizes cancer cells to chemotherapy (Meurette *et al*, 2005; Murphy *et al*, 2008; Abdalla, 2011; Ortega *et al*, 2011; Traverso *et al*, 2013).

Several studies suggest that increased levels of glutathione (GSH) and its enzymes is considered as one of the major contributing factors to increase chemo-resistance in cancer cells

(Wilson *et al*, 2006; Abdalla, 2011; Ortega *et al*, 2011; Traverso et al, 2013). Cisplatin, oxaliplatin, doxorubicin, and 5-fluorouracil are examples of chemotherapeutic agents which have an ability to conjugate with the thiol glutathione (GSH) (Wilson *et al*, 2006; Traverso et al, 2013). From earlier studies, the proposed mechanisms for the role of GSH in regulating chemotherapy agents' resistance are: (1) GSH may serve as a cofactor in facilitating multidrug resistance protein (MRP)-mediated chemotherapy agents efflux in cells; (2) GSH may serve as a redox regulating cytoprotector based on the observations that many chemotherapy agents such as cisplatin and doxorubicin-resistant cells overexpress GSH; (3) high GSH levels in cancer cells can increase the rate of conjugation and detoxification of chemotherapy agents; and (4) GSH may function as a copper (Cu) chelator, thus reducing their effectiveness (Wilson *et al*, 2006; Abdalla, 2011; Ortega *et al*, 2011; Brechbuhl *et al*, 2012; Traverso et al, 2013). These reports suggested that in order to increasing chemotherapy efficacy and limit multi-drug resistance (MDR), the GSH levels may be depleted in cancer cells (Wilson *et al*, 2006; Abdalla, 2011; Ortega *et al*, 2011; Brechbuhl *et al*, 2012; Traverso et al, 2013).

Pervious work has shown isothiocyanates such as sulforaphane and β-phenylethyl isothiocyanate (Andelová *et al*, 2007; Hu *et a*l, 2010; Traverso *et al*, 2013; Irwin *et al*, 2013); and polyphenols such as quercetin (Tsao,2010; Khan *et al*, 2012) are the strongest natural inducer of phase II detoxifying enzymes such as GST (Andelová *et al*, 2007; Hu *et a*l, 2010; Tsao,2010; Khan *et al*, 2012; Traverso *et al*, 2013; Irwin *et al*, 2013). It has been shown that both isothiocyanate and polyphenol can serve as either antioxidants or pro-oxidants depending on cellular conditions, requirement and doses; thus they can help in the modulation of antioxidant redox system such as glutathione (GSH) (Andelová *et al*, 2007; Hu *et a*l, 2010; Tsao,2010; Khan *et al*, 2012; Traverso *et al*, 2013; Irwin *et al*, 2013). In addition, it has been reported that the antioxidant activity of isocyanates and polyphenols play a crucial role in their chemopreventive effect; whilst their pro-oxidant action may be important for their anti-cancerous effects (Andelová *et al*, 2007; Hu *et a*l, 2010; Khan *et al*, 2012; Traverso *et al*, 2013; Irwin *et al*, 2013).

Currently, all the present studies reported that GSH depletion in cells is strongly correlated to restore apoptosis induction, and this action could be very useful to increase the therapeutic efficacy of cancer treatment (Franco *et al*, 2007; Franco and Cidlowski, 2009; Ortega *et al*, 2011; Traverso *et al*, 2013; Irwin *et al*, 2013; Rocha *et al*, 2014). They reported that GSH depletion has been shown to regulate both extrinsic and intrinsic apoptotic signalling cascades at distinct checkpoints (Franco *et al*, 2007; Franco and Cidlowski, 2009; Ortega *et al*, 2011). Particularly, GSH depletion can predispose cells to apoptosis or directly trigger cell death by modulation of both the permeability transition pore formation and the activation of execution caspases (Franco *et al*, 2007; Franco and Cidlowski, 2009; Ortega *et al*, 2011). Also, it has

been reported that a reduction in the GSH content is necessary for the formation of the apoptosome and activates the intrinsic apoptotic pathway initiator by its oxidation-dependent dimerization (Franco *et al*, 2007; Franco and Cidlowski, 2009; Ortega *et al*, 2011). Furthermore, GSH depletion has also been shown to trigger cytochrome *c* release and it has been proposed that released cytochrome *c* from the mitochondria requires to be oxidized for its pro-apoptotic action, which would need cytosolic GSH levels to be depleted (Franco and Cidlowski, 2009). Also, it is reported that the GSH depletion during apoptosis can occur through distinct mechanisms: (1) by its extrusion across the plasma membrane through GSH transporters or pumps, which can lead then to activate the death receptors or caspases; (2) by its oxidation to GSSG which can lead to activate the intrinsic pathways of apoptosis because of the generation of reactive oxygen species (ROS) that arise from impairment of mitochondrial function; (3) by both its direct oxidation and/or conjugation, which then activated the apoptotic signals arising from pro-oxidant stimuli mediate GSH depletion; or finally by (4) impairment of GSH synthesis and recycling, which might also contribute to induce apoptosis (Wilson *et al*, 2006; Traverso *et al*, 2013). Many studies showed that the antioxidant property of GSH is strongly linked to overexpression of anti-apoptotic Bcl-2, which inhibits mitochondrial-induced apoptosis (Franco *et al*, 2007; Franco and Cidlowski, 2009; Abdalla, 2011; Ortega *et al*, 2011).

In fact, the modulation of cellular GSH is a double-edged sword, both sides of which have been exploited for potential therapeutic benefits (Abdalla, 2011; Traverso *et al*, 2013; Irwin *et al*, 2013; Rocha *et al*, 2014). Enhancing the capacity of GSH and its associated enzymes, in order to protect cells from redox related changes or environmental toxins, represents a persistent aim in the search for cytoprotective strategies against cancer (Abdalla, 2011; Traverso *et al*, 2013; Irwin *et al*, 2013; Rocha *et al*, 2014). On the contrary, the strategy of depleting GSH and GSH-related detoxification pathways is aimed at sensitizing cancer cells to chemotherapy, the so-called chemosensitization (Abdalla, 2011; Traverso *et al*, 2013; Irwin *et al*, 2013; Rocha *et al*, 2014). Finally, all these studies suggested that understanding the GSH-driven molecular pathways could be crucial to design new therapeutic strategies for overcoming cancer cell progression and chemoresistance, and increasing chemosensitivity (Abdalla, 2011; Traverso et al, 2013; Irwin et al, 2013; Rocha et al, 2014). To date, little work has looked at the GSH levels in leukaemia cells; and how these are affected by polyphenols alone and in combination with standard chemotherapy agents. Here a study of GSH levels is undertaken in order to determine whether GSH is a key to mechanism of action of polyphenols and chemotherapy agents in leukaemia cells and whether it can be used to explain the synergistic and antagonist effects observed when polyphenols and chemotherapy agents are used in combination.

4.1.3 Hallmark of DNA Damage: γ-H2AX Foci Formation

It is known that DNA wraps around an octameric complex of core histones H2A, H2B, H3 and H4 to form nucleosomes (Kuo and Yang, 2008; Fragkos *et al*, 2009; Rajendran *et al*, 2011). These histones are highly basic proteins that complex with DNA to form chromatin (Kuo and Yang, 2008; Fragkos *et al*, 2009; Rajendran *et al*, 2011). H2AX is a member of the H2A histone family and its phosphorylation is considered as a hallmark of DNA damage (Solier and Pommier, 2009; Rajendran *et al*, 2011). Studies have reported when cells are exposure to DNA damaging agents such as ionizing radiation, UV light or chemotherapies (e.g. cyclophosphamid, cisplatin, etoposide and doxorubicin) this causes a rapid phosphorylation of a serine-139 residue (also known as γ-H2AX) (Rakiman *et al*, 2008; Kuo and Yang, 2008; Fragkos *et al*, 2009; Rajendran *et al*, 2011). This directly recruits clusters of proteins at the site of DNA damage, called DNA damage response foci (Rakiman *et al*, 2008; Kuo and Yang, 2008; Fragkos *et al*, 2009; Rajendran *et al*, 2011). The rapid phosphorylation of serine 139 (γ-H2AX) recruits a variety of proteins commonly associated with DNA damage/repair and cell cycle check points (Fragkos *et al*, 2009; Rajendran *et al*, 2011). Indeed, H2AX is phosphorylated by phosphatidylinositol-3 kinase (PI3K)-like kinases, including ATM, ATM-Rad3-related (ATR), ATM related kinase (ATX) and other cell cycle checkpoint factors such as Chk1 and Chk2 (Fragkos *et al*, 2009; Rajendran *et al*, 2011). So, phosphorylated γH2AX plays an important role the recruitment and localization of DNA proteins, and cell cycle checkpoint factors to the DNA-damaged sites; the latter being the main effector for the induction of apoptosis (Kuo and Yang, 2008; Fragkos *et al*, 2009; Solier and Pommier, 2009; Rajendran *et al*, 2011). Thus the presence of γ-H2AX foci are an excellent marker of DNA damage caused by cytotoxic agents or cancer treatments (Kuo and Yang, 2008; Fragkos *et al*, 2009; Solier and Pommier, 2009; Rajendran *et al*, 2011).

Some earlier studies in solid tumours have reported that dietary agents including: isothiocyanates (such as sulforaphane) (Singh *et al*, 2004; Zhang et al, 2006; Andelová *et al*, 2007; Sekine-Suzuki *et al,* 2008) and polyphenols (such as quercetin, genistein, curcumin and resveratrol) (Tan *et al*, 2009; Ouyang *et al*, 2009; Sahu *et al*, 2009; Leone *et al,* 2010) can cause DNA damage response, resulting in the formation of γ-H2AX foci (Singh *et al*, 2004; Zhang et al, 2006; Andelová *et al*, 2007; Sekine-Suzuki *et al,* 2008; Tan *et al*, 2009; Ouyang *et al*, 2009; Sahu *et al*, 2009; Leone *et al,* 2010; Rajendran *et al*, 2011). For example, sulforaphane has been shown to induce DNA damage associated with the elevation in the expression of γ-H2AX levels, which also caused an increase in ROS generation, a depletion of GSH, an activation of G_2/M checkpoint and cell death in cancer cell lines of prostate (PC-3) (Singh *et al*, 2004), colon (SW620 and HCT116) (Andelová *et al*, 2007) and cervical (HeLa) (Sekine-Suzuki *et al,* 2008). Likewise, the polyphenols: quercetin, genistein, curcumin and

resveratrol have been shown to cause direct DNA damage in association with an increase of H2AX phosphorylation through the activation of the ATM/ATR and CHK1/2 pathway-mediated S or G_2/M cell cycle arrest and apoptosis; in cancer cell lines of lung (A549) (Tan *et al*, 2009), ovarian (HO-8910 and Ovcar-3) (Tyagi *et al*, 2005; Ouyang *et al*, 2009) and pancreas (BxPC-3) (Sahu *et al*, 2009).

Rajendran *et al*, (2011) suggested that combination agents could be very effective and produced synergistic actions when used to target DNA damage and interfer with DNA repair in cancer cells (Rajendran *et al*, 2011). There are only four studies examining the effects of combination anti-cancer agents on DNA damage. The combination used included: benzyl isothiocyanate and γ-irradiation (Sahu *et al*, 2009), β-phenylethyl isothiocyanate and vorinostat (Hu *et al,* 2010), sulforaphane and the HDAC inhibitor, panobinostat (LBH589) (Pettazzoni *et al*, 2011) and resveratrol and the purine analogs (fludarabine or cladribine) (Podhorecka *et al*, 2011). Each combination caused a synergistic cytotoxicity, G_2/M phase arrest and apoptosis associated with increase the expression of γ-H2AX in pancreatic cancer cell lines (BxPC-3) (Sahu *et al*, 2009), a prostate cancer cell lines (PC3) (Pettazzoni *et al*, 2011) a leukaemia cell lines (HL60) (Hu *et al,* 2010) and chronic lymphocytic leukaemia (CLL) cells taken from a patients (Podhorecka *et al*, 2011). It was reported that the synergestic effect seen in each study was attributed to an increase in γ-H2AX foci which occurred through the activation of ATM/ATR, Chk2, Cdc25C, and Cdk-1, and induction of p21 (Sahu *et al*, 2009; Hu *et al,* 2010; Pettazzoni *et al*, 2011; Podhorecka *et al*, 2011); and the inhibition of GSH (Hu *et al,* 2010). Therefore the measurement of γ-H2AX foci is useful indicator to identify the most effective agents that induce the DNA damage (in particular DSBs) (Kuo and Yang, 2008). γ-H2AX can also be useful for elucidating the pharmacodynamics of cytotoxic drugs and tumour killing drugs (Kuo and Yang, 2008). The foci can be measured to elucidate whether or not drugs reach the tumour, whether or not drugs reach their activated forms, and whether or not the drugs affect the DNA (Kuo and Yang, 2008). Finally, agents that targeted the DNA and then caused DNA damage through an increased in the formation of γ-H2AX foci can be very effective for cancer treatment; because presence of γ-H2AX foci after treatment indicate the induction of cell cycle arrest and/or apoptosis in cancer (Kuo and Yang, 2008; Rajendran *et al*, 2011).

4.1.4 Hypothesis

It is hypothesised that the induction of apoptosis observed following polyphenol treatment is caused by the activation of the intrinsic apoptotic pathway through the up-regulation of caspase 9 and/or the extrinsic apoptotic pathway via the up-regulation of caspase 8 in part, via reduction of glutathione, which normally protects cells against oxidative stress and apoptosis.

Further to this, it is hypothesised that polyphenols alone and in combination with chemotherapy agents can alter DNA damage and subsequent apoptosis.

4.1.5 Aims

The major aims of this study were to investigate the effects of the polyphenols and chemotherapeutic agents alone or in combination on: (1) activity of initiator caspase 8 and 9 of apoptosis using Caspase-Glo 8 and 9 luminescent assays, (2) glutahione (GSH) levels using the GSH-Glo™ glutathione luminescent assay and CellTracker GSH-Green 5-chloromethylfluorescein diacetate (CMFDA) stain and (3) γ-H2AX foci formation using immuneflurocant staining called Alexa Fluor® 647 Mouse anti-H2AX (pS139) in two human lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) and two human myeloid leukaemia cell lines (THP-1 and KG1a).

4.2 Methods and Materials

4.2.1 Experimental Design

The effects of polyphenols alone (quercetin, apigenin, chrysin, emodin, aloe-emodin, rhein, cis-stilbene and trans-stilbene) and in combination with standard chemotherapy agents were investigated to determine effects on caspase 8 and 9 activity; glutathione (GSH) levels and the induction of DNA damage. Initially, the effect of each polyphenol alone (quercetin, apigenin, chrysin, emodin, aloe-emodin, rhein, *cis*-stilbene and *trans*-stilbene) was examined on the levels of caspase 8 and 9 activity, glutathione (GSH) levels and DNA damage.

For the polyphenol/chemotherapy agent combination work, only those combinations which induced a synergistic reduction in ATP levels and induction of apoptosis were investigated further these included: quercetin (QUE); apigenin (AP); emodin (EMO); rhein (RH) and *cis*-stilbene (CIS) (Sigma) (Chapter 2). Likewise only those chemotherapy agents which significantly interacted with polyphenols which were identified in Chapter 3 were investigated further. These included: two topoisomerase inhibitor agents (doxorubicin (DOX) and etoposide, (ETP)), two alkylating agents (cyclophosphamide (CYCLO) and cisplatin (CSP)), and two anti-metabolite agents (methotrexate (MTX) and 6-mercaptopurine (6-MP)) (Sigma). All polyphenols were prepared as described in Chapter 2. All chemotherapy agents were prepared as described in Chapter 3. The effect of these selected polyphenols and chemotherapy agents were examined alone and in combination, on the caspase 8 and 9 activity, glutathione (GSH) levels and DNA damage.

Four human leukaemia cell lines were used for this study: two lymphoid leukaemia cell lines: JURKAT and CCRF-CEM, and two myeloid leukaemia cell lines: THP-1 and KG-1a (Chapter 2). All cell lines were tested regularly for mycoplasma contamination using the MycoAlert TM

mycoplasma detection kit (Lonza) and were all tested negative throughout the study. Cells were cultured as previously described (Chapter 2) and incubated at 37°C with 5% CO_2 in a humidified incubator.

4.2.2 Caspases-Glo ® Luminescent 8 and 9 Assays

Caspases play a key role in the induction of apoptosis (Lavrik *et al*, 2005; Belloc *et al,* 2000). Caspase 3 which was shown to be up-regulated by polyphenols (Chapter 2) is commonly initiated and activated by either caspase 8 via the extrinsic pathway, or by caspase 9 via the intrinsic pathway (Belloc *et al,* 2000). Thus here Caspase-Glo 8 and 9 assays (Promega) were used to determine the effects of polyphenols and chemotherapy agents on the induction of caspases 8 and 9 to determine whether apoptosis was activated via the intrinsic and/or extrinsic pathway. The Caspase-Glo 8 and 9 assays (Promega) contains a luminogenic substrate: tetrapeptide Z-LETD-aminoluciferin for caspase 8 and tetrapeptide (Z-LEHD-aminoluciferin for caspase 9. These substrates combine with a thermostable luciferase in the presence of caspase 8 or caspase 9; this causes cell lysis and the release of ATP. Each released ATP combines with a free aminoluciferin molecule, which in the presence of oxygen (O_2), magnesium (Mg^{2+}) and luciferase is catalysed to oxyluciferin, AMP, PPi and carbon dioxide. The energy formed during this reaction produces a luminescent signal (Figure 4.1) which can be measured by a luminometer. The resulting luminescent signal is directly proportional to the amount of caspase 8 or 9 activity.

Caspase-Glo® 8 and 9 assays (Promega) were used to examine: 1) the effect of all polyphenols alone (quercetin, apigenin, chrysin, emodin, aloe-emodin, rhein, *cis*-stilbene and *trans*-stilbene (Sigma)) in four leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) following 6 and 24 h. Quercetin, apigenin, emodin, rhein and *cis*-stilbene were investigated at their NSD, LSD (as determined earlier in Chapter 3), and their AP_{50} doses (Chapter 2). The less potent polyphenols: chrysin, aloe-emodin and *trans*-stilbene were tested only at their AP_{50} doses (Chapter 2); 2) the effects of selected polyphenols (quercetin (QUE), apigenin (AP), emodin (EMO), rhein (RH) and *cis*-stilbene (CIS)) (Sigma) were investigated in combination with four chemotherapy drugs (etoposide, doxorubicin, cyclophosphamide, cisplatin (CSP)) (Sigma). Doses were selected as the LSDs that could induce apoptosis from the results from Chapter 3. A summary of the treatment regimens is shown in Table 4.1. Cells were seeded into white 96-well plates (Fisher Scientific) at 2.5 X 10^3 cells per well and treated. All treatments were performed in triplicate. An ethanol vehicle controls at 0.1 % (v/v) ethanol was also included.

Following treatments Caspase 8 and 9 activity was measured using the Caspase-Glo 8 and 9 assays (Promega). Plates containing cells were removed from the incubator and equilibrated to room temperature for 30 minutes. Fifty microlitres Caspase-Glo reagent (Promega) with

proteasome inhibitor (MG-132) were added to each well and gently mixed on a plate shaker at 400 rpm for 30 seconds. The plate was then incubated at room temperature for 90 min. The luminescence was measured using a Wallac Victor 2 1420 luminescence detector. The average luminescence readings were calculated and all treated samples were normalised to the vehicle controls.

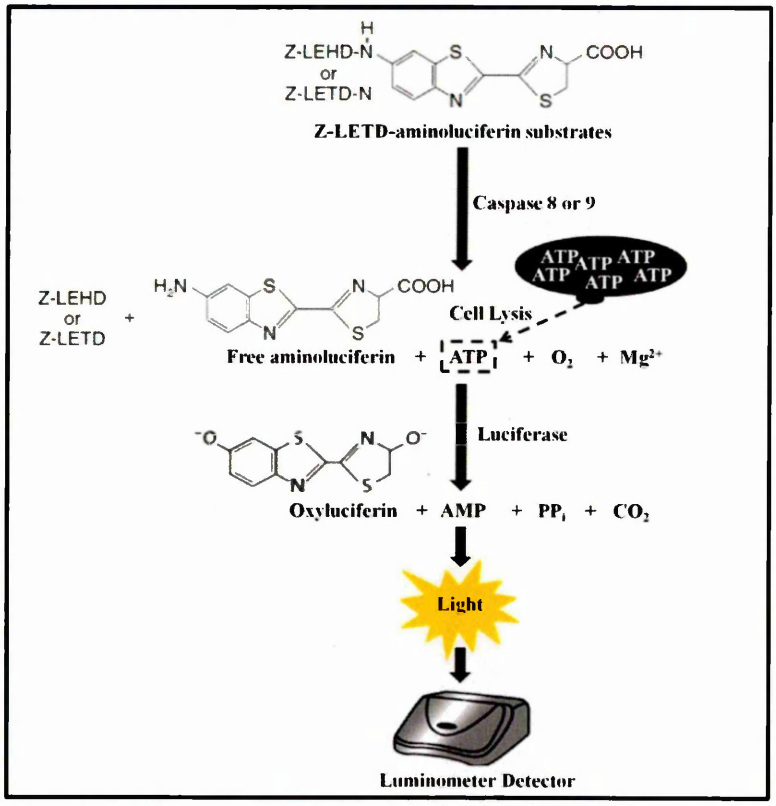


Figure 4.1: Schematic of the Caspases-Glo 8 and 9 Assays. Caspase 8 or 9 cleaves their luminogenic substrate (Z-LETD or Z-LEHD respectively) causing the production of free aminoluciferin. The free aminoluciferin reactions with ATP, oxygen (O_2) and magnesium ($Mg2^+$) in the presence of luciferase to production luminescence, which can be detected by a luminometer.

The Polyphenols/Chemotherapy Combinations Used to Investigate Caspase 8 and 9 Activity.				
Combination Treatments	**Lymphoid Leukaemia Cell Lines**		**Myeloid Leukaemia Cell Lines**	
	JURKAT	**CCRF-CEM**	**THP-1**	**KG-1a**
Doxorubicin (DOX) & Polyphenols	DOX + QUE DOX + AP DOX + EMO DOX + RH DOX + CIS	DOX + QUE DOX + AP DOX + EMO DOX + RH DOX + CIS	DOX + QUE DOX + AP	DOX + QUE DOX + AP
Etoposide (ETP) & Polyphenols	ETP + QUE ETP + AP ETP + EMO ETP + RH ETP+ CIS	ETP + QUE ETP + AP ETP + EMO ETP + RH ETP+ CIS	ETP + QUE ETP + AP	ETP + QUE ETP + AP
Cyclophosphamide (CYCLO) & Polyphenols	CYCLO + QUE CYCLO+ AP CYCLO + EMO CYCLO + RH	CYCLO + QUE CYCLO+ AP CYCLO + EMO CYCLO + RH	CYCLO + AP	CYCLO + AP
Cisplatin (CSP) & Polyphenols	CSP + QUE CSP + AP CSP + EMO CSP + RH CS + CIS	CSP + QUE CSP + AP CSP + EMO CSP + RH CS + CIS	CSP + QUE CSP + AP	CSP + QUE CSP + AP

Table 4.1: A summary of the polyphenols/chemotherapy combinations used to investigate caspase 8 and 9 activity. The LSDs for each agent was studied in four leukaemia cell lines, following 24 h treatment. Only those combinations that had previously induced a synergistic effect on caspase 3 activity and the morphological changes of apoptosis (Chapter 3) were studied.

4.2.3 Glutathione (GSH) Assays

The detection of GSH is important in understanding the mechanisms of action of any cancer treatment. For this reason two glutathione (GSH) assays: 1) GSH-Glo™ Glutathione Assay (Promega); and 2) CellTracker GSH-Green 5-chloromethylfluorescein diacetate (CMFDA) stain were used to screen the effects of polyphenols and chemotherapies alone and in combination. The CellTracker™ Green CMFDA- staining for GSH was used to confirm the GSH-Glo™ Glutathione assay results and enable visualisation of GSH in pre-apoptotic cells.

4.2.3.1 GSH-Glo™ Glutathione Assay

The GSH-Glo™ glutathione assay has two steps (Figure 4.2): 1) Initially the luciferin derivative substrate proluminogenic Luciferin-NT is converted into luciferin in the presence of glutathione S-transferase (GST) which causes cell lysis. During this process, GST detaches a non-functional group from a luciferin derivative and couples this to the reduced glutathione. 2) Luciferase is then added which converts luciferin in the presence of ATP, oxygen (O_2) and magnesium (Mg^{2+}) to the oxyluciferin, AMP, carbon dioxide and energy in the form of a luminescent signal. This signal can now be measured by a luminometer, and is directly proportional to the level of glutathione (GSH) present in the cell.

The GSH-Glo™ Glutathione Luminescent assay (Promega) was optimised using buthionine sulphoximine (BSO) as negative control (GSH inhibitor agent) and N-acetylcysteine (NAC) as positive control (GSH induction agent) in four leukaemia cell lines (CCRF-CEM, JURKAT, KG1a, THP-1) and two normal cell lines ($CD133^+$ HSC and $CD34^+$ HSC) following 24 h.

Using this assay the basal GSH levels was determined for the four leukaemia cell lines (CCRF-CEM, JURKAT, KG1a, THP-1) and the two normal cell lines ($CD133^+$ HSC and $CD34^+$ HSC).

Initially the GSH-Glo™ Glutathione Luminescent assay (Promega) was then used to determine the effect of all polyphenols alone (quercetin, apigenin, chrysin, emodin, aloe-emodin, rhein, *cis*-stilbene and *trans*-stilbene (Sigma)) on GSH levels in four leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) at 24 h. Quercetin, apigenin, emodin, rhein and *cis*-stilbene were examined at their NSD and LSD that reduced ATP levels (as determined in Chapter 3); and their dose ranges used to determine the IC_{50} values (Chapter 2). For chrysin, aloe-emodin and *trans*-stilbene these were tested at the dose ranges used to determine their IC_{50} values (Chapter 2).

For the polyphenol/chemotherapy combination work the effect of selected polyphenols (quercetin (QUE), apigenin (AP), emodin (EMO), rhein (RH) and *cis*-stilbene (CIS)) (Sigma), and six chemotherapy drugs (etoposide, doxorubicin, cyclophosphamide, cisplatin (CSP),

methotrexate (MTX) and 6-mercaptopurine (6-MP)) (Sigma) were investigated alone and in combination on GSH levels using their LSDs that reduced ATP levels (Chapter 3) in four leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) at 24 h. These polyphenols and chemotherapy drugs were selected as they had previously caused synergistic or antagonistic effects when used in combination (Chapter 3). A summary of the treatment regimens is shown in Table 4.2. Cells were seeded into white 96-well plates (Fisher Scientific) at 2.5 X 10^3 cells per well. Following treatment cells were incubated at 37^0C with 5% CO_2 for 24 h. All treatments were performed in triplicate. An ethanol vehicle controls at 0.1 % (v/v) ethanol was also included.

Following treatment glutathione levels were measured using the GSH-Glo™ glutathione luminescent assay (Promega). Treated cells were initially washed with PBS, resuspended in 50 µl of PBS in a white 96-well plate (Fisher Scientific). Fifty microliters of GSH-Glo™ reagent containing Luciferin-NT substrate and glutathione S-transferase (diluted 1:50 in GSH-Glo™ reaction buffer) (Promega) was added to each well. The content was then gently mixed, and incubated at RT for 30 min. Next, 100µl of reconstituted luciferin detection reagent containing the luciferase enzyme (Promega) was added to each well. The content was then gently mixed using a shaker for 2 min and then further incubated at RT for a further 15 min. The luminescent signal was measured using a Wallac Victor 2 1420 luminescence detector. The luminescent signal is proportional to the levels of GSH in the sample. The average luminescence readings were calculated and all treated samples were normalised to the control.

The Polyphenols/Chemotherapy Combinations Used to Investigate Glutathione (GSH) Levels and DNA Damge (γ-H2AX Foci Formation)

The Combination Treaments	Lymphoid Leukaemia Cell Lines		Myeloid Leukaemia Cell Lines	
	JURKAT	CCRF-CEM	THP-1	KG-1a
Doxorubicin (DOX) & Polyphenols	DOX + QUE DOX + AP DOX + EMO DOX + RH DOX + CIS	DOX + QUE DOX + AP DOX + EMO DOX + RH DOX + CIS	DOX + QUE DOX + AP DOX + EMO DOX + RH DOX + CIS	DOX + QUE DOX + AP DOX + EMO DOX + RH DOX + CIS
Etoposide (ETP) & Polyphenols	ETP + QUE ETP + AP ETP + EMO ETP + RH ETP+ CIS	ETP + QUE ETP + AP ETP + EMO ETP + RH ETP+ CIS	ETP + QUE ETP + AP ETP + EMO ETP + RH ETP+ CIS	ETP + QUE ETP + AP ETP + EMO ETP + RH ETP+ CIS
Cyclophosphamide (CYCLO) & Polyphenols	CYCLO + QUE CYCLO+ AP CYCLO + EMO CYCLO + RH CYCLO + CIS	CYCLO + QUE CYCLO+ AP CYCLO + EMO CYCLO + RH CYCLO + CIS	CYCLO + QUE CYCLO+ AP CYCLO + EMO CYCLO + RH CYCLO + CIS	CYCLO + QUE CYCLO+ AP CYCLO + EMO CYCLO + RH CYCLO + CIS
Cisplatin (CSP) & Polyphenols	CSP + QUE CSP + AP CSP + EMO CSP + RH CS + CIS	CSP + QUE CSP + AP CSP + EMO CSP + RH CS + CIS	CSP + QUE CSP + AP CSP + EMO CSP + RH CS + CIS	CSP + QUE CSP + AP CSP + EMO CSP + RH CS + CIS

Table 4.2: A summary of the polyphenols/chemotherapy combinations used to investigate glutathione (GSH) levels and the formation of γ-H2AX Foci in four leukaemia cell lines, following at treatment with the LSDs for 24 h. Note that only those combinations that produced a synergistic, additive, competitive antagonistic or antagonistic effects at reduction of ATP levels; or caused an interactive, non-Interactive or antagonistic effects on cell cycle (Chapter 3) were investigated.

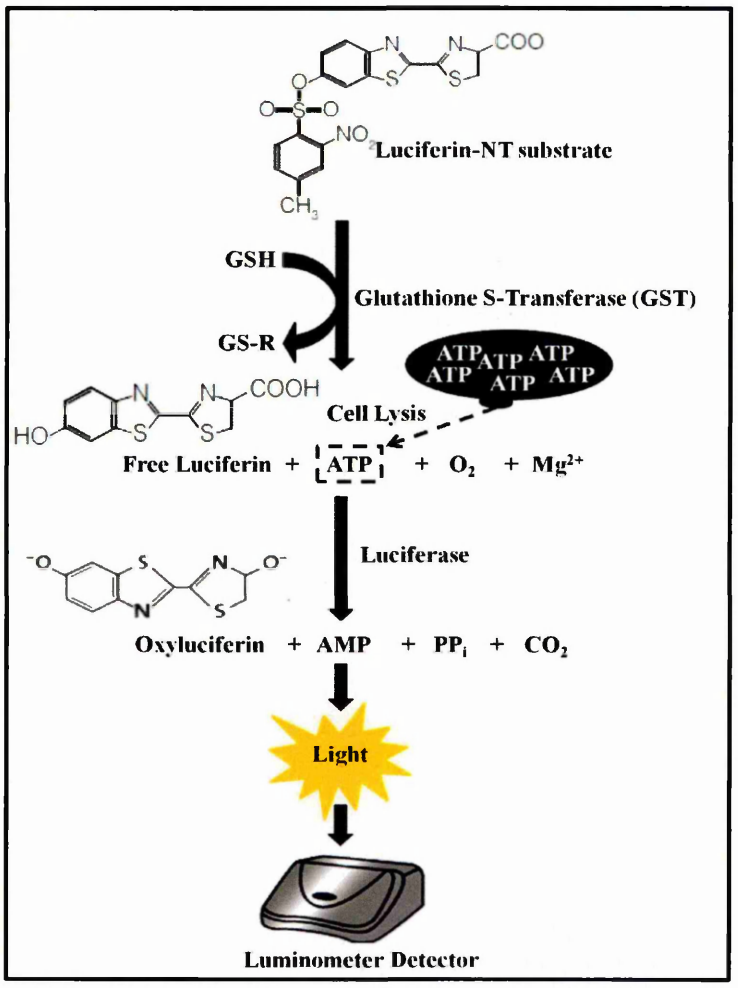


Figure 4.2: Schematic of the GSH-Glo™ Glutathione Assay. The assay is based on the conversion of a luciferin NT-substrate into luciferin in the presence of glutathione and glutathione S-transferase (GST). Free luciferin is converted in the presence of luciferase, ATP, oxygen (O_2) and magnesium (Mg^{2+}) to oxyluciferin, AMP, carbon dioxide (CO_2) and energy in the form of a luminescent signal which can be measured by luminometer.

4.2.3.2 Cell Tracker™ Green 5-Chloromethylfluorescein Diacetate (CMFDA)-Glutathione Staining and Fluorescence Microscopy.

The Cell Tracker Green 5-chloromethyl fluorescein diacetate (CMFDA) dye is an excellent tool for long-term measurement of intracellular thiol levels to assess cell viability, cytotoxicity and drugs sensitivity and resistance (West *et al*, 2001; Christian *et al*, 2008). Cell Tracker Green CMFDA dye (Molecular Probe) is a thiol-reactive cytoplasmic fluorescent probe (Lantz et al, 2001; West *et al*, 2001). The CMFA stain freely diffuses through the cell membrane of live cells (Lantz et al, 2001; West *et al*, 2001; Christian *et al*, 2008). Once in the cell, the acetate group of the CMFDA stain is cleaved by cytoplasmic esterases. The chloromethyl group of CMFDA then reacts with the sulfhydryl (SH) group of any glutathione in the presence of glutathione transferase (GST). This makes the cell impermeable, trapping green fluorescent 5-chloromethyl-fluorescein dye in the cell cytoplasm which can be visualised by fluorescence microscope (Figure 4.3) (Lantz et al, 2001; West *et al*, 2001; Christian *et al*, 2008). It is important to note that the levels of GST affect the levels of staining and fluorescence (Lantz et al, 2001). However the CMFDA dye does not fluorescence until it is cleaved by cytoplasmic

esterase and conjugation with glutathione (West *et al*, 2001). This dye has a relatively low pKa value which exhibits bright green fluorescence in the cytoplasm at all physiological pH levels (Lantz et al, 2001; West *et al*, 2001). CMFDA is commonly used in conjunction with Hoechst 33342 staining, as this is an effective way of analysing intracellular glutathione levels using the CMFDA, as well as being able to discriminate between live and apoptotic cells which have reduced intracellular glutathione and the morphological characteristic of apoptosis which is shown by the CMFDA and Hoechst 3342 staining respectively (Lantz et al, 2001; West *et al*, 2001; Christian *et al*, 2008).

The Cell Tracker™ Green 5-chloromethylfluorescein diacetate (CMFDA)-GSH stain was used to measure the percentage of cells with GSH activity, when treated with selected polyphenols (quercetin (QUE), apigenin (AP), emodin (EMO), rhein (RH) and *cis*-stilbene (CIS)) (Sigma); and chemotherapy agents (doxorubicin (DOX), etoposide (ETP), cyclophosphamide (CYCLO), cisplatin (CSP), methotrexate (MTX) and 6-mercaptopurine (6-MP)) (Sigma) when used alone, and in combination at their LSDs (Chapter 3). The selected polyphenols and chemotherapy drugs were selected as they had previously demonstrated a synergistic or antagonistic effect when used in combination (Chapter 2). A summary of the treatment regimens is shown in Table 4.2.This investigation was made in four leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) following 24 h treatment. Cells were seeded into white 24-well plates (Fisher Scientific) at 1.0 X 10^3 cells per well and treated. Finally, treated cells incubated at 37^0C with 5% CO_2 for 24 h. All treatments were performed in triplicate, an ethanol vehicle controls at 0.1 % (v/v) ethanol was also included.

Following polyphenol and chemotherapy treatments, cells were stained with Cell Tracker™ Green CMFDA (Molecular Probes) to detect and quantify GSH levels (specificity 95%) and counterstained with Hoechst 33342 stain (Sigma) to localize the cell nuclei. The cells were harvested and transferred to labelled centrifuge tube and spun at 400 g for 5 min. The cells were then gently resuspended in 2 ml of 0.5 µM pre-warmed Cell Tracker™ Green CMFDA dye working solution prepared in serum-free medium and incubation at $37C^0$ with 5% CO_2 for 45 min, this was then replaced with 2 ml fresh pre-warmed serum-free medium and incubation at $37C^0$ with 5% CO_2 for a further 30 min. The cells were then washed twice in PBS and transferred to a 96 well plate (Fisher Scientific). Ten microliteres of 2 µg/ml of Hoechst 33342 (Sigma) was added in each well and incubated for 15 min. Stained cells were observed and analysed under inverted fluorescence microscopy (Olympus, 1x2-UCB) using $Cell^F$ imaging software (Olympus). CMFDA and Hoechst 33342 stains were examined using the FITC (green) and DAPI (blue) fluorescent filters. The excitation wavelengths were 488 nm for CMFDA and 364 nm for Hoechst 33342. The emission wavelengths for fluorescence detection were 510–540 nm for CMFDA and 380– 485 nm for Hoechst 33342.

Stained cells were examined under an inverted fluorescence microscopy (Olympus, 1x2-UCB). Using the monocamera and dual excitation settings images were captures using the FITC filter for CMFDA green staining and the DAPI filter for Hoechst 33342 staining (Figure 4.4). Images were captured using Cell^F imaging software (Olympus). CMFDA green staining was localised mainly in cytoplasm, as well in nucleus of the live cells only, which commonly have high glutathione level, while it is depleted in apoptotic and dead cells (Figure X). Hoechst 33342 blue staining was commonly localised in the nuclei of both live and dead cells (Figure X). Two hundred cells (green and blue) were counted and then percentage of (green stained) GSH positive cells was determined for each sample.

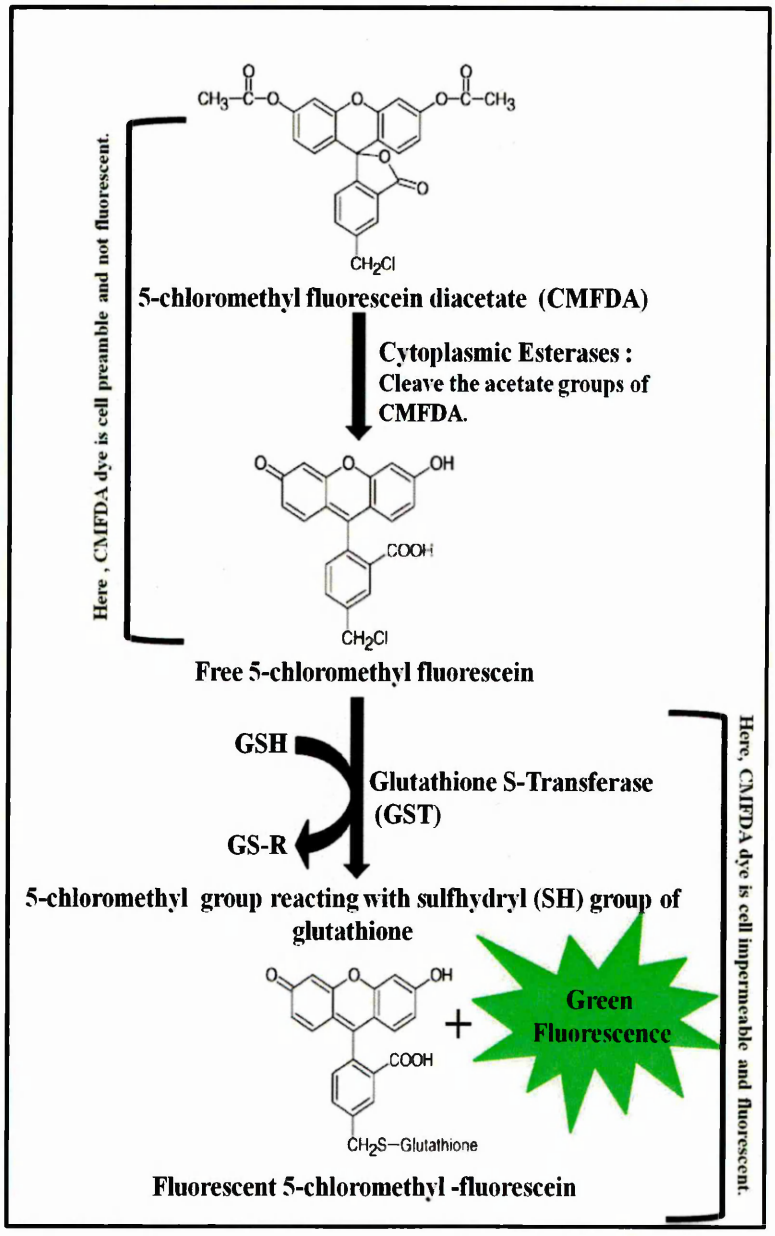


Figure 4.3: Schematic of the CellTracker™ 5-chloromethyl fluorescein diacetate (CMFDA) stain. CMFDA dye freely diffuses through the cell membrane of live cells. Once the dye diffused into the cells, cytoplasmic esterases cleave the acetate groups of CMFDA dye, then the chloromethyl group of CMFDA reacting with sulfhydryl (SH) group of glutathione in the presence of glutathione transferase (GST) making a cell impermeable and trapping 5-chloromethyl–fluorescein dye within the cell which fluorescence green and can be visualised using a fluorescence microscope.

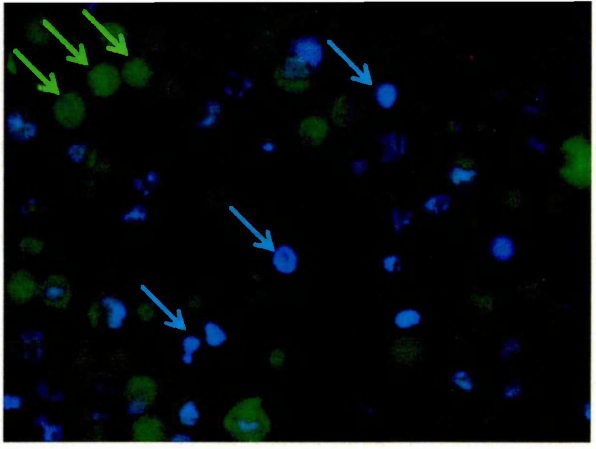


Figure 4.4: An example of glutathione (GSH) assessment uses dual staining of CMFDA (green) and Hoechst 33342 (blue) for THP-1 myeloid cell lines after treated with a combination of DOX and QUE at their LSDs for 24 h. From the picture, it can be seen that Hoechst 33342 blue staining (blue arrows) localised the nuclei of both living and dead cells, while CMFDA green staining (green arrows) was localised in the cell cytoplasm and nucleus of only live cells.

4.2.4 γ-H2AX Immunofluorescent Detection using Alexa Fluor® 647 Mouse anti-H2AX (pS139) Staining and Fluorescence Microscopy

Cytotoxic agents and radiation commonly cause DNA damage. They cause double-stranded breaks (DSBs) in DNA which is followed by rapid formation of thousands of gamma-H2AX foci. These trigger serine 139 phosphorylation in the chromatin flanking the DSB site. This is normally excised during post-translational processing and is included in amino acid sequence numbering. Immunofluorescent staining can be used to identify H2AX (pS139)-containing foci. As such, γ-H2AX (pS139) immunofluorescence localization serves as a biomarker for DNA damage. This is the most sensitive method to detect the γ-H2AX and enables the quantification of single foci in individual cells. Here by using γ-H2AX immunofluorescent staining, it was possible to confirm the previous cell cycle results, by quantifying the cells with foci in each sample after treatment with polyphenols and chemotherapy alone and in combination.

Alexa Fluor® 647 Mouse anti-H2AX (pS139) is specific designed for the phosphorylation of Ser-139 at the C-terminal region of H2AX enabling the visualisation of γH2AX by immunofluorescence microscopy. This direct immunofluorescence method uses a single primary antibody which recognizes and binds to the targeted antigen epitope (Figure 4.5). The antibody is conjugated to a fluorophore, which can be excited and visualised by fluorescent microscope. This immunofluoresence can be used in combination with non-antibody methods of fluorescent staining such as Hoechst 33342 to label DNA.

The γ-H2AX immunofluorescent stain (BD Pharmingen™) was used to measure the DNA damage (measured as γ-H2AX foci) in four leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a), when treated with selected polyphenols (quercetin (QUE), apigenin (AP), emodin (EMO), rhein (RH) and cis-stilbene (CIS)) (Sigma); and six chemotherapy drugs (doxorubicin (DOX), etoposide (ETP), cyclophosphamide (CYCLO), cisplatin (CSP), methotrexate (MTX) and 6-mercaptopurine (6-MP)) (Sigma) alone and in combination at their

LSDs (Chapter 3) for 24 h. These polyphenols and chemotherapy treatments were selected as they previously caused a synergistic or antagonistic effect on ATP levels and apoptosis; plus they had an interactive or antagonistic effect on cell cycle (Chapter 3). Table 4.2 summarises the combination treatments investigated here. All cells were seeded into a BD Falcon™ 96-well Imaging Plate (BD Pharmingen™) at 1.0 X 10^3 cells per well and treated. Treated cells incubated at 37^0C with 5% CO_2 for 24 h. All treatments were performed in triplicate. An ethanol vehicle control at 0.1 % (v/v) ethanol was also included.

Following treatments, the cells were removed from the wells, centrifuged at 400g for 10 minutes and washed twice with 100 µl of PBS (Sigma). Cells were fixed for 10 minutes in 100 µl BD Cytofix™ fixation buffer (BD Pharmingen™). The fixative was removed, and cells washed twice with 100 µl of PBS. Cells were then permeabilised using 100 µl of -20°C 90% methanol (Sigma) and incubated at RT for 5 min, permeabilisation buffer was then removed, and the cells were washed twice in 100 µl of PBS. Fifty microliters of diluted Alexa Fluor® 647 Mouse anti-H2AX (pS139) (1:10 v/v) (BD Pharmingen™) was added to the cells and they were incubated at RT for 60 min in the dark. Following incubation cells were washed three times in PBS. After the final wash, cells were counter-stained in 100 µl 2 mg/ml Hoechst 33342 (Sigma) and incubated for 15 min before imaging. Stained cells were visualised using an inverted fluorescence microscopy (Olympus, 1x2-UCB) using the monocamera using dual excitation settings for Texas Red (for γ-H2AX foci) which were stained red and for DAPI (for Hoechst 33342) blue staining of the cell nuclear material (Figure 4.6). Those cells with 6 and more γ-H2AX foci were considered as cells with DNA damage (Rakiman *et al*, 2008). Two hundred cells (cells with ≥6 foci and cells with <6 foci) were counted and the percentage of cells with ≥6 foci was determined for each sample. Images were captured using (Cell-F software, Olympus).

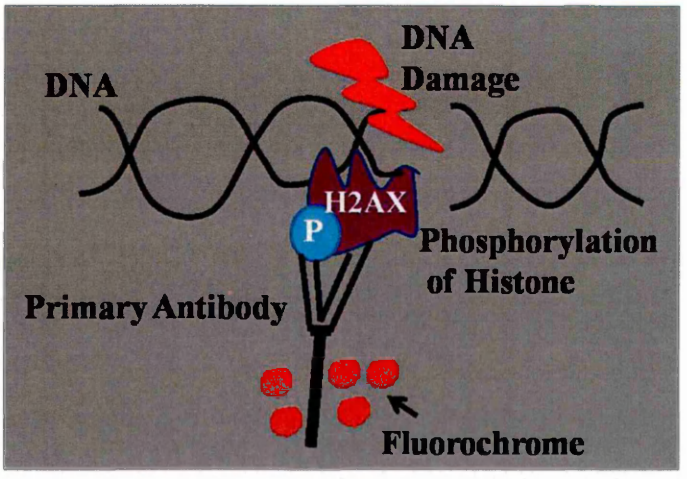


Figure 4.5: The Principle Method of H2AX Immunofluorescent Detection.

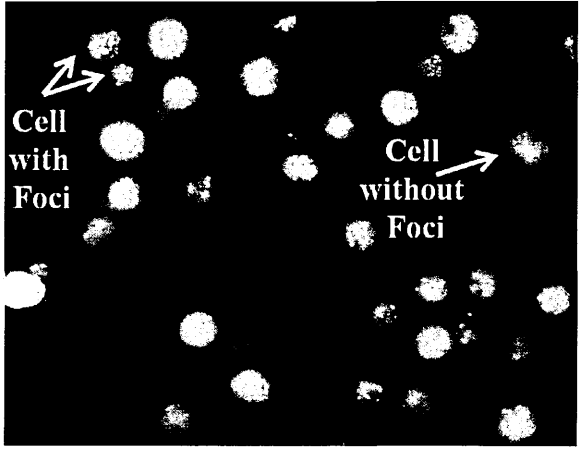


Figure 4.6: Immunofluorescent detection of DNA damage (measured as γ-H2AX foci) assessment using Alexa Fluor® 647 Mouse anti-H2AX (pS139) and Hoechst 33342 staining of JURKAT lymphoid cell lines after treatment with a combination of ETP and QUE at their LSD for 24 h.

4.2.5 Statistical Analysis

Using the Stats Direct software a Shapiro-Wilke test was used to determine whether the data was parametric or non-parametric. As the data was non-parametric, a Kruskal-Wallis and Conover-Inman post hoc test was used. Statistical significance was set at $p \leq 0.05$.

4.2.5.1 Analysis of Effect of Polyphenols Alone on Caspase Activity and GSH Levels

The caspase 8 and 9 activity and GSH level following polyphenol treatment alone was normalised to the vehicle-only control, and each data point (n=3) was plotted. A statistical comparison was made between the polyphenols treatment and the vehicle controls.

4.2.5.2 Analysis of Effect of Combination Treatments on Caspase Activity, GSH Levels and γ-H2AX Foci Formation

Data of combination work was normalised to the vehicle-only control. The median and ranges were calculated for all the assays. The effects of the combination treatments were categorised using the following criteria:

- An increase in the levels of caspase (8 or 9) activity or depletion of GSH levels or induction of γ-H2AX foci formation by a polyphenol = **X**
- An increase the levels of caspase (8 or 9) activity or depletion of GSH levels or induction of γ-H2AX foci formation by chemotherapy agent = **Y**
- An increase the levels of caspase (8 or 9) activity or depletion of GSH levels or induction of γ-H2AX foci formation by combined polyphenol and chemotherapy agent treatment = **Z**

X+Y were calculated and described as our **Expected Value.** The effect of combination chemotherapy and polyphenols treatments was classified as: **Additive** or **Synergistic** or **Competitive Antagonistic** or **Antagonistic** according to their statistical analysis. The combination effects were classified and defined as desribed in Chapter 3 and Section 3.2.6.

4.3.1 Caspase 8 and 9 Activity

4.3.1.1 Effect of Polyphenols on Caspase 8 and 9 Activity

Flavonoid compounds: quercetin, apigenin and chrysin caused a significant increased in both caspase 8 and 9 activity at 24 h in all lymphoid and myeloid leukaemia cell lines (Figure 4.7, 4.8, 4.9 and Table 4.3). Following 6 and 24 h, both quercetin and apigenin significantly increased caspase 8 and 9 activity in the lymphoid leukaemia cell lines ($p \leq 0.05$), whilst in myeloid leukaemia cell lines a significant increase was only seen at 24 h ($p \leq 0.05$) (Figure 4.7, 4.8 and Table 4.3). Chrysin, however did not significantly affect caspase 8 or 9 activity at either 6 or 24 h in KG-1a cells ($p \leq 0.05$); while it caused a significant increase in caspase 8 and 9 activity at 6 and 24 h in Jurkat cells ($p \leq 0.05$) (Figure 4.9 and Table 4.3). Whilst CCRF-CEM and THP-1 had both caspase 8 and 9 activity at 24h, but not at 6 h ($p \leq 0.05$) (Figure 4.9 and Table 4.3).

Anthraquinone compounds: emodin, rhein and aloe-emodin in general did not affect caspase 8 activity; but significantly increased caspase 9 activity in all the leukaemia cell lines investigated ($p \leq 0.05$) (Figure 4.10, 4.11, 4.12 and Table 4.3). In particular following 6 h and 24 h, emodin, rhein (used at their NSD, LSD and IC_{50} doses), and aloe-emodin (used at its IC_{50}) significantly increased caspase 9 but had no effect on caspase 8 activity in Jurkat, CCRF-CEM and THP-1 cells ($p \leq 0.05$) (Figure 4.10, 4.11, 4.12 and Table 4.3). Whist KG1a cells treated with emodin for 6 and 24 h did not affect either caspase 8 or 9 activity (Figure 4.10 and Table 4.3). Similarly, KG1a cells and THP-1 cells were treated with rhein at the NSD and LSD at 6 h and 24 h, there was no significant effect on caspase 8 or 9 activity. However there was a significant effect increase in caspase 9 activity when rhein was used at its IC_{50} treatment dose at both 6 and 24 h ($p \leq 0.05$) (Figure 4.11 and Table 4.3). Aloe-emodin stimulation at its IC_{50} dose did not affect caspase 8 activity, but significantly increased caspase 9 activity in all the leukaemia cell lines investigated at both 6 h and 24 h ($p \leq 0.05$) (Figure 4.12 and Table 4.3).

Stilbene compounds: *cis*-stilbene and *trans*-stilbene in general caused a significant increase in both caspase 8 and 9 activity following 24 h in all leukaemia cell lines investigated (Figure 4.13, 4.14 and Table 4.3). In particularly *cis*-stilbene over all doses significantly increased caspase 8 and 9 activity in the lymphoid leukaemia cell lines following 6 h and 24 h, ($p \leq 0.05$); and in the myeloid leukaemia cell lines following 24 h ($p \leq 0.05$) (Figure 4.13 and Table 4.3). Trans-stilbene was only investigated at the IC_{50} doses, which did not show any effect on caspase activity at 6 h, but it did significantly increase both caspase 8 and 9 activity at 24 h in all leukaemia cell lines ($p \leq 0.05$) (Figure 4.14 and Table 4.3).

In summary, flavonoids (quercetin, apigenin and chrysin) and stilbenes (*cis*-stilbene and *trans*-stilbene) increased caspase 8 and 9 activity in all leukaemia cell lines, most significantly at 24 h (Table 4.3). The anthraquinone compounds: emodin, rhein and aloe-emodin increased caspase 9 activity following both 6 h and 24 h in lymphoid cell lines, and following 24 h in the myeloid cell lines (Table 4.3).

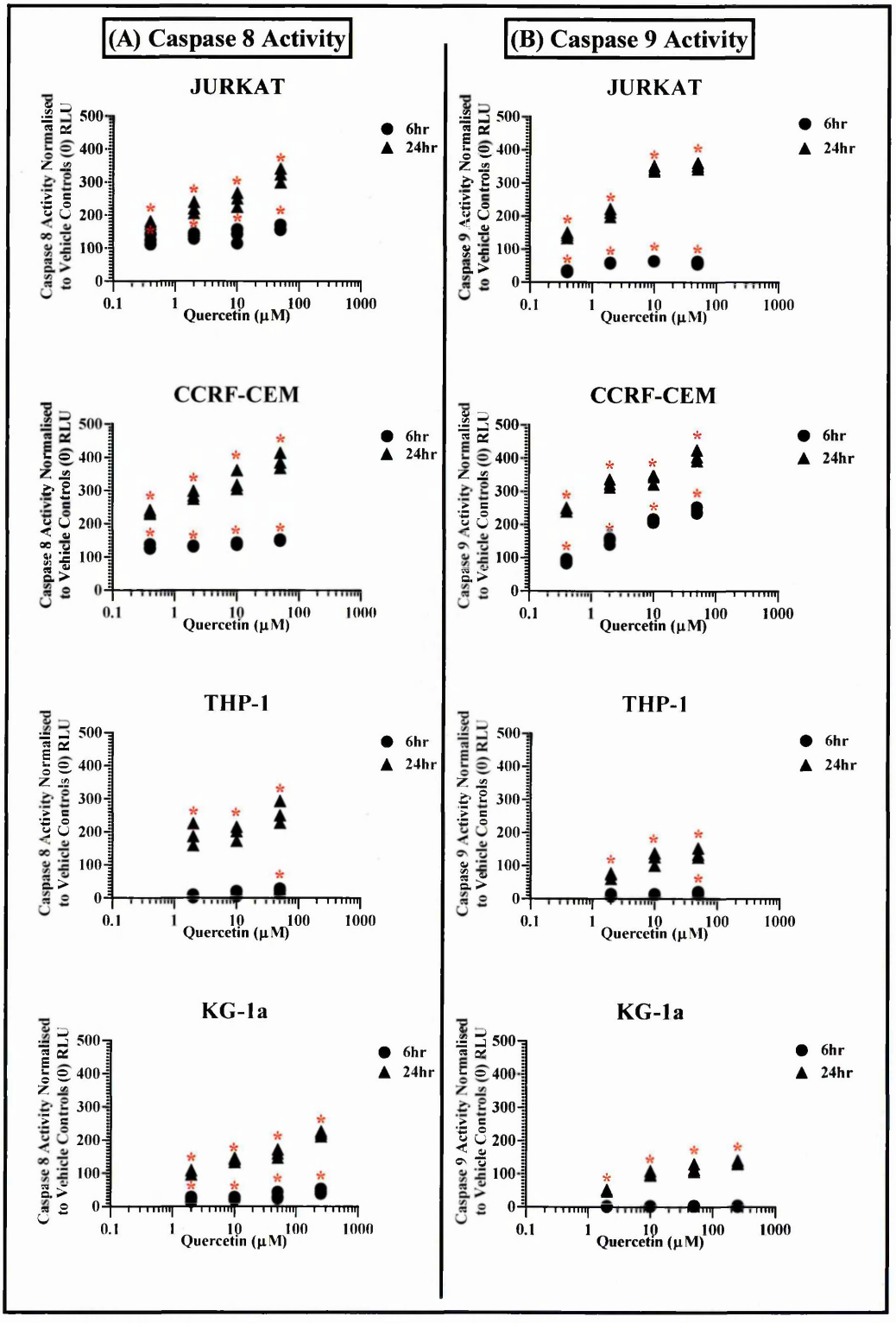


Figure 4.7: Effect of quercetin on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

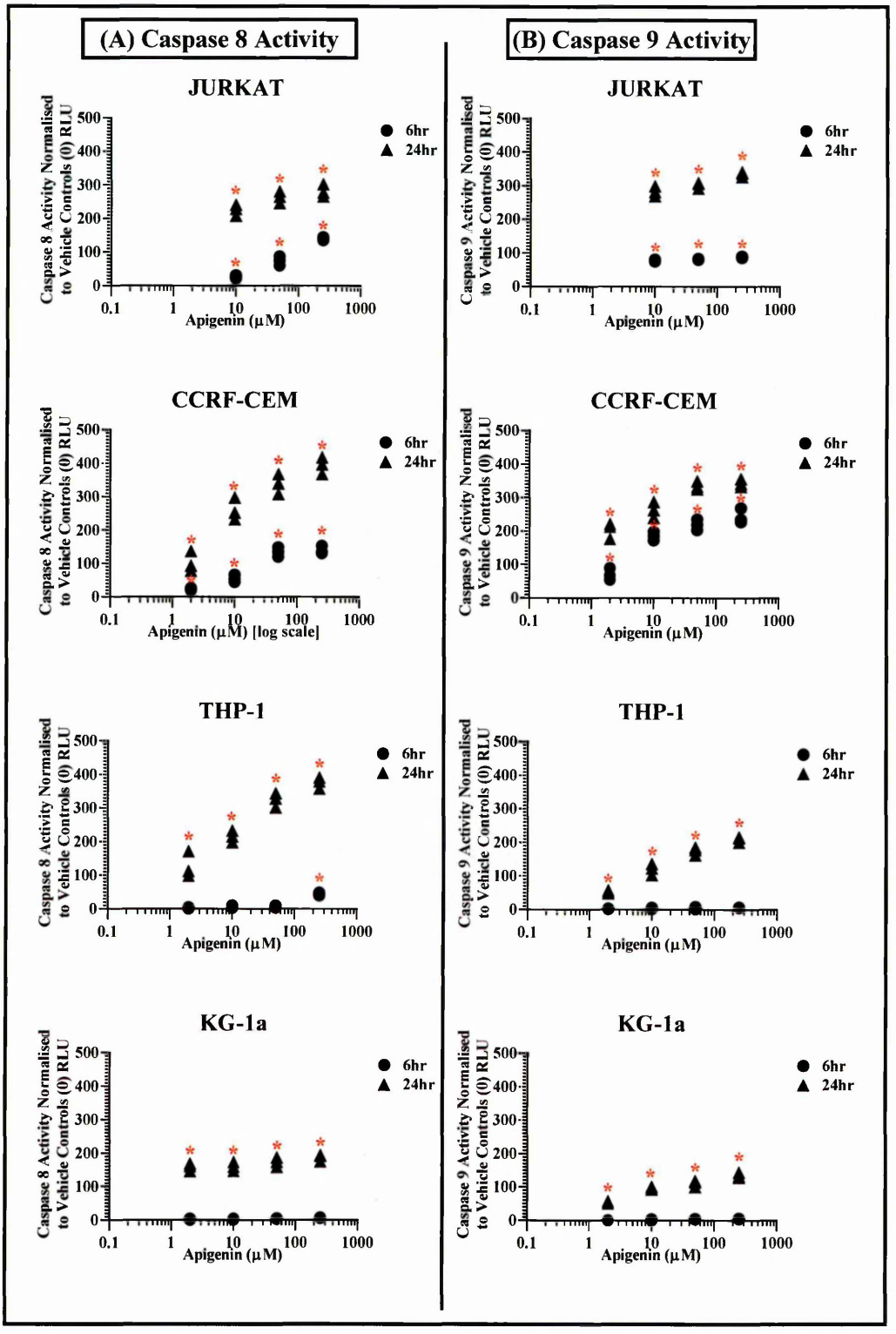


Figure 4.8: Effect of apigenin on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

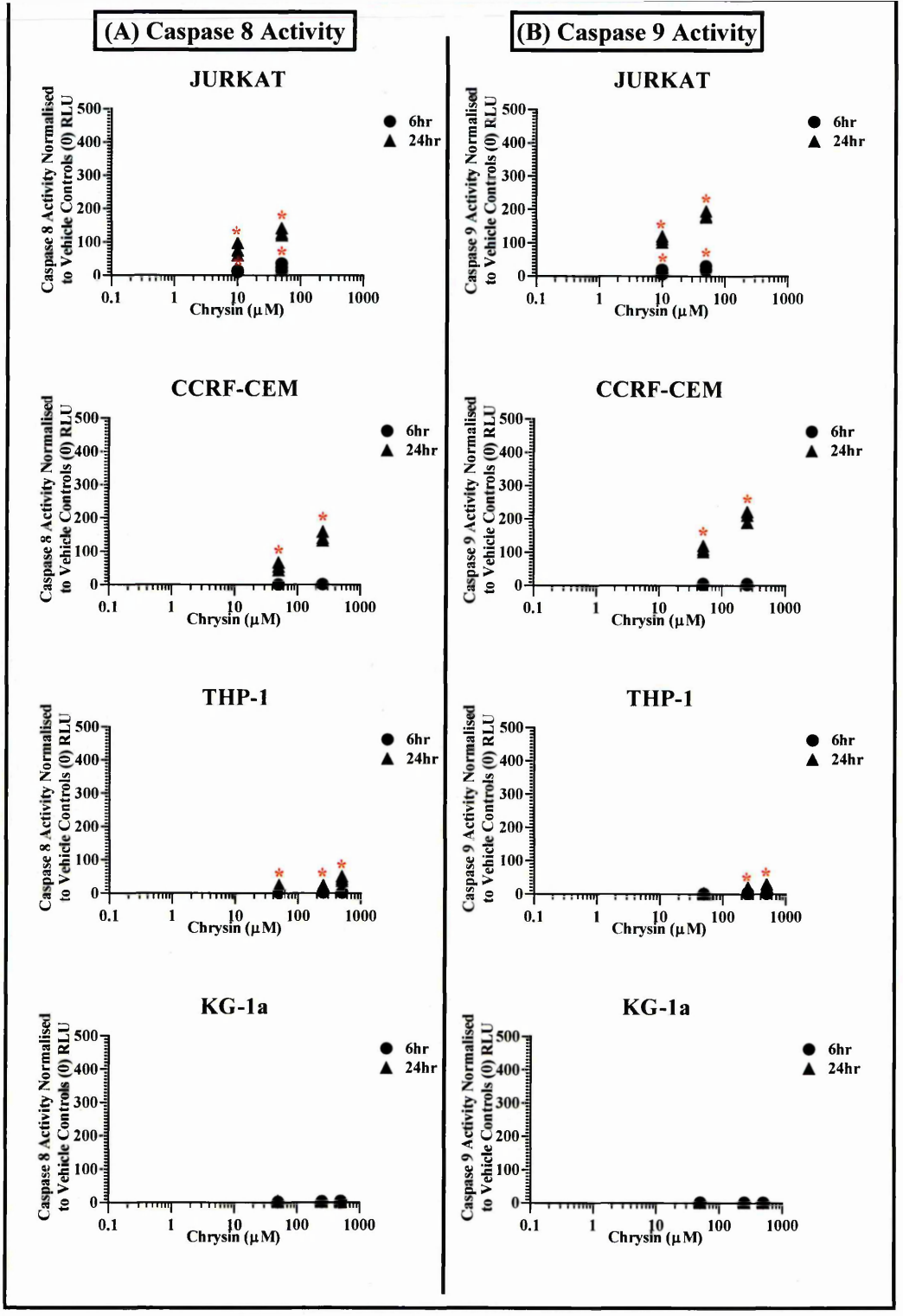


Figure 4.9: Effect of chrysin on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

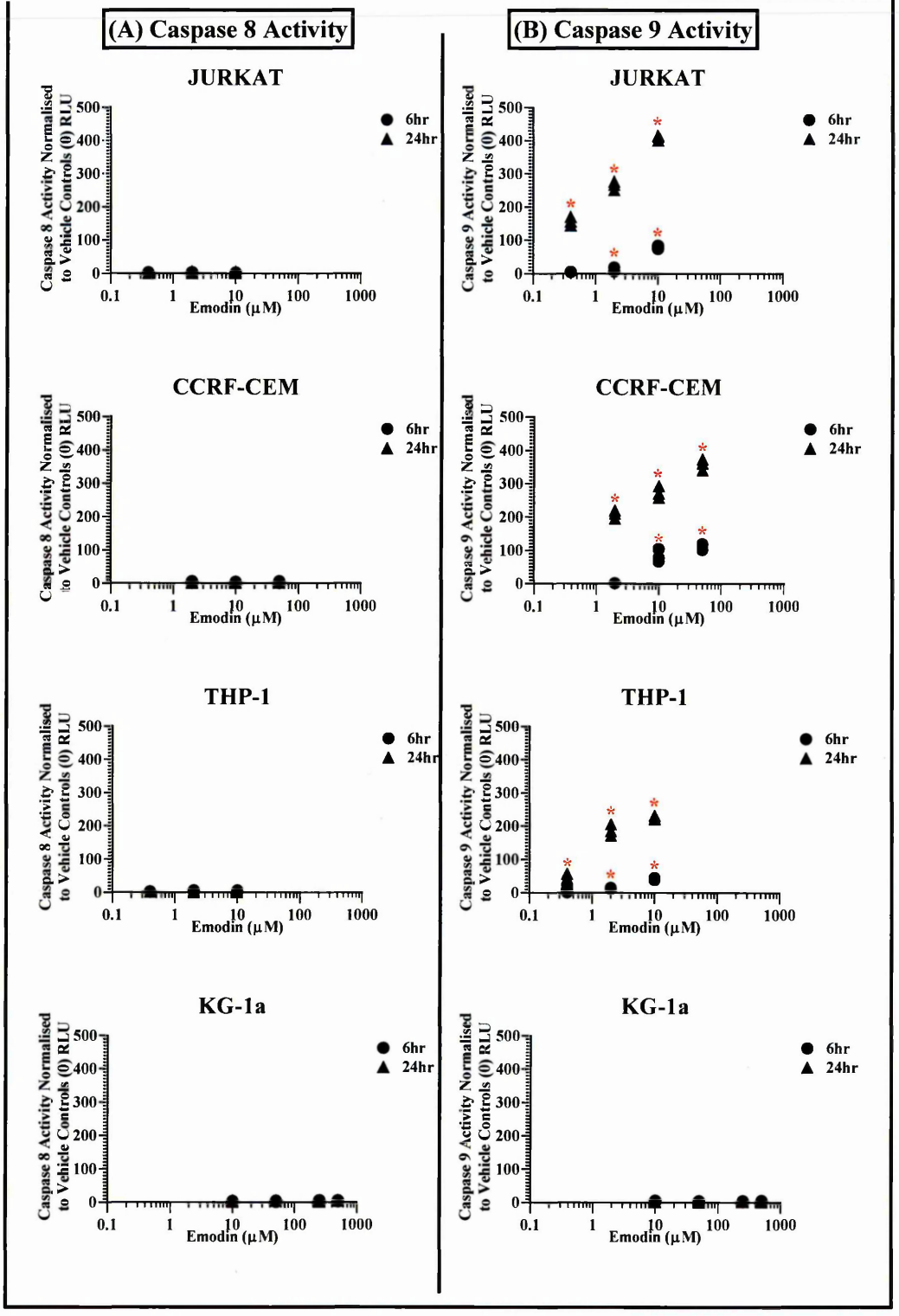


Figure 4.10: Effect of emodin on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

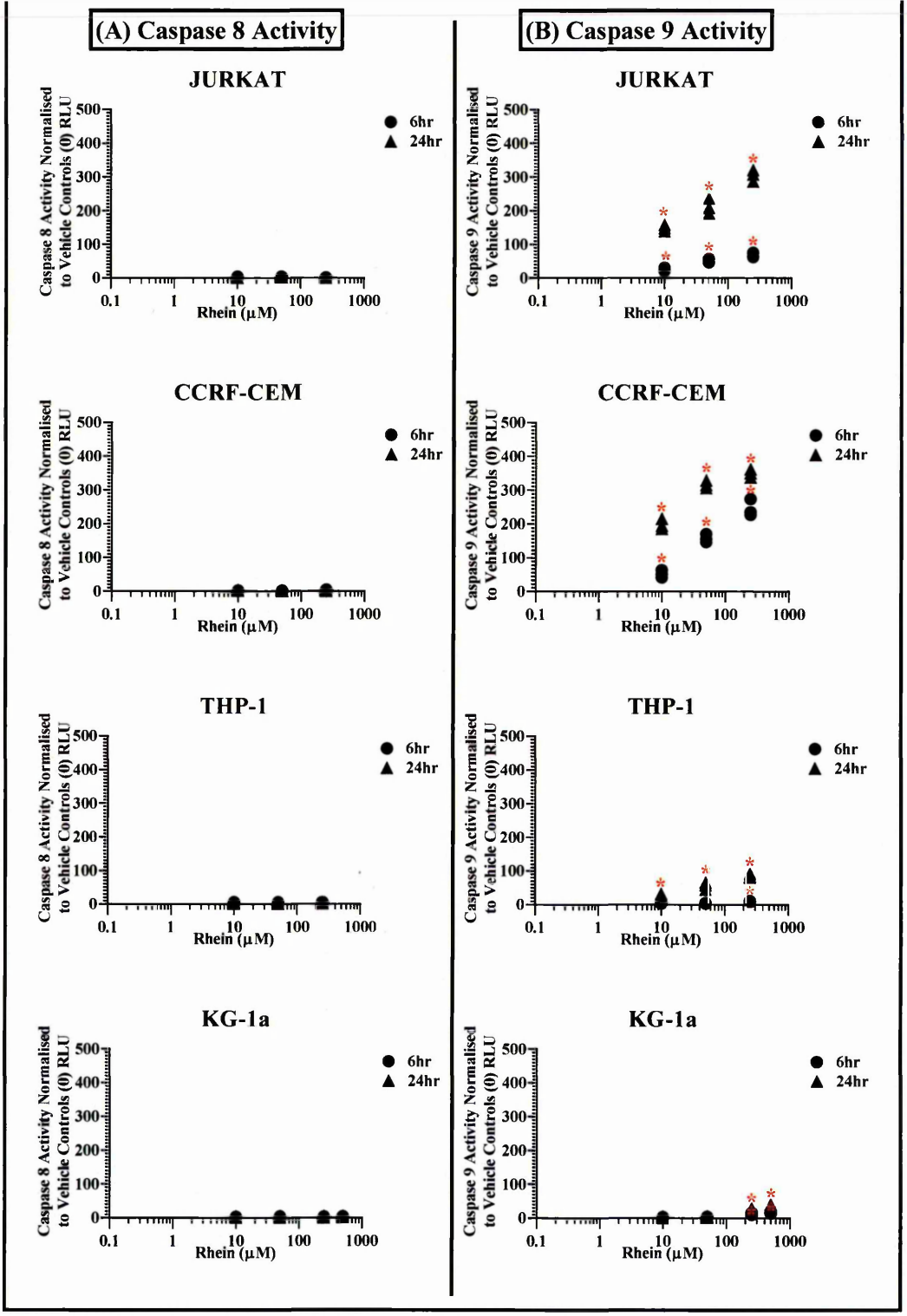


Figure 4.11: Effect of rhein on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

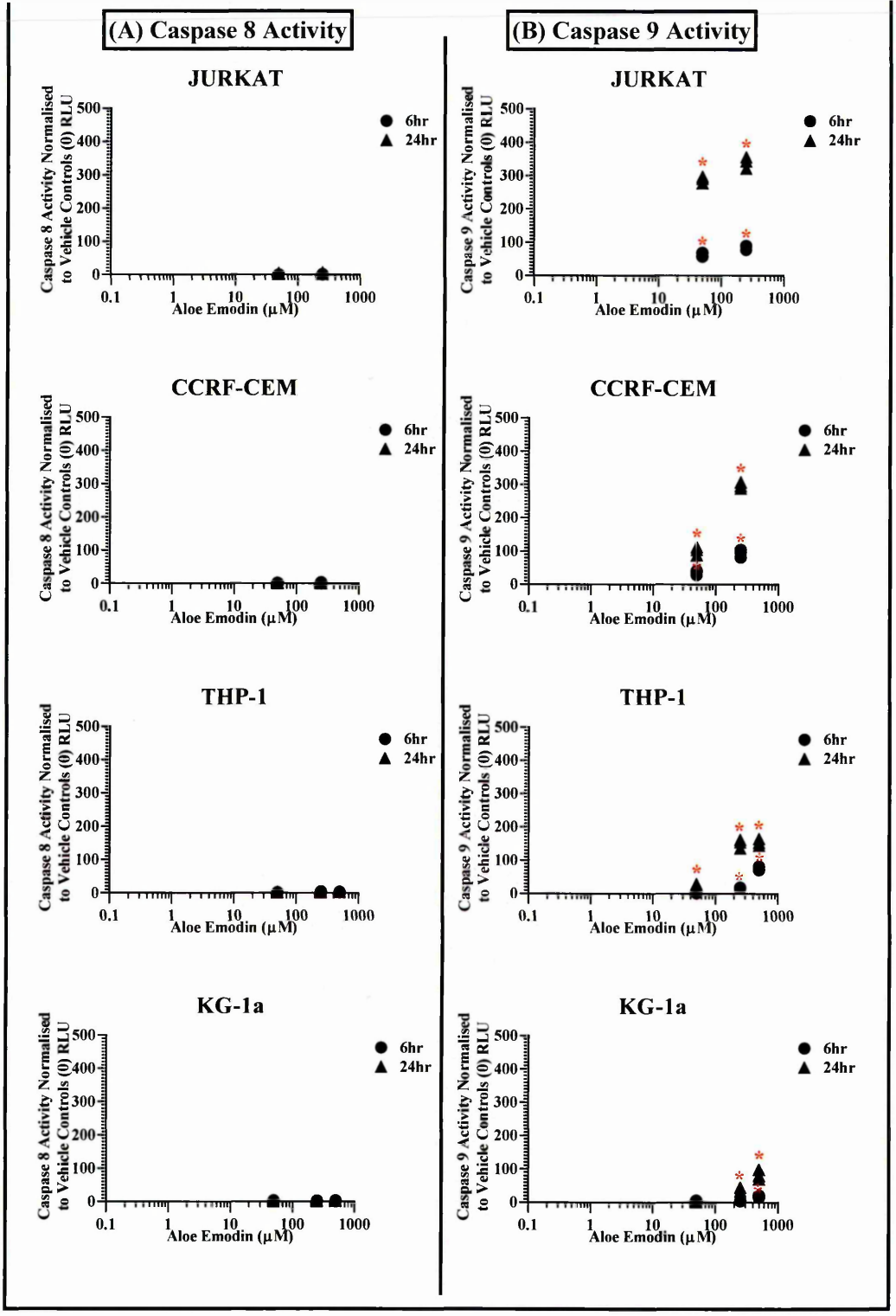


Figure 4.12: Effect of aloe-emodin on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

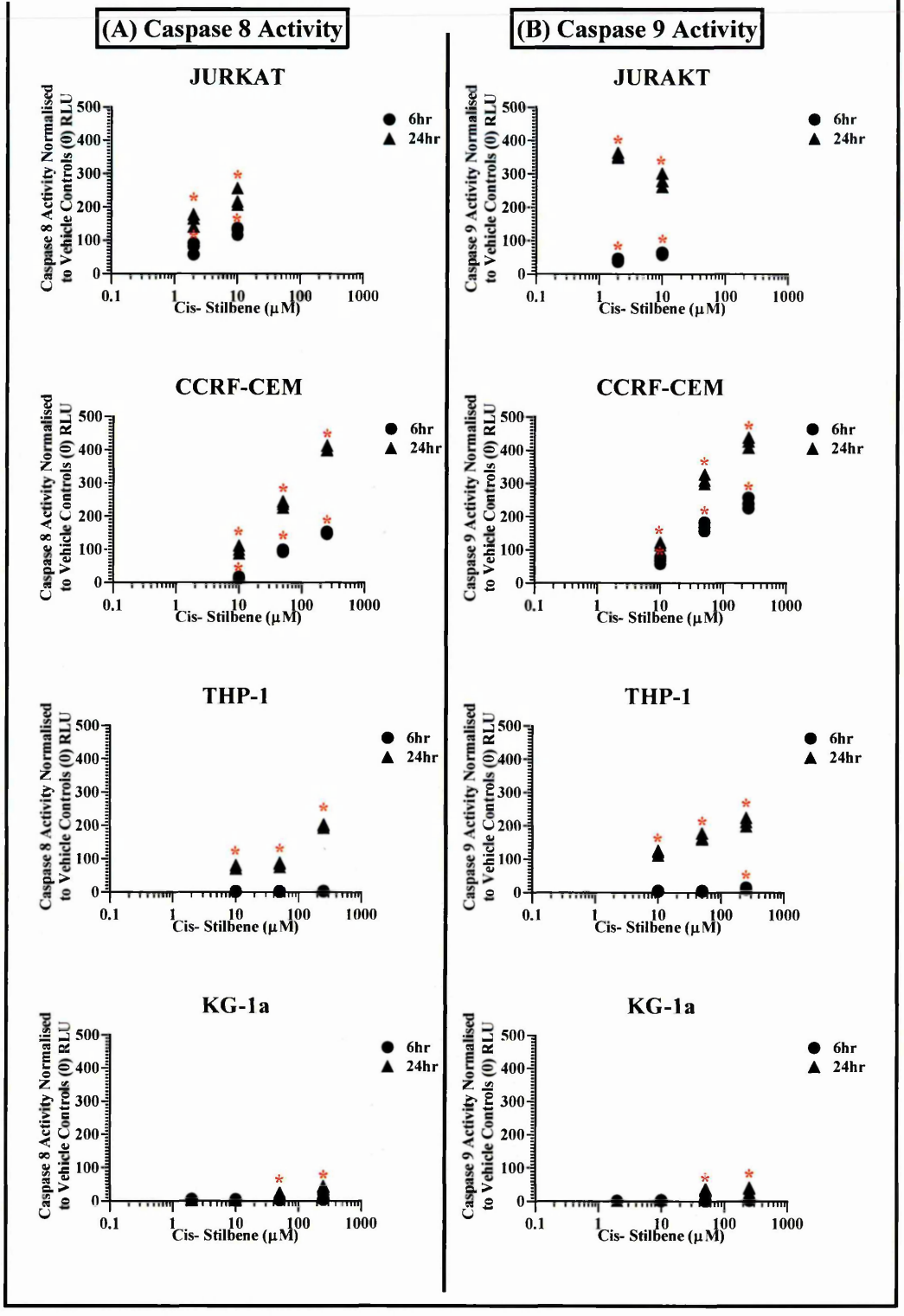


Figure 4.13: Effect of *cis*-stilbene on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

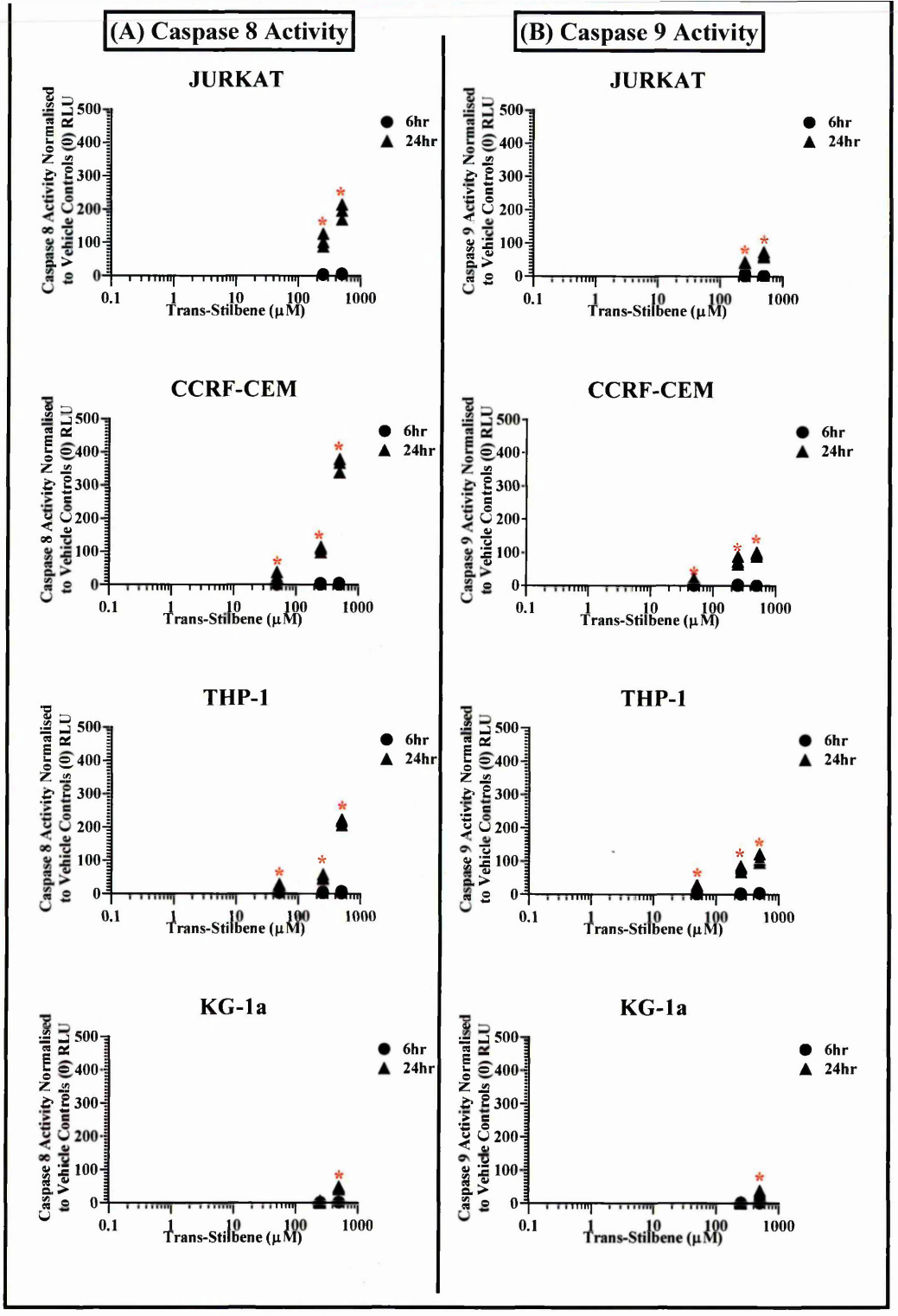


Figure 4.14: Effect of *trans*-stilbene on initiator caspases 8 (A) or 9 (B) in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines following 6 and 24 h. Data normalised to vehicle-control which was set to 0 RLU, and each data point plotted (n=3). * = $p \leq 0.05$ compared to vehicle control.

Polyphenols	Targets	Doses	The Significant Effects of Polyphenols Alone on Initiator Caspase 8 and 9 Activity following 6 h and 24 h in Leukaemia Cell Lines.							
			Lymphoid Leukaemia				Myeloid Leukaemia			
			JURKAT		CCRF-CEM		THP-1		KG-1a	
			6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
Quercetin	Caspase 8 Activity	NSD	*	*	*	*	—	*	*	*
		LSD	*	*	*	*	—	*	*	*
		IC_{50}	*	*	*	*	*	*	*	*
	Caspase 9 Activity	NSD	*	*	*	*	—	*	—	*
		LSD	*	*	*	*	—	*	—	*
		IC_{50}	*	*	*	*	*	*	—	*
Apigenin	Caspase 8 Activity	NSD	*	*	*	*	—	*	—	*
		LSD	*	*	*	*	—	*	—	*
		IC_{50}	*	*	*	*	*	*	—	*
	Caspase 9 Activity	NSD	*	*	*	*	—	*	—	*
		LSD	*	*	*	*	—	*	—	*
		IC_{50}	*	*	*	*	—	*	—	*
Chrysin	Caspase 8 Activity	IC_{50}	*	*	—	*	—	*	—	—
	Caspase 9 Activity	IC_{50}	*	*	—	*	—	*	—	—
Emodin	Caspase 8 Activity	NSD	—	—	—	—	—	—	—	—
		LSD	—	—	—	—	—	—	—	—
		IC_{50}	—	—	—	—	—	—	—	—
	Caspase 9 Activity	NSD	—	*	—	*	—	*	—	—
		LSD	*	*	*	*	*	*	—	—
		IC_{50}	*	*	*	*	*	*	—	—
Rhein	Caspase 8 Activity	NSD	—	—	—	—	—	—	—	—
		LSD	—	—	—	—	—	—	—	—
		IC_{50}	—	—	—	—	—	—	—	—
	Caspase 9 Activity	NSD	*	*	*	*	—	*	—	—
		LSD	*	*	*	*	—	*	—	—
		IC_{50}	*	*	*	*	*	*	*	*
Aloe -Emodin	Caspase 8 Activity	IC_{50}	—	—	—	—	—	—	—	—
	Caspase 9 Activity	IC_{50}	*	*	*	*	*	*	*	*
Cis- Stilbene	Caspase 8 Activity	NSD	*	*	*	*	—	*	—	—
		LSD	*	*	*	*	—	*	—	—
		IC_{50}	*	*	*	*	—	*	—	*
	Caspase 9 Activity	NSD	*	*	*	*	—	*	—	—
		LSD	*	*	*	*	—	*	—	—
		IC_{50}	*	*	*	*	*	*	—	*
Trans-Stilbene	Caspase 8 Activity	IC_{50}	—	*	—	*	—	*	—	*
	Caspase 9 Activity	IC_{50}	—	*	—	*	—	*	—	*

Table 4.3: A summary of the significant effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*-stilbene and *trans*-stilbene) alone on initiator caspase 8 and caspase 9 activity in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG1a) cell lines at 6 h and 24 h. This was evaluated by the Caspase-Glo® 8 and 9 Assays. Quercetin, apigenin, emodin, rhein and *cis*-stilbene were investigated at their NSD, LSD and IC_{50} treatment ranges, while chrysin, aloe-emodin and *trans*-stilbene were investigated only at their IC_{50} treatment ranges. * = $p \leq 0.05$ (Kruskal-Wallis and Conover-Inman post hoc test). — = no activity.

4.3.1.2 Effects of Topoisomerase Inhibitors Combined with Polyphenols on Caspase 8 and 9 Activity

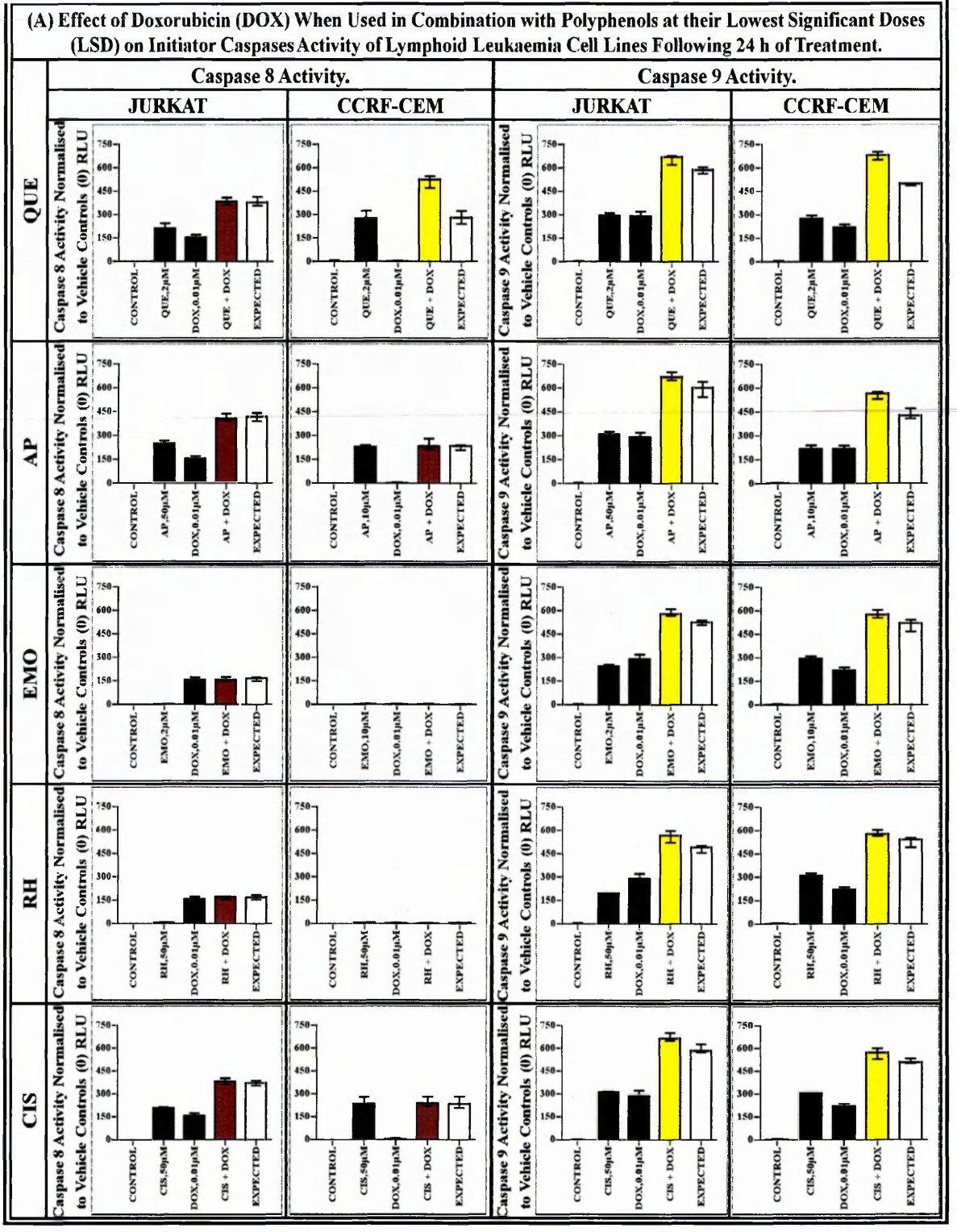
(A) Effects of Doxorubicin (DOX) Combined with Polyphenols on Initiator Caspase Activity

DOX alone used at its LSD for 24 h significantly increased both caspase 8 and caspase 9 activity in most leukaemia cell lines (JURKAT, THP-1 and KG1a) ($p<0.05$) (Figure 4.15). However in the CCRF-CEM lymphoid cell lines, DOX alone significantly increased caspase 9 activity ($p<0.05$); there was no change in caspase 8 activity (Figure 4.15). DOX treatment combined with QUE or AP treatment generally induced an additive induction in caspase 8 activity and a synergistic induction in caspase 9 activity in all leukaemia cell lines ($p<0.05$) (Figure 4.15 and Table 4.4). With the exception that CCRF-CEM cells treated with DOX together with QUE induced a synergistic increase in caspase 8 and caspase 9 activity ($p<0.05$) (Figure 4.15 and Table 4.4). Furthermore, when DOX was used in combined with EMO, RH or CIS there was a additive increase in caspase 8 activity, and synergistic increase in caspase 9 activity in both lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.15 and Table 4.4).

(B) Effects of Etoposide (ETP) Combined with Polyphenols on Initiator Caspase Activity

ETP alone used at its LSD for 24 h significantly increased both caspase 8 and caspase 9 activity in most leukaemia cell lines (JURKAT, THP-1 and KG1a) ($p<0.05$) (Figure 4.16). However in CCRF-CEM lymphoid cell lines, ETP alone only significantly increased caspase 9 activity ($p<0.05$) (Figure 4.16). ETP when combined with QUE or AP mainly caused an additive increase in caspase 8 activity, and a synergistic increase in caspase 9 activity in all the leukaemia cell lines ($p<0.05$) (Figure 4.16 and Table 4.5). ETP when used in combination with EMO or RH caused an additive increase in caspase 8 activity on three leukaemia cell lines (JURKAT, THP-1 and KG-1a), whilst it caused a synergistic increase in caspase 8 activity in CCRF-CEM cell line ($p<0.05$) (Figure 4.16 and Table 4.5). Additionally, ETP when used in combination with EMO or RH caused a synergistic increase in caspase 9 activity in the lymphoid leukaemia cell lines (JURJAT and CCRF-CEM) ($p<0.05$) (Figure 4.16 and Table 4.5). Likewise, when ETP was used in combined with CIS it caused an additive increase in caspase 8 activity, and a synergistic increase in caspase 9 activity in the lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) ($p<0.05$) (Figure 4.16 and Table 4.5).

(A) Effect of Doxorubicin (DOX) When Used in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Initiator Caspases Activity of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.



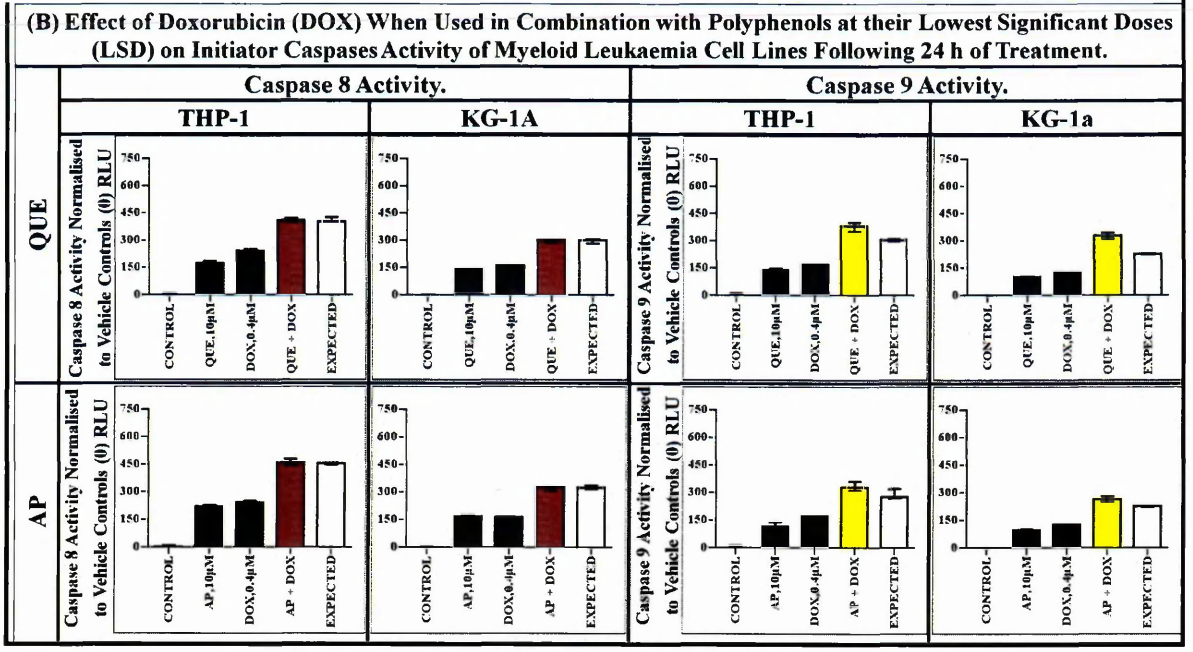
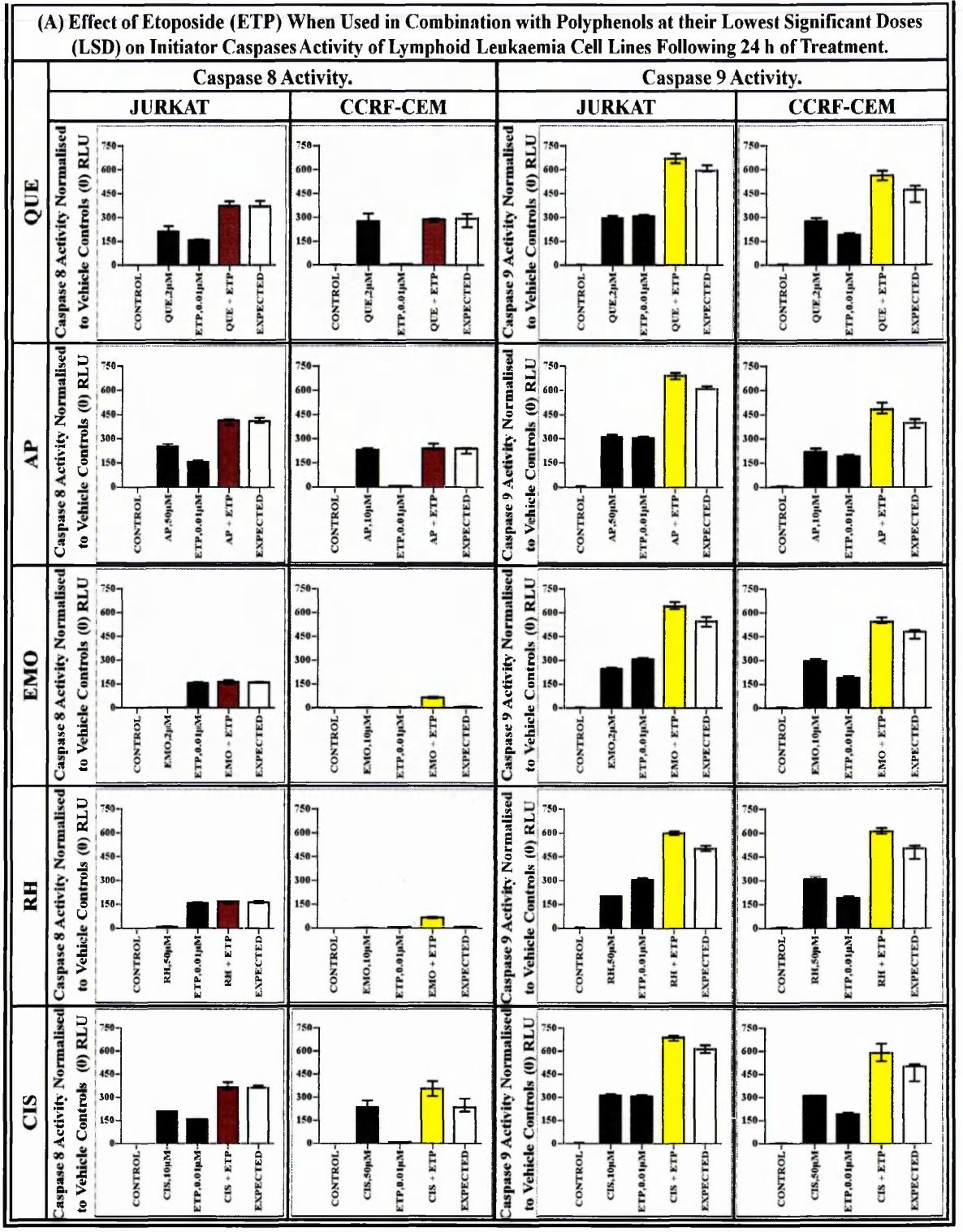


Figure 4.15: The effect of DOX on caspase 8 and 9 activity: (A) When used in combination with QUE, AP, EMO, RH or CIS in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM); and (B) When used in combination with QUE or AP in myeloid leukaemia cell lines (THP-1 and KG-1a). Cells were treated with DOX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-control, which was assigned a 0 RLU. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 8 and 9 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.


(A) Effect of Etoposide (ETP) When Used in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Initiator Caspases Activity of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.
Caspase 8 Activity.
Caspase 9 Activity.
JURKAT
CCRF-CEM
JURKAT
CCRF-CEM
QUE
AP
EMO
RH
CIS
Caspase 8 Activity Normalised to Vehicle Controls (0) RLU
Caspase 9 Activity Normalised to Vehicle Controls (0) RLU
CONTROL
QUE,2μM
AP,50μM
AP,10μM
EMO,2μM
EMO,10μM
RH,50μM
CIS,10μM
CIS,50μM
ETP,0.01μM
ETP,0.0μM
QUE + ETP
AP + ETP
EMO + ETP
RH + ETP
CIS + ETP
EXPECTED

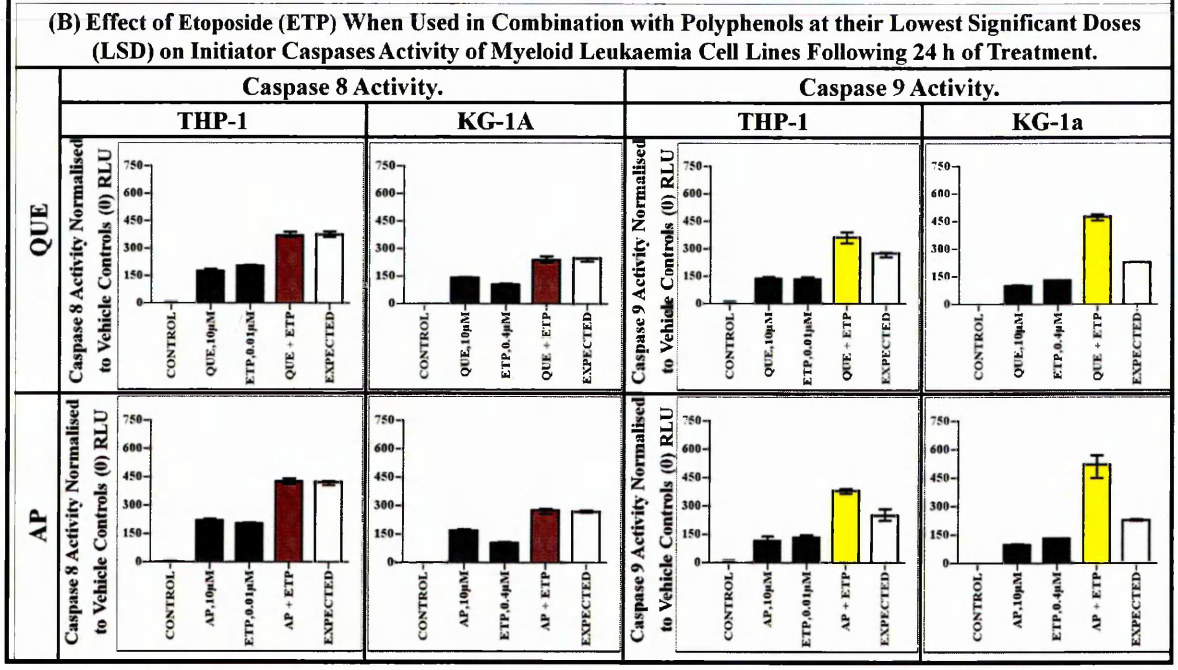


Figure 4.16: The effect of ETP on caspase 8 and 9 activity: (A) When used in combination with QUE, AP, EMO, RH or CIS in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM); and (B) When used in combination with QUE or AP in myeloid leukaemia cell lines (THP-1 and KG-1a). Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-control, which was assigned a 0 RLU. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 8 and 9 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

4.3.1.3 Effects of Alkylating Chemotherapy Agents Combined with Polyphenols on Caspase 8 and 9 Activity

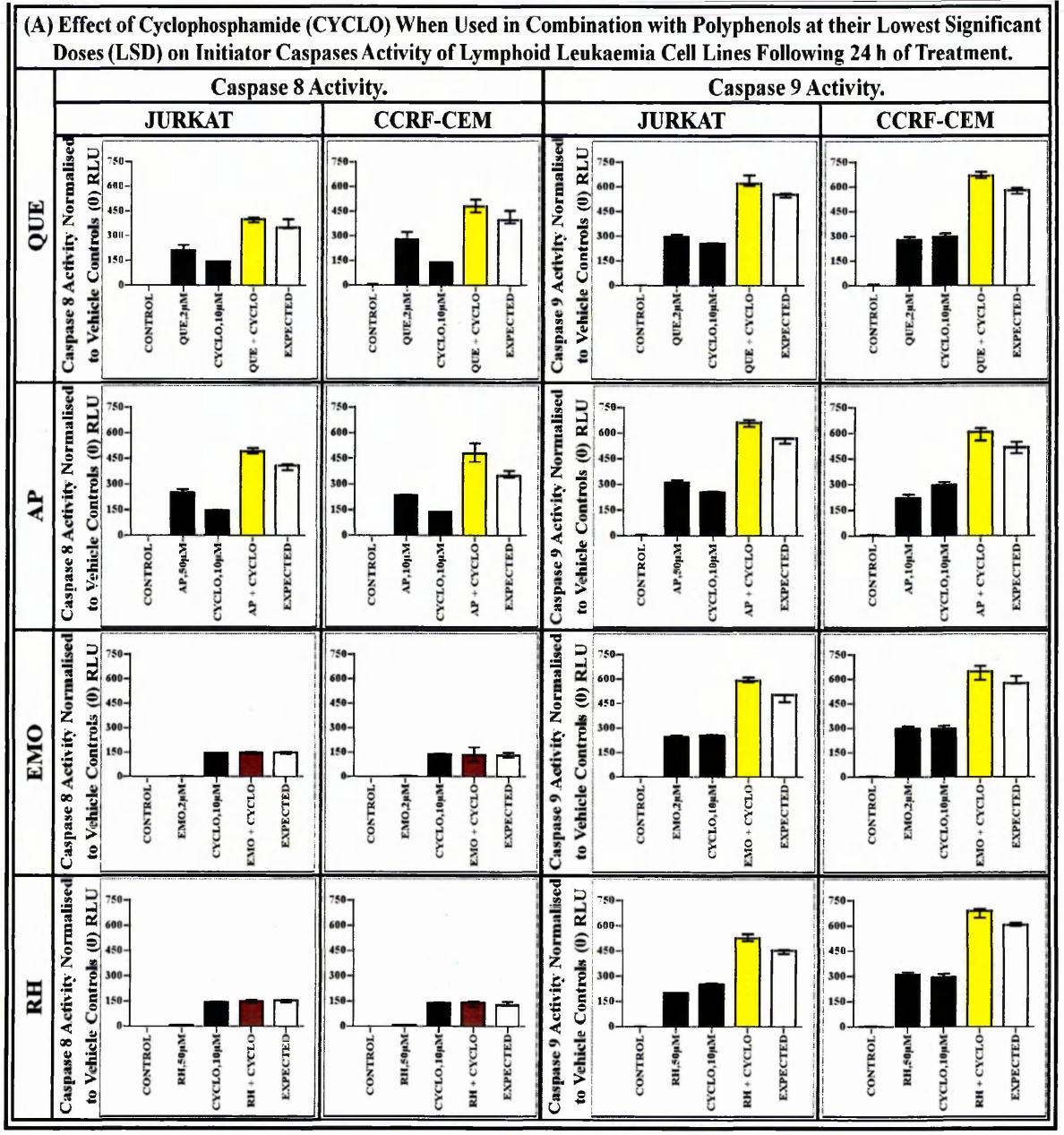
(A) Effects of Cyclophosphamide (CYCLO) Combined with Polyphenols on Initiator Caspase Activity

CYCLO alone used at its LSD for 24 h significantly increased both caspase 8 and caspase 9 activity in all leukaemia cell lines ($p<0.05$) (Figure 4.17). CYCLO when used in combination with QUE or AP caused a synergistic increase in both caspase 8 and caspase 9 activity in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.17 and Table 4.6). However, when CYCLO was used in combination with EMO or RH there was an additive increase in caspase 8 activity, and a synergistic increase in caspase 9 activity in the lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) ($p<0.05$) (Figure 4.17 and Table 4.6).

(B) Effects of Cisplatin (CSP) Combined with Polyphenols on Initiator Caspase Activity

CSP alone used at its LSD for 24 h significantly increased both caspase 8 and caspase 9 activity in all leukaemia cell lines ($p<0.05$) (Figure 4.18). CSP when used in combined with QUE or AP caused a synergistic increase in both caspase 8 and caspase 9 activity in all leukaemia cell lines ($p<0.05$) (Figure 4.18 and Table 4.7). Whilst CSP in combined with CIS caused a synergistic increase in caspase 8 and caspase 9 activity only in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.18 and Table 4.7). When CSP was combined with EMO or RH there was a additive increase in caspase 8 activity, and a synergistic increase caspase 9 activity in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.18 and Table 4.7).

(A) Effect of Cyclophosphamide (CYCLO) When Used in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Initiator Caspases Activity of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.



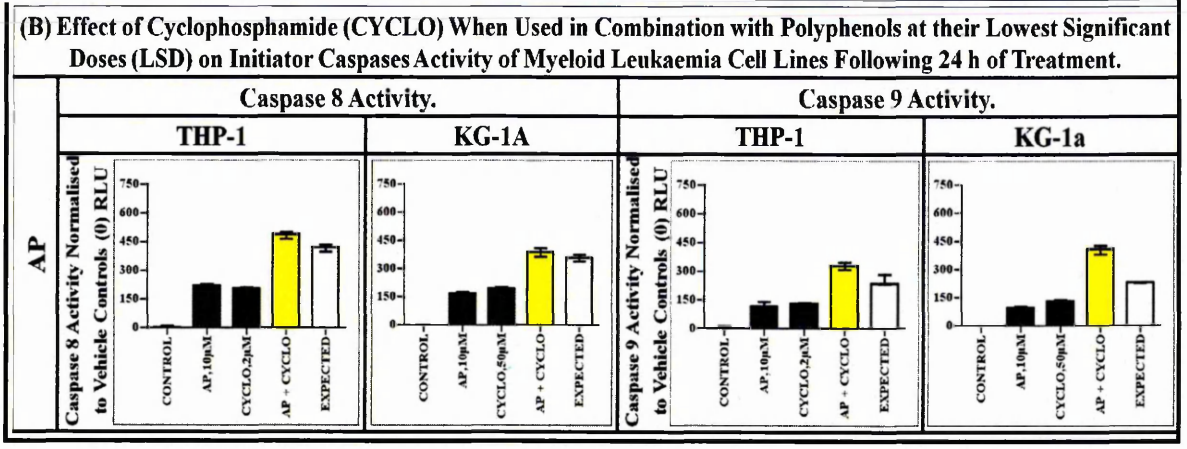
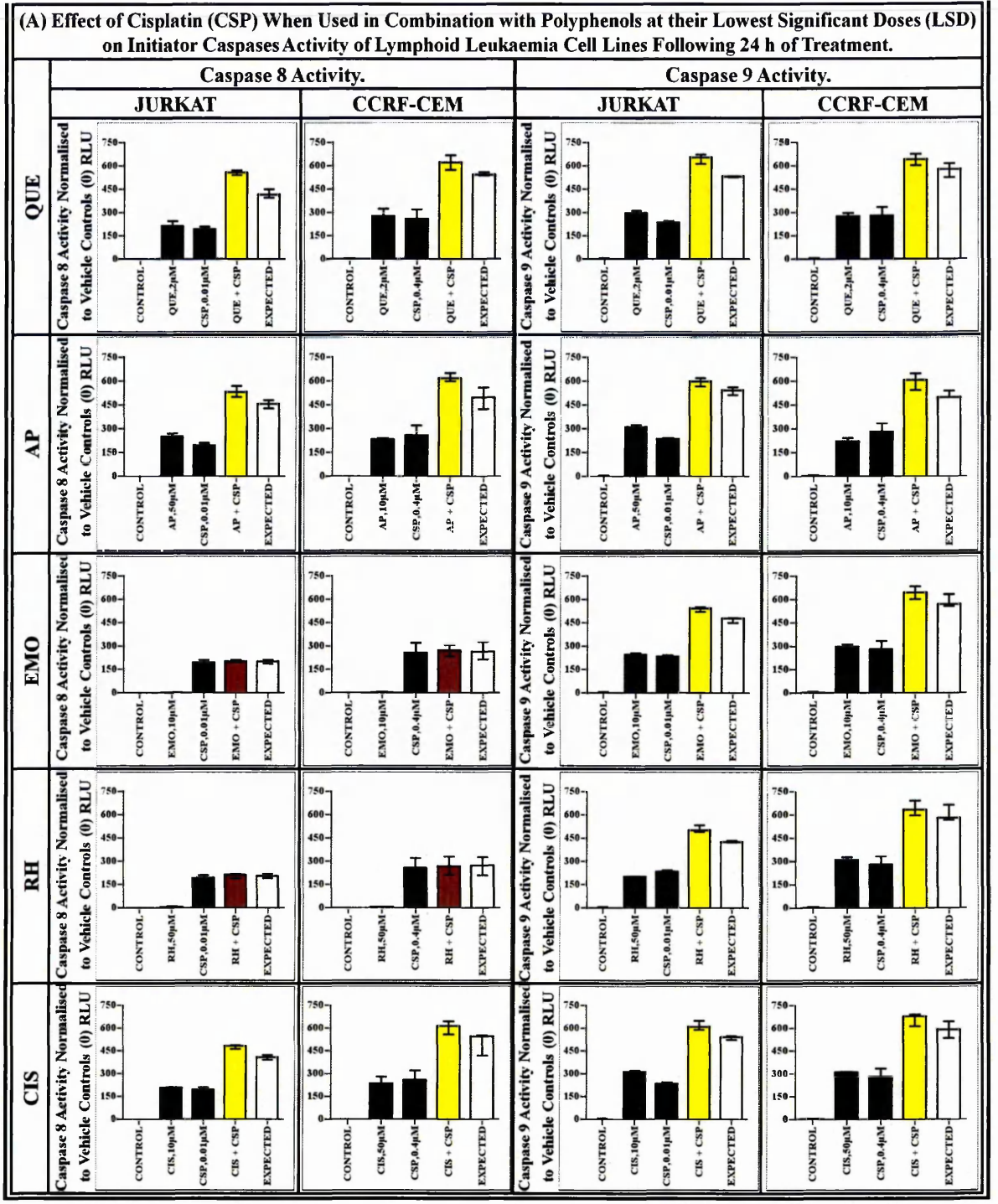


Figure 4.17: The effect of CYCLO on caspases 8 and 9 activity: (A) when used in combination with QUE, AP, EMO or RH in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM); and (B) When used in combination with AP in myeloid leukaemia cell lines (THP-1 and KG-1a). Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-control, which was assigned a 0 RLU. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 8 and 9 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.


(A) Effect of Cisplatin (CSP) When Used in Combination with Polyphenols at their Lowest Significant Doses (LSD) on Initiator Caspases Activity of Lymphoid Leukaemia Cell Lines Following 24 h of Treatment.
Caspase 8 Activity.
Caspase 9 Activity.
JURKAT
CCRF-CEM
JURKAT
CCRF-CEM
QUE
AP
EMO
RH
CIS
Caspase 8 Activity Normalised to Vehicle Controls (0) RLU
Caspase 9 Activity Normalised to Vehicle Controls (0) RLU
CONTROL
QUE,2μM
AP,50μM
AP,10μM
EMO,10μM
RH,50μM
CIS,10μM
CIS,50μM
CSP,0.01μM
CSP,0.4μM
QUE + CSP
AP + CSP
EMO + CSP
RH + CSP
CIS + CSP
EXPECTED

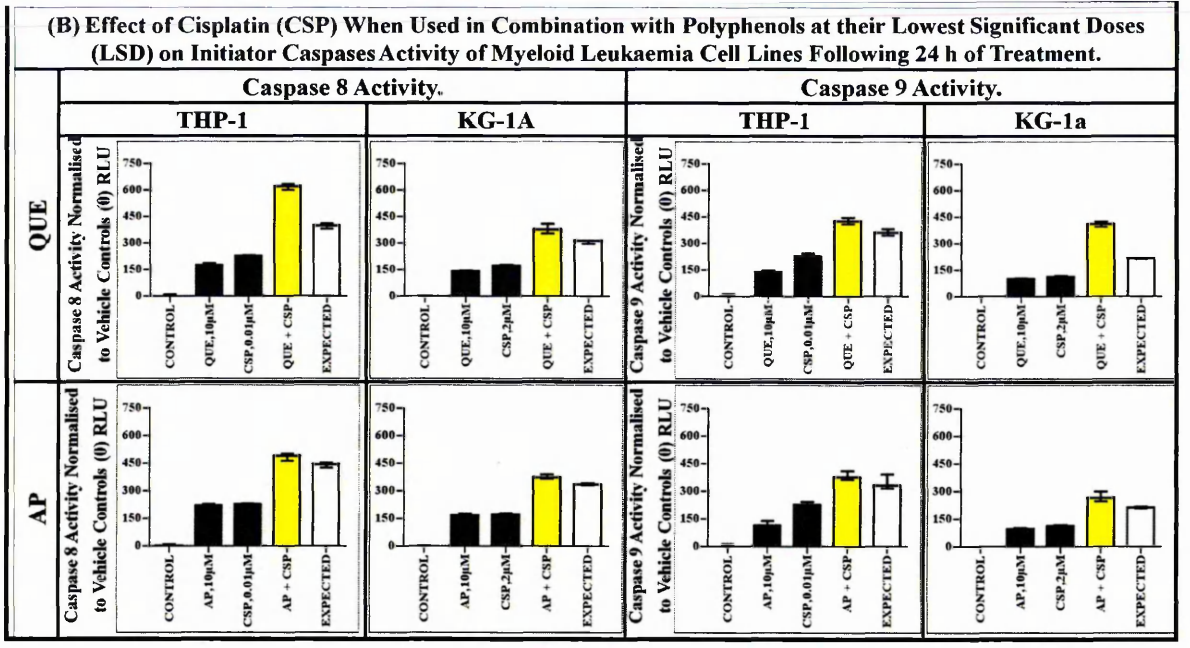


Figure 4.18: The effect of CSP on caspases 8 and 9 activity: (A) when used in combination with QUE, AP, EMO, RH or CIS in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM); and (B) When used in combination with QUE or AP in myeloid leukaemia cell lines (THP-1 and KG-1a). Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle-control, which was assigned a 0 RLU. The data was expressed as medians with ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar shows the expected effect of the combined treatments on caspase 8 and 9 activity; the coloured bars indicate significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

4.3.2 Gluathione (GSH) Activity

4.3.2.1 Basal Glutathione (GSH) Levels in Normal and Leukaemia Cell Lines

In order to evaluate the significance of any changes in glutathione (GSH) levels it is essential to establish the basal GSH level presence in each untreated cell line. It was found that he GSH levels varied between cell lines. These cell lines could be ranked in order of GSH levels from the lowest to the highest levels: CCRF-CEM < JURKAT < $CD34^+$ HSC < THP-1 < KG-1a < $CD133^+$ HSC ($p \leq 0.05$) (Figure 4.19). As a result, the lymphoid leukaemia cell lines had significantly lower GSH levels compared to myeloid leukaemia cell lines and normal cells ($CD34^+$ HSC and $CD133^+$ HSC) ($p \leq 0.05$) (Figure 4.19).

In addition, when leukaemia cell lines and normal cells were treated with 50 µM GSH inhibitor (buthionine sulfoximine (BSO)) for 24 h, there was a significant decrease in GSH levels in all leukaemia and normal cell lines when compared to untreated cells ($p \leq 0.05$) (Figure 4.19). Alternatively, when cells were treated with GSH inducer agent (N-acetyle cysteine (NAC)) for 24 h, there was a significant increase in GSH levels in the lymphoid leukaemia cell lines only when compared to untreated cells ($p \leq 0.05$) (Figure 4.19).

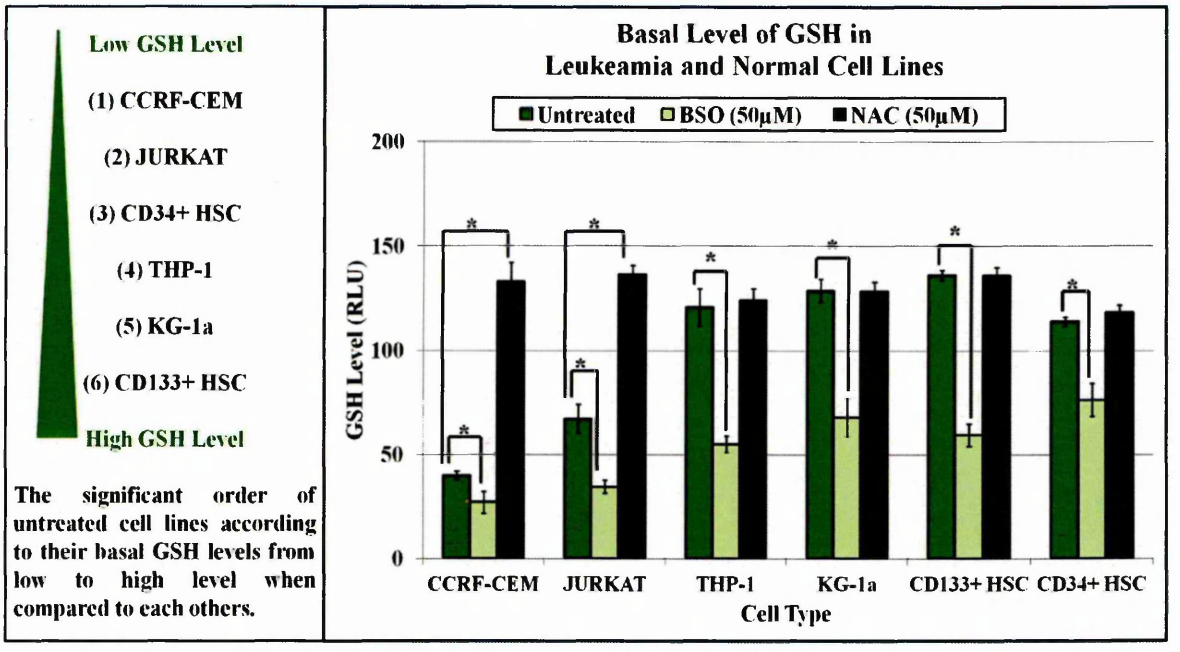
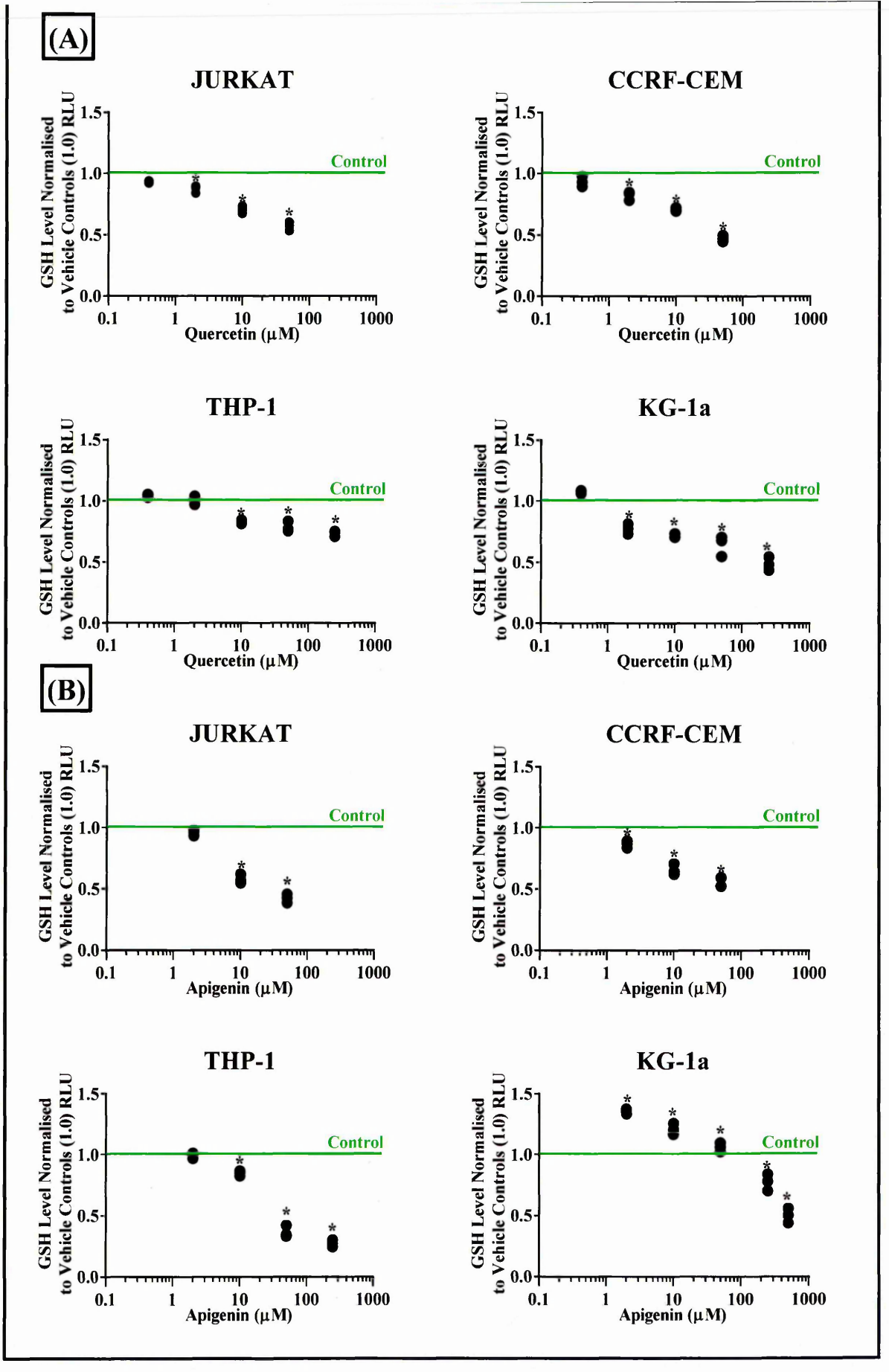


Figure 4.19: Basal level of glutathione (GSH) in two lymphoid leukaemia (CCRF-CEM and JURKAT), two myeloid leukaemia (THP-1 and KG-1a) cell lines; and two normal ($CD133^+$HSC and $CD34^+$ HSC) cell lines. Cells were untreated, treated with GSH inhibitor agent (buthionine sulfoximine (BSO)) and with GSH inducer agent (N-acetyle cysteine (NAC)) for 24 h. This was evaluated by the GSH-Glo™ Glutathione Assay. The data is expressed as median with range in triplicate. * = $p \leq 0.05$.

4.3.2.2 Effect of Polyphenols Alone on Glutathione (GSH) Levels

The NSDs and LSDs of polyphenols showed differential effects on GSH levels within the leukaemia cell lines at 24 h (Table 4.8). At the NSDs of quercetin (Figure 4.20), apigenin (Figure 4.20), emodin (Figure 4.21) and rhein (Figure 4.21) there was no significant effect on GSH levels. However, the LSDs caused a significant decrease GSH levels in the lymphoid cell lines and one myeloid cell line (THP-1) ($p \leq 0.05$) (Table 4.8). Quercetin was the only polyphenol when used at its LSD caused a significant depletion on GSH level in the KG-1a myeloid cell lines ($p \leq 0.05$) (Figure 4.20 and Table 4.8). In contrast, apigenin (Figure 4.20), emodin (Figure 4.21) and rhein (Figure 4.21) significantly increased GSH levels in one myeloid (KG-1a) cell line, when used at its NSDs and LSDs ($p \leq 0.05$). Cis-stilbene when used at the NSDs and LSDs did not show any significant effect on GSH levels within the two lymphoid cell lines (Figure 4.22 and Table 4.8); but did significantly increase GSH levels in the two myeloid cell lines ($p \leq 0.05$) (Figure 4.22 and Table 4.8).

At the IC_{50} treatment doses, all polyphenols (quercetin, apigenin, chrysin (Figure 4.20), emodin, rhein, aloe-emodin (Figure 4.21), cis-stilbene and trans-stilbene (Figure 4.22)) significantly depleted the GSH levels in all the leukaemia cell lines ($p \leq 0.05$) (Table 4.8). The only exception was seen with trans-stilbene which was significantly elevated GSH levels in KG-1a cells when compared to vehicle-control ($p \leq 0.05$) (Figure 4.22 and Table 4.8).


(A)
JURKAT
CCRF-CEM
THP-1
KG-1a
GSH Level Normalised to Vehicle Controls (1.0) RLU
Control
Quercetin (μM)
(B)
Apigenin (μM)
0.0
0.5
1.0
1.5
0.1
1
10
100
1000

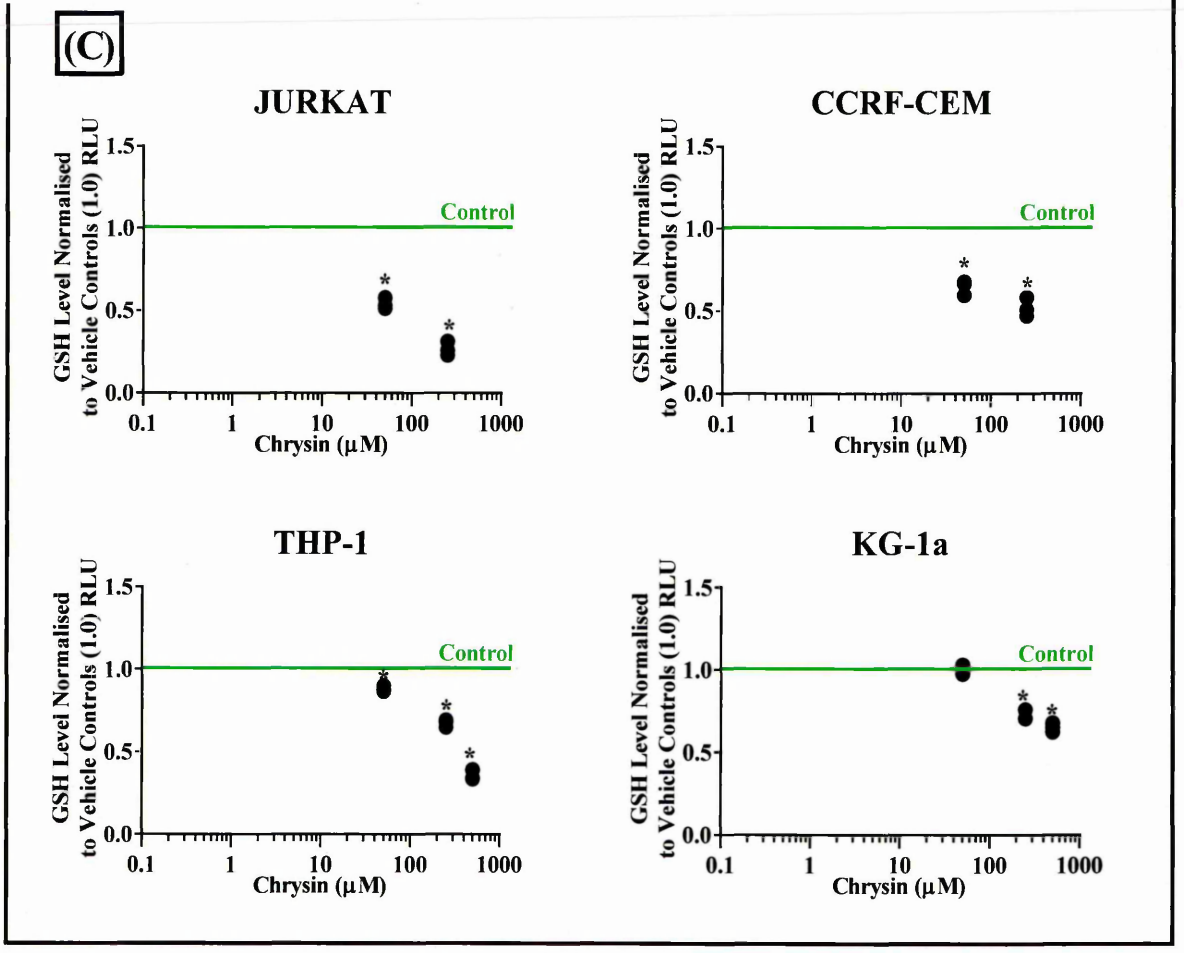
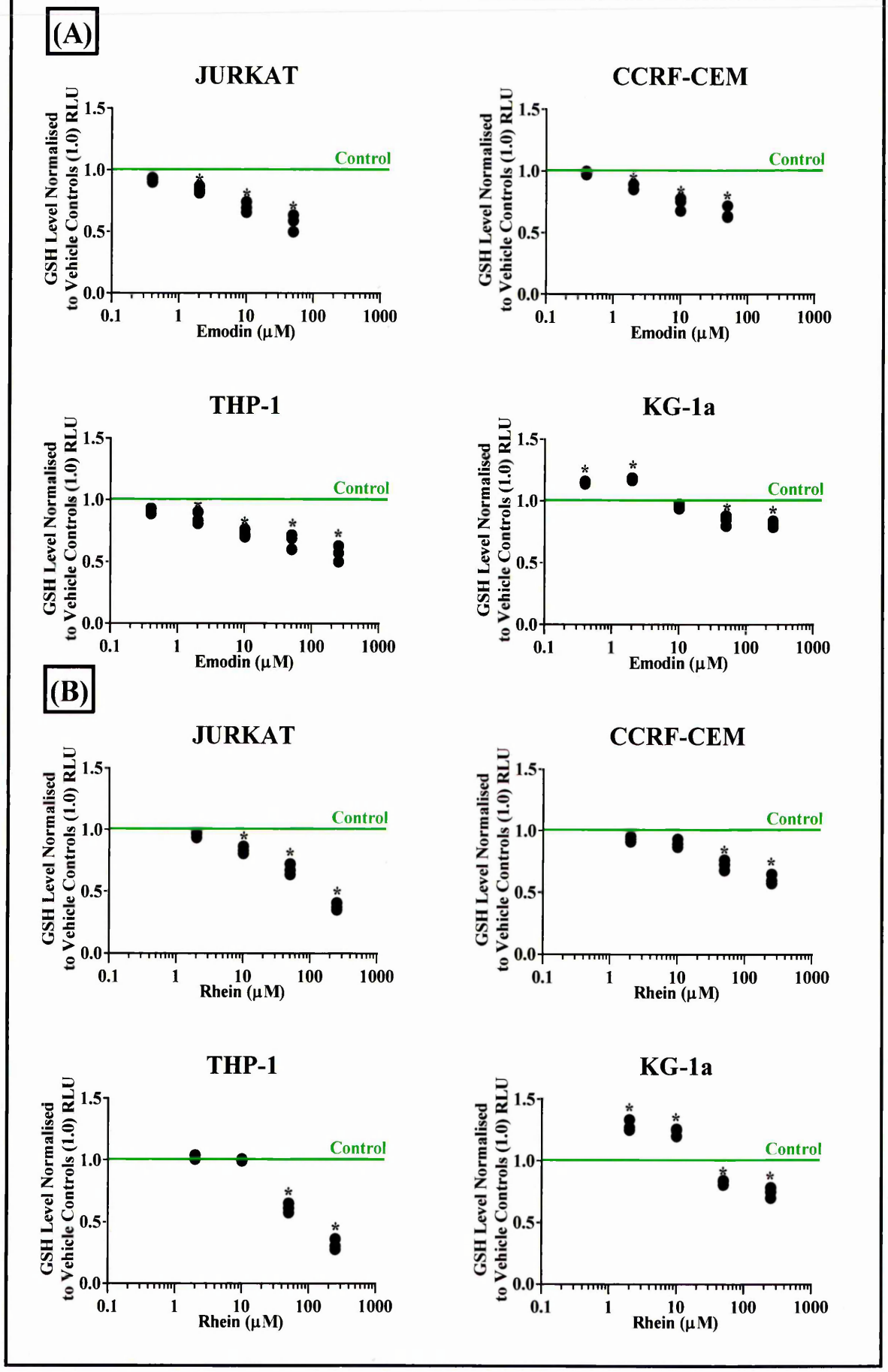


Figure 4.20: Effect of polyphenols: (A) quercetin, (B) apigenin and (C) chrysin on GSH levels in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines. Cells were treated with quercetin and apigenin at their NSD, LSD and IC_{50} treatment ranges; while chrysin was only used at its IC_{50} treatment ranges for 24 h. This was evaluated by the GSH-Glo™ Glutathione Assay. Data was normalised to the vehicle-control which was assigned 1.0 RLU of GSH level, and each data point plotted (n=3). * = $p \leq 0.05$.


(A)
JURKAT
CCRF-CEM
THP-1
KG-1a
GSH Level Normalised to Vehicle Controls (1.0) RLU
Control
Emodin (μM)
(B)
Rhein (μM)
0.0
0.5
1.0
1.5
0.1
1
10
100
1000

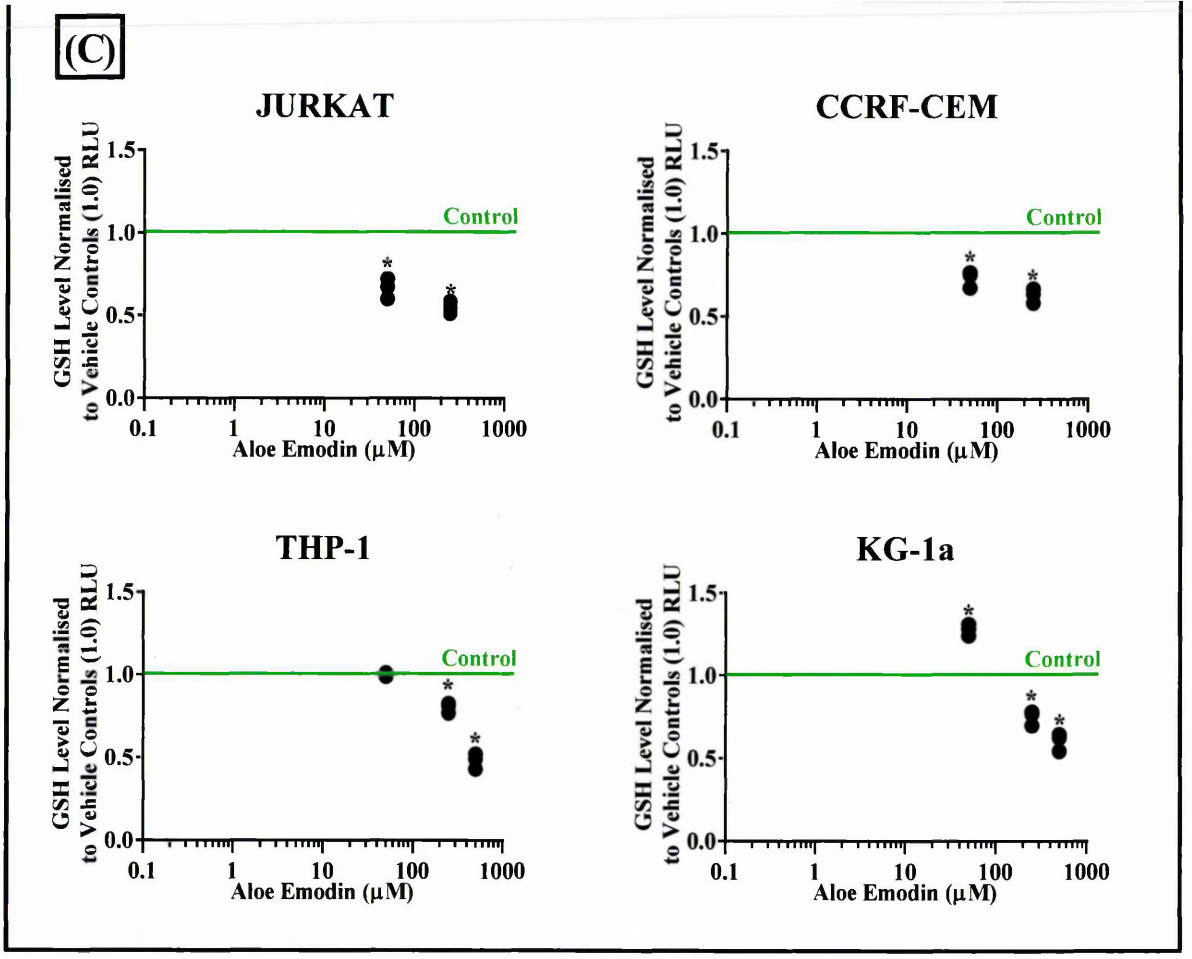


Figure 4.21: Effect of polyphenols: (A) emodin, (B) rhein and (C) aloe-emodin on GSH levels in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines. Cells were treated with emodin and rhein at their NSD, LSD and IC_{50} treatment ranges; while aloe-emodin was only used at its IC_{50} treatment ranges for 24 h. This was evaluated by the GSH-Glo™ Glutathione Assay. Data was normalised to the vehicle-control which was assigned 1.0 RLU of GSH level, and each data point plotted (n=3). * = $p \leq 0.05$.

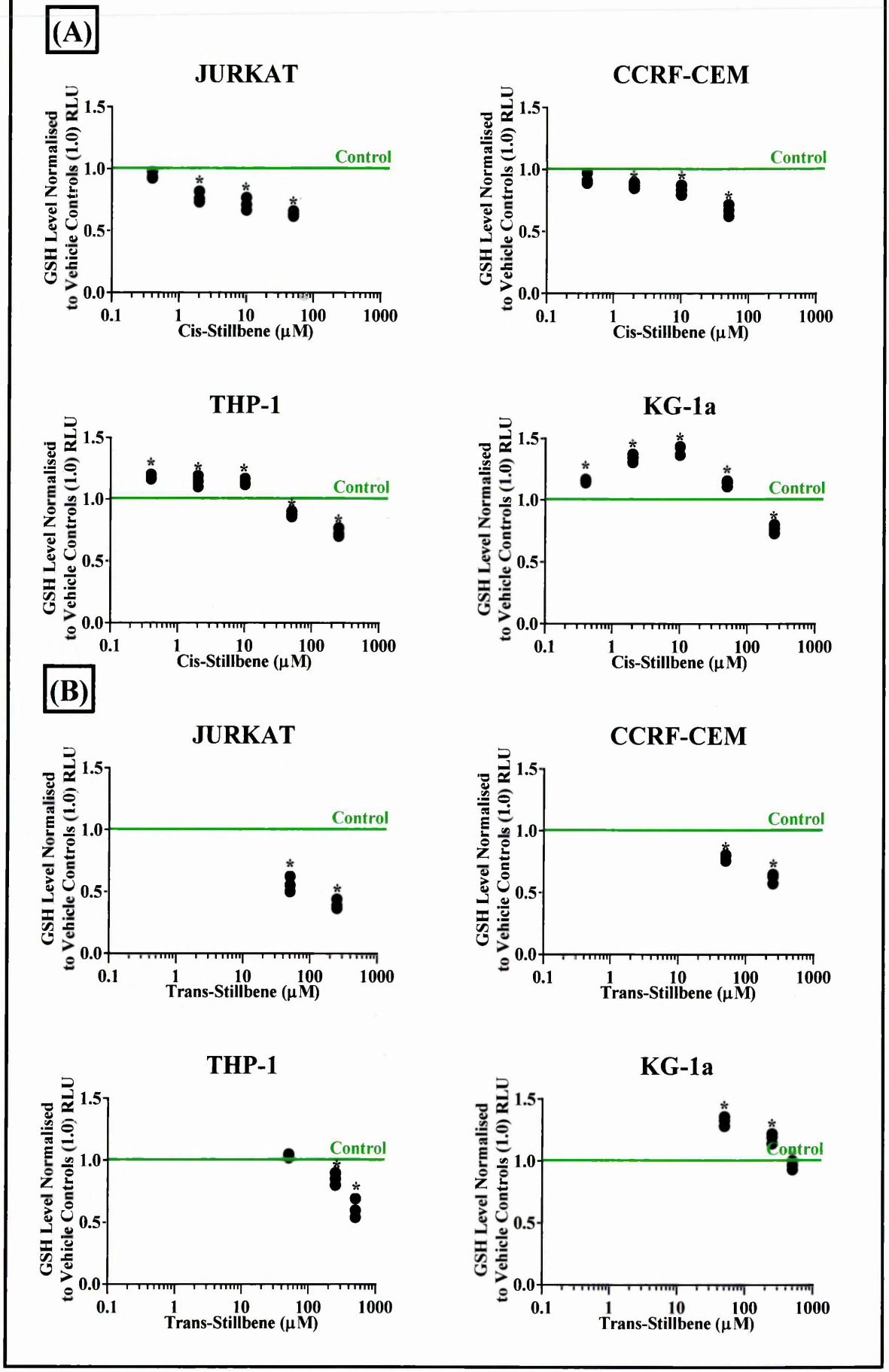

(A)
JURKAT
CCRF-CEM
THP-1
KG-1a
GSH Level Normalised to Vehicle Controls (1.0) RLU
Control
Cis-Stillbene (µM)
(B)
JURKAT
CCRF-CEM
THP-1
KG-1a
GSH Level Normalised to Vehicle Controls (1.0) RLU
Control
Trans-Stillbene (µM)

Figure 4.22: Effect of polyphenols: (A) *cis*-stilbene and (B) *trans*-stilbene on GSH levels in two lymphoid leukaemia (JURKAT and CCRF-CEM), and two myeloid leukaemia (THP-1 and KG-1a) cell lines. Cells were treated with *cis*-stilbene at their NSD, LSD and IC_{50} treatment ranges; while *trans*-stilbene was only used at its IC_{50} treatment ranges for 24 h. This was evaluated by the GSH-Glo™ Glutathione Assay. Data was normalised to the vehicle-control which was assigned 1.0 RLU of GSH level, and each data point plotted (n=3). * = $p \leq 0.05$.

Polyphenols	Investigated Doses	The Significant Effects of 24 h Polyphenol Treatment on GSH Levels.			
		Lymphoid Leukaemia		Myeloid Leukaemia	
		JURKAT	CCRF-CEM	THP-1	KG1a
Quercetin	NSD	—	—	—	—
	LSD	↓	↓	—	↓
	IC_{50}	↓	↓	↓	↓
Apigenin	NSD	—	↓	—	↑
	LSD	↓	↓	↓	↑
	IC_{50}	↓	↓	↓	↓
Chrysin	IC_{50}	↓	↓	↓	↓
Emodin	NSD	—	—	—	↑
	LSD	↓	↓	↓	↑
	IC_{50}	↓	↓	↓	↓
Rhein	NSD	—	—	—	↑
	LSD	↓	—	—	↑
	IC_{50}	↓	↓	↓	↓
Aloe-emodin	IC_{50}	↓	↓	↓	↓
Cis-Stilbene	NSD	—	—	↑	↑
	LSD	↓	↓	↑	↑
	IC_{50}	↓	↓	↓	↓
Trans-Stilbene	IC_{50}	↓	↓	↓	↑

Table 4.8: A summary of the significant effect of eight polyphenols (quercetin, apigenin, chrysin, rhein, emodin, aloe-emodin, *cis*-stilbene and *trans*-stilbene) on GSH levels in two lymphoid leukaemia cell lines (JURKAT and CCRF-CEM), and two human myeloid leukaemia cell lines (THP-1 and KG1a) at 24 h. This was evaluated by the GSH-Glo™ Glutathione Assay. Quercetin, apigenin, emodin, rhein and *cis*-stilbene were investigated at their NSD, LSD and IC_{50} treatment ranges, while chrysin, aloe-emodin and *trans*-stilbene were investigated only at their IC_{50} treatment ranges. — (no effect), ↓ (significant decrease in GSH levels) and ↑ (significant increase in GSH levels).

4.3.2.3 Effects of Topoisomerase Inhibitors Alone and in Combination with Polyphenols on GSH Levels

(A) Effects of Doxorubicin (DOX) Combined with Polyphenols on Glutathione (GSH) Levels

DOX alone significantly reduced the level of GSH in all leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 4.23, 4.24, 4.25). DOX in combination with QUE or AP caused a synergistic decrease in GSH levels in all lymphoid and myeloid cell lines ($p<0.05$) (Figure 4.23, 4.24 and Table 4.4). When DOX was used in combination with EMO, RH or CIS it also caused a synergistic reduction in GSH levels, but only in the lymphoid leukaemia cell lines (Figure 4.23, 4.24 and Table 4.4). In contrast, in the myeloid cell lines, DOX was combined with EMO or RH it produced a competitive antagonistic effect, significantly increasing GSH levels ($p<0.05$) (Figure 4.23, 4.24 and Table 4.4). When DOX was used in combination with CIS it produced an additive reduction in GSH levels in the myeloid leukaemia cell lines ($p<0.05$) (Figure 4.23, 4.24 and Table 4.4).

(B) Effects of Etoposide (ETP) Combined with Polyphenols on (Glutathione) GSH Levels

ETP alone significantly reduced the GSH levels in all the leukaemia cell lines (JURKAT, CCRF-CEM, THP-1 and KG-1a) ($p<0.05$) (Figure 4.26, 4.27, 4.28). ETP when used in combination with QUE or AP produced a synergistic reduction in GSH levels ($p<0.05$) in all the leukaemia cell lines (Figure 4.26, 4.27 and Table 4.5). ETP when used in combination with EMO produced a synergistic reduction in GSH level in the lymphoid leukaemia cell lines only (Figure 4.26, 4.27 and Table 4.5); and an additive effects in myeloid leukaemia cell lines ($p<0.05$) (Figure 4.26, 4.27 and Table 4.5). ETP when used in combination with RH or CIS produced a synergistic reduction in GSH levels in the lymphoid leukaemia cell lines only (Figure 4.26, 4.27 and Table 4.5). Whilst in the myeloid leukaemia cell lines ETP combined with RH or CIS displayed competitively antagonistic or antagonistic effects with a significantly increase GSH levels ($p<0.05$) (Figure 4.26, 4.27 and Table 4.5).

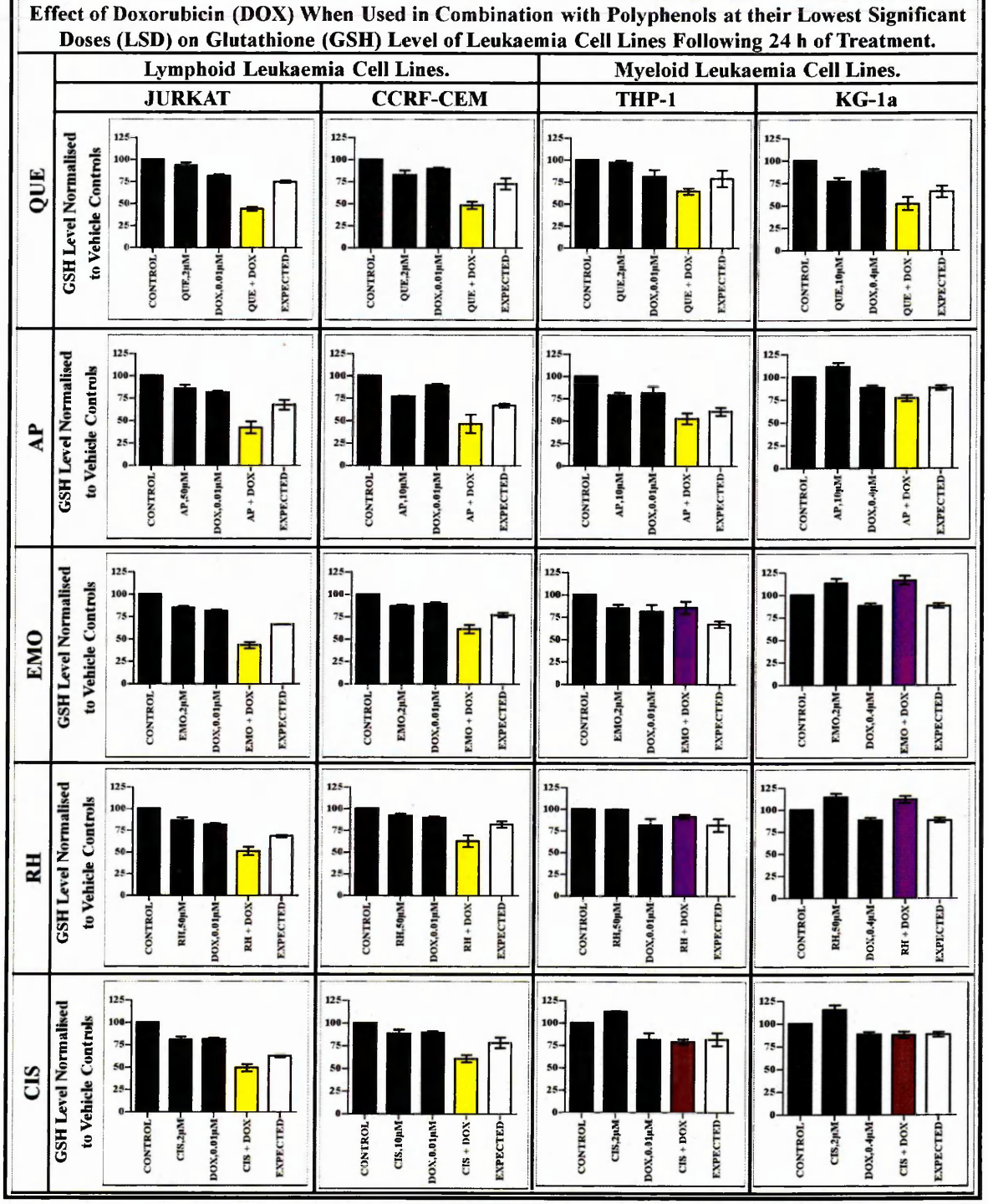


Figure 4.23: The effect of DOX used in combination with QUE, AP, EMO, RH or CIS on GSH levels of leukaemia cell lines. This was evaluated by the GSH-Glo™ Glutathione Assay. Cells were treated with DOX and polyphenols alone and in combination for 24 h using their LSD. Data was normalised to the vehicle-control which was assigned 100% of GSH Level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle-controls and treatments alone; the white bar shows the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive reduction of GSH levels in brown, synergistic reduction in GSH levels in yellow, a competitive antagonistic increase in GSH levels in purple and an antagonistic increase in GSH levels in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

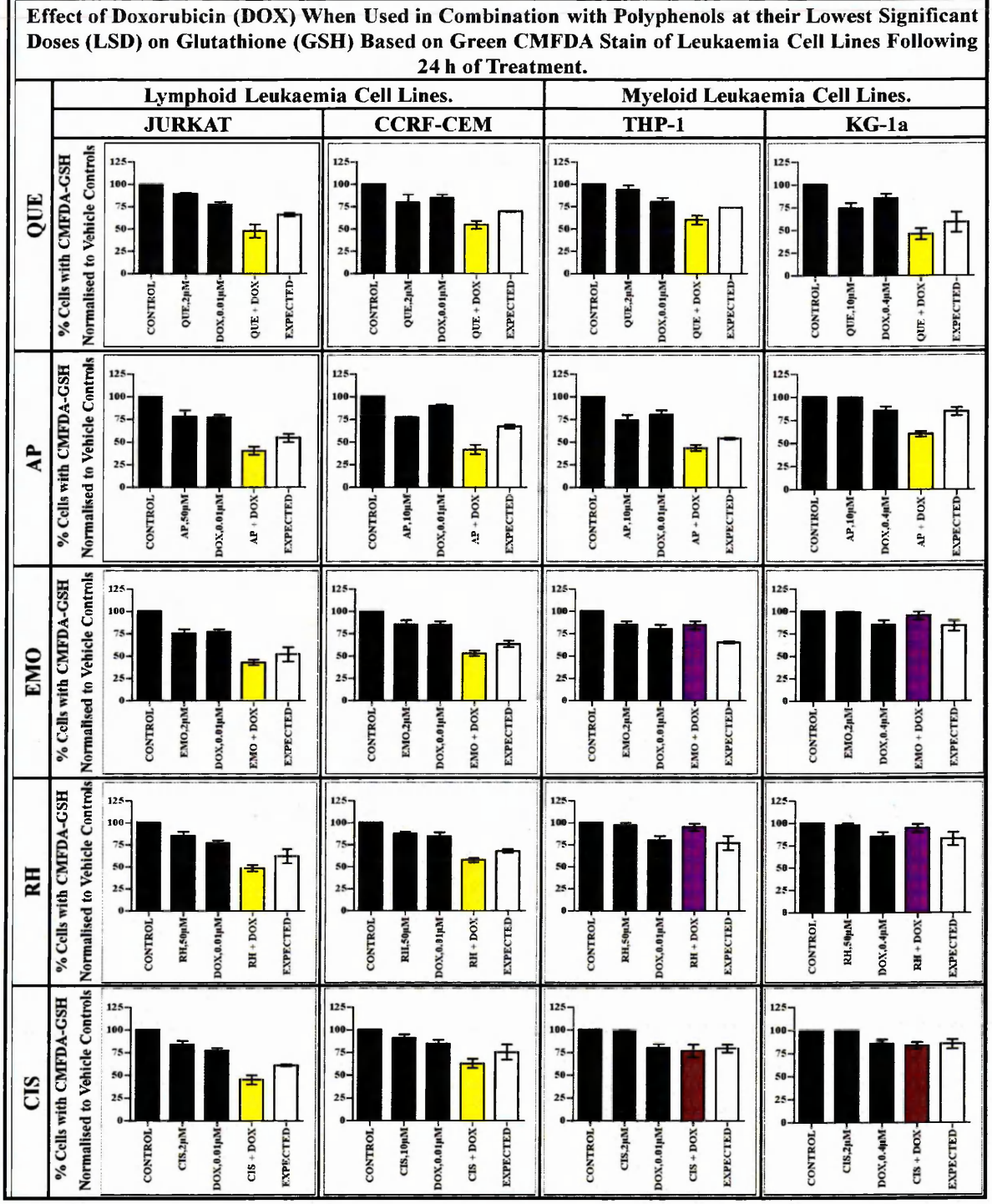


Figure 4.24: The effect of DOX used in combination with QUE, AP, EMO, RH or CIS on intracellular GSH levels of leukaemia cell lines. This was evaluated by double staining with the CellTracker GSH- Green CMFDA (5-chloromethylfluorescein diacetate) and blue Hoechst 33342 using fluorescence microscopy. Cells were treated with DOX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

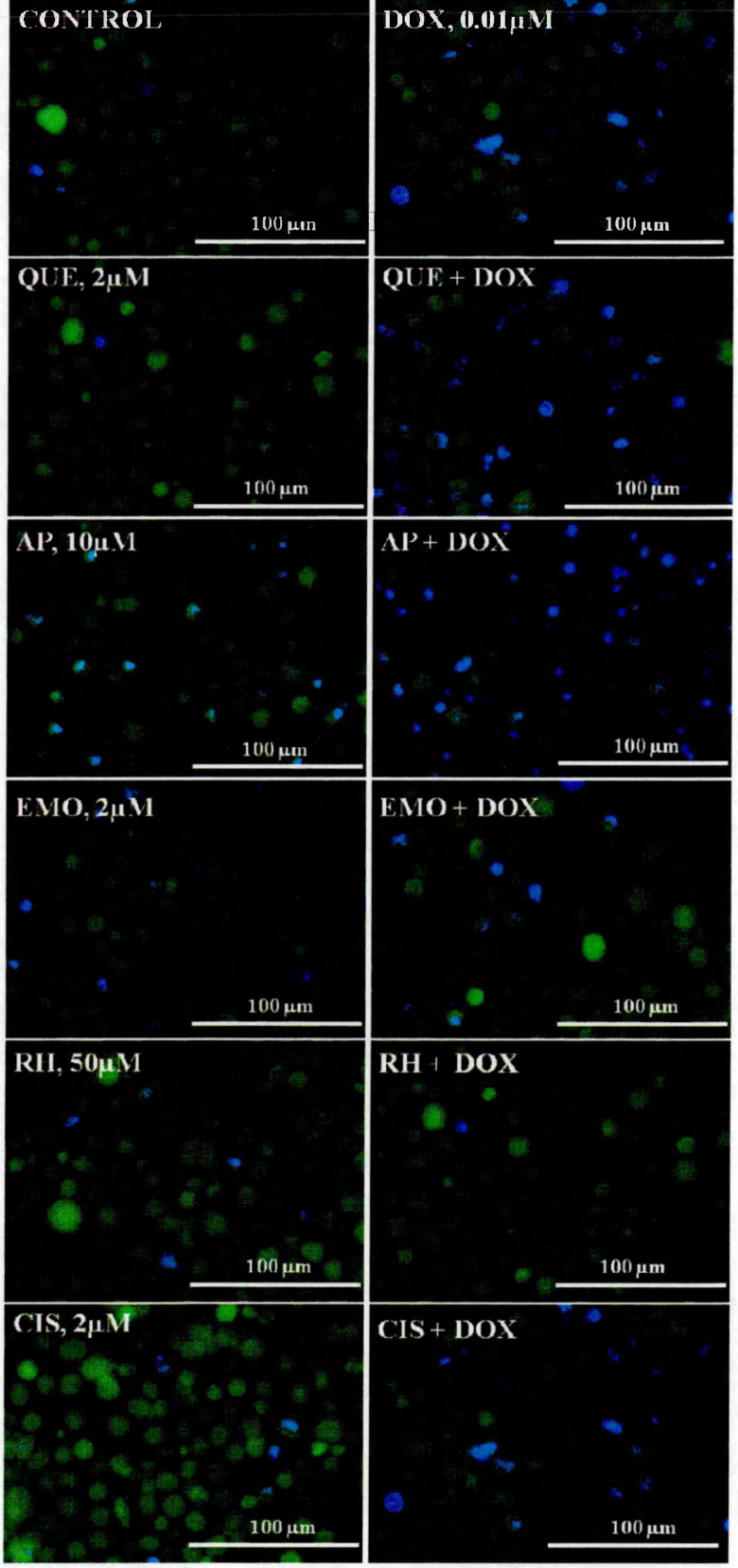


Figure 4.25: An example of fluorescent detection of cellular glutathione (GSH) for THP-1 myeloid leukaemia cells when treated with LSDs of DOX and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination for 24 h; using the CellTracker GSH- Green 5-chloromethylfluorescein diacetate (CMFDA) and blue Hoechst 33342 stains. Cells with green-CMFDA staining indicate the presence of GSH, while cells with blue Hoechst 33342 staining indicate a decrease in GSH. . Scale bar = 100 µm.

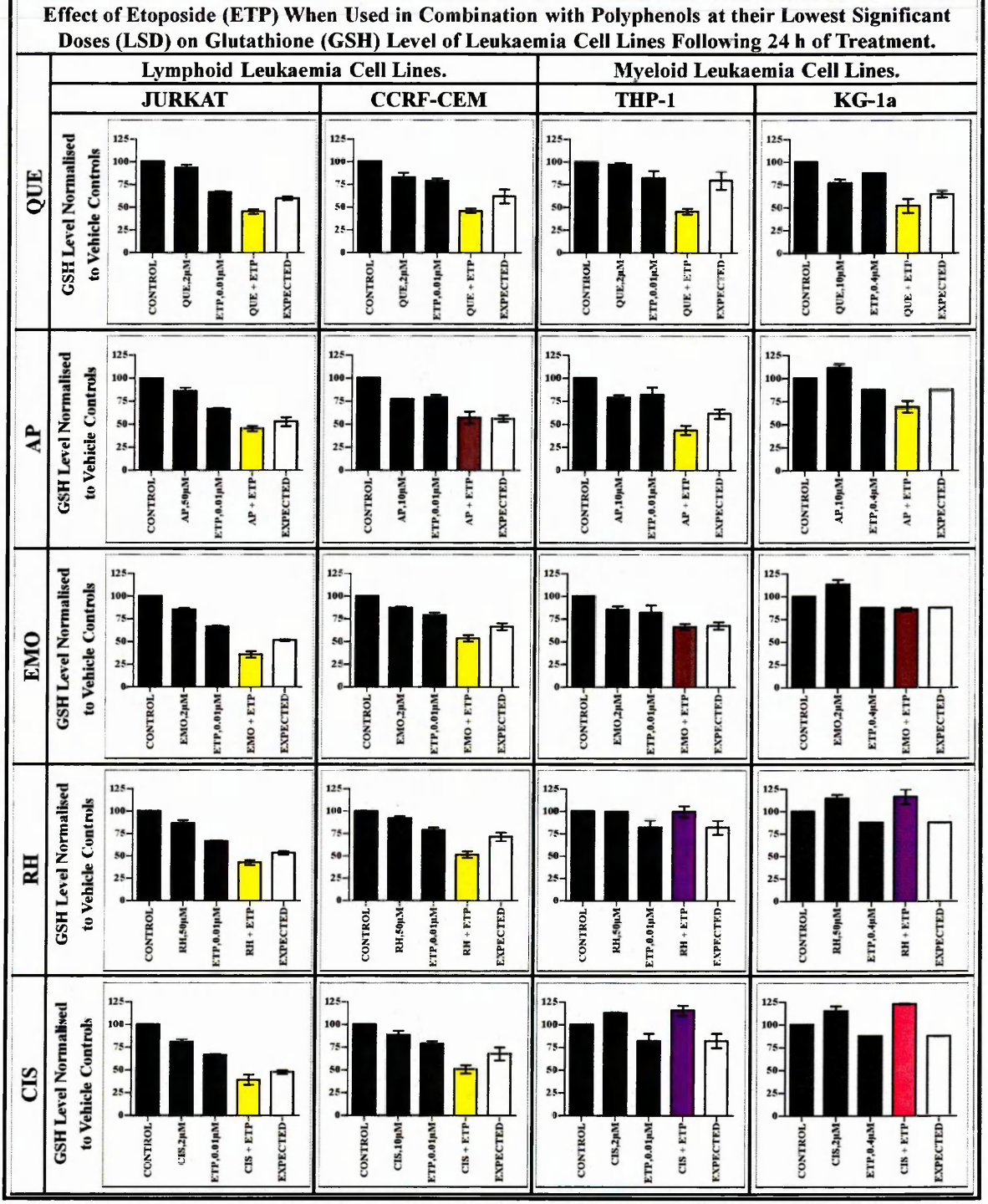


Figure 4.26: The effect of ETP used in combination with QUE, AP, EMO, RH or CIS on GSH levels of leukaemia cell lines. This was evaluated by the GSH-Glo™ Glutathione Assay. Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical signficant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

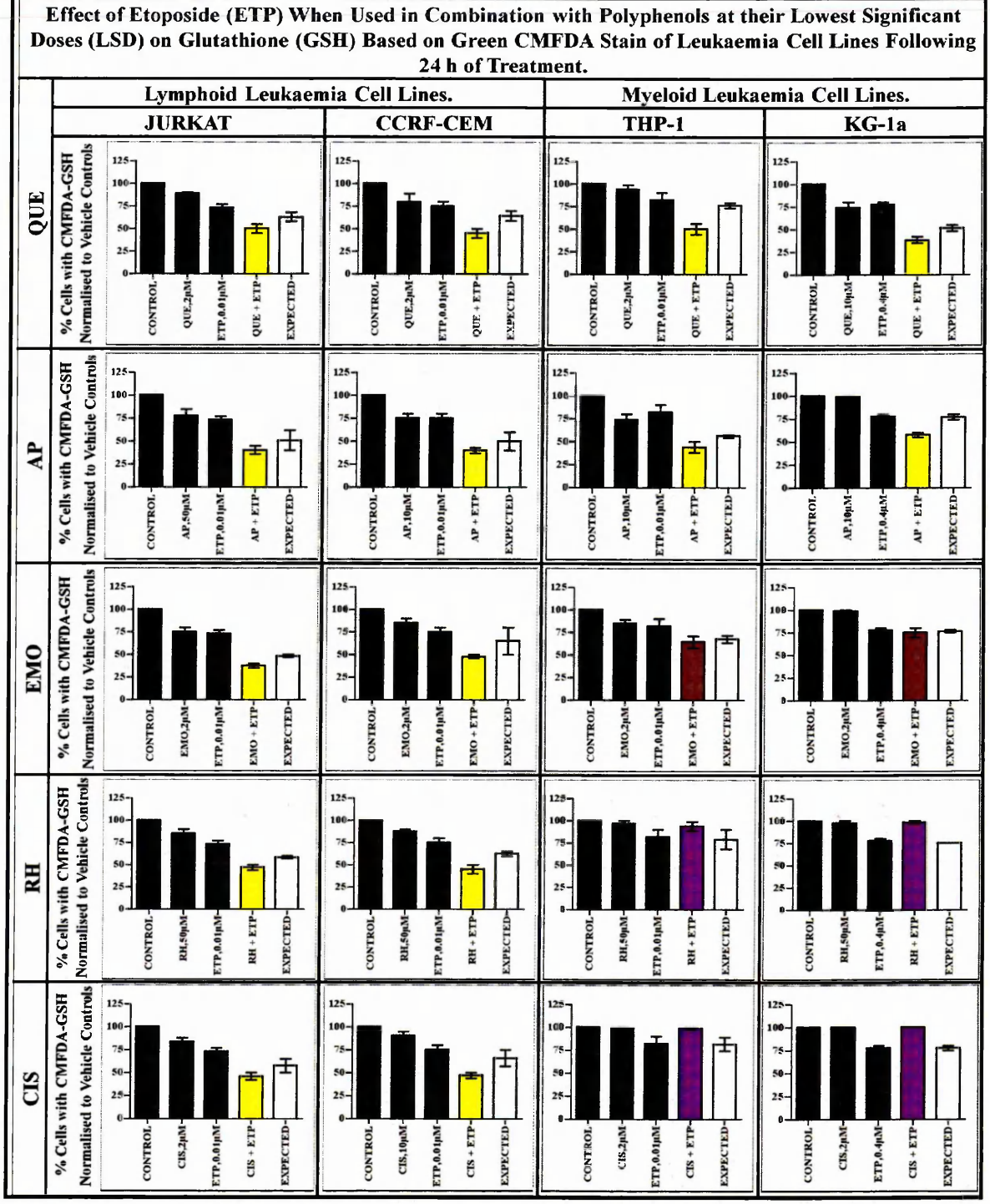


Figure 4.27: The effect of ETP used in combination with QUE, AP, EMO, RH or CIS on intracellular GSH levels of leukaemia cell lines. This was evaluated by double staining with the CellTracker GSH- Green CMFDA (5-chloromethylfluorescein diacetate) and blue Hoechst 33342 using fluorescence microscopy. Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

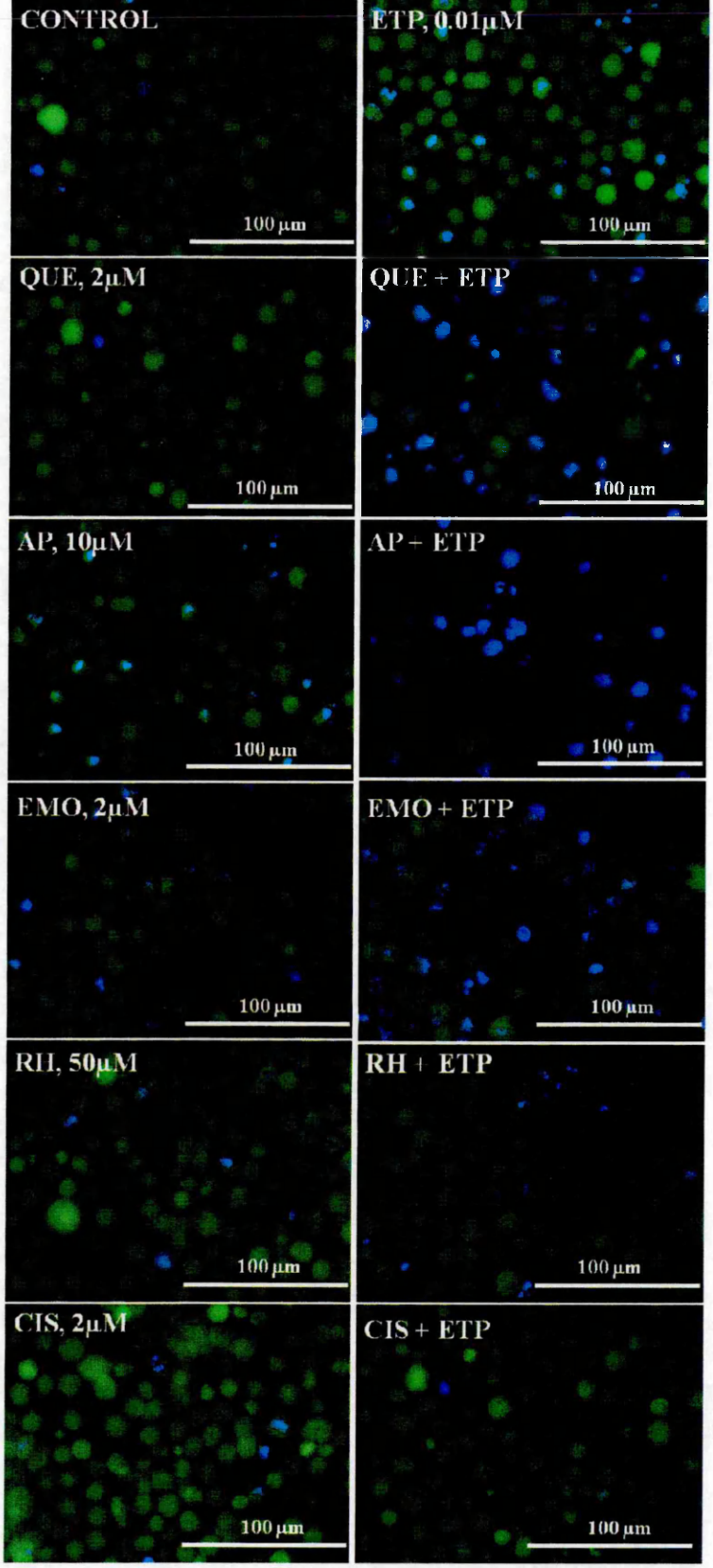


Figure 4.28: An example of fluorescent detection of cellular glutathione (GSH) for THP-1 myeloid leukaemia cells when treated with LSDs of ETP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination for 24 h; using the CellTracker GSH- Green 5-chloromethylfluorescein diacetate (CMFDA) and blue Hoechst 33342 stains. Cells with green-CMFDA staining indicate the presence of GSH, while cells with blue Hoechst 33342 staining indicate a decrease in GSH. . Scale bar = 100 µm.

4.3.2.4 Effects of Alkylating Chemotherapy Alone and in Combination with Polyphenols on GSH Levels

(A) Effects of Cyclophosphamide (CYCLO) Alone and in Combination with Polyphenols on Glutathione (GSH) Levels

CYCLO alone significantly reduced the level of GSH in three leukaemia cell lines (JURKAT, CCRF-CEM and THP-1) ($p<0.05$) (Figure X, X), but had no effect on KG-1a cells (Figure 4.29, 4.30, 4.31). CYCLO when used in combination with AP caused a synergistic reduction in GSH levels ($p<0.05$) in all leukaemia cell lines (Figure 4.29, 4.30 and Table 4.6). However, when CYCLO was combined with QUE, EMO or RH a synergistic reduction in GSH levels was seen in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.29, 4.30 and Table 4.6); whilst in myeloid leukaemia cell lines a competitively antagonistic or antagonistic effect and a significant increase in GSH levels was observed ($p<0.05$) (Figure 4.29, 4.30 and Table 4.6). Likewise, when CYCLO used in combination with CIS a competitive antagonistic or antagonistic effect was seen in all the leukaemia cell lines ($p<0.05$) (Figure 4.29, 4.30 and Table 4.6).

(B) Effects of Cisplatin (CSP) Alone and in Combination Polyphenols on Glutathione (GSH) Levels

CSP alone significantly reduced the levels of GSH in all the leukaemia cell lines ($p<0.05$) (Figure 4.32, 4.33, 4.34). CSP combined with QUE, resulted in a synergistic reduction in GSH levels in all leukaemia cell lines ($p<0.05$) (Figure 4.32, 4.33 and Table 4.7). Likewise, when CSP was combined with AP, it produced a synergistic effect on GSH levels in lymphoid leukaemia cell lines ($p<0.05$); as well a synergistic effect or an additive effect on GSH levels in myeloid leukaemia cell lines ($p<0.05$) (Figure 4.32, 4.33 and Table 4.7). Additionally, when CSP was used in combination with EMO, RH, or CIS, it caused a synergistic reduced in GSH levels in the lymphoid leukaemia cell lines only ($p<0.05$) (Figure 4.32, 4.33 and Table 4.7). However, in myeloid leukaemia cell lines, when CSP was combined with EMO, RH, or CIS, a competitive antagonistic or antagonistic effect as seen significantly increasing GSH levels ($p<0.05$) (Figure 4.32, 4.33 and Table 4.7).

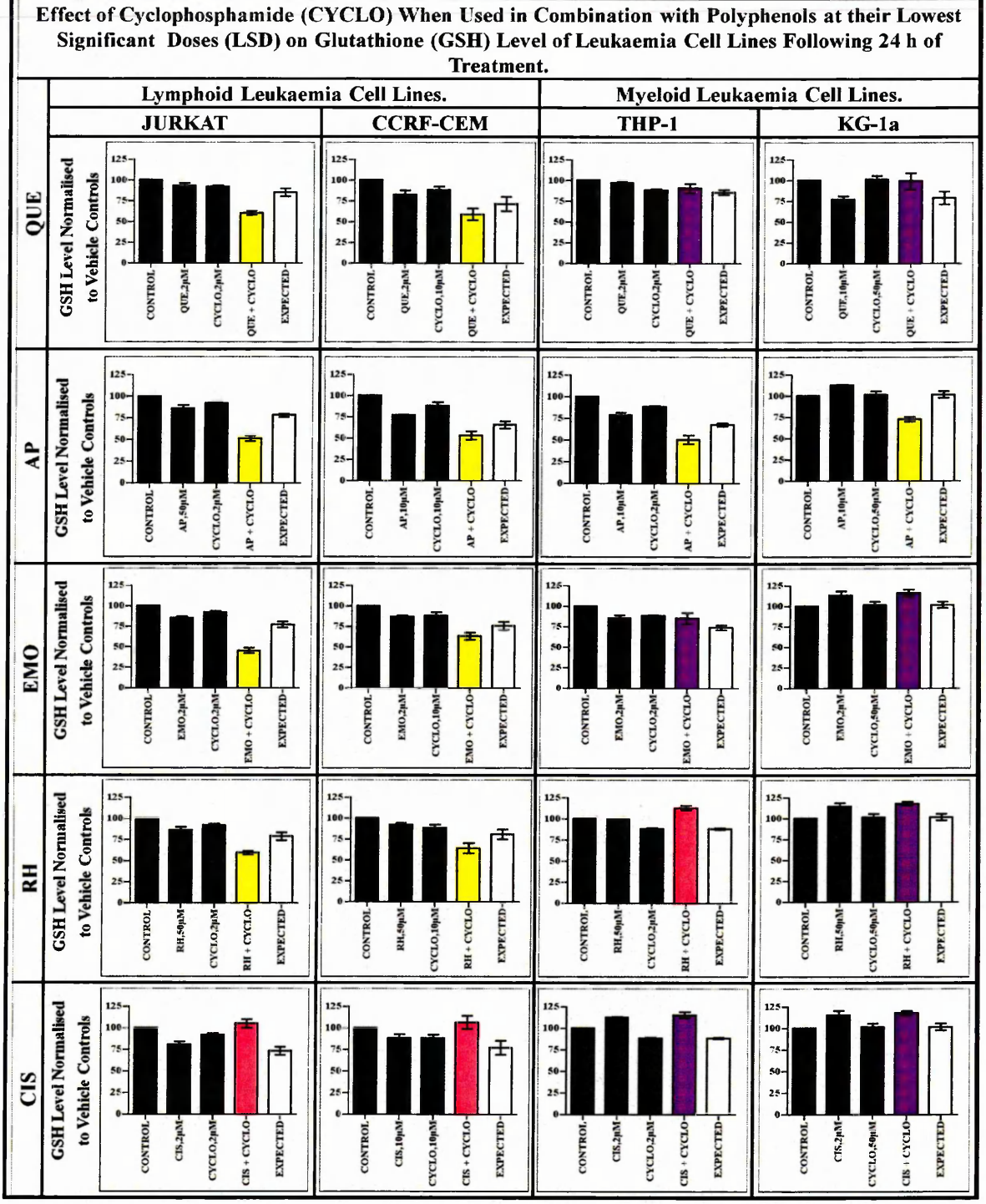


Figure 4.29: The effect of CYCLO used in combination with QUE, AP, EMO, RH or CIS on GSH levels of leukaemia cell lines. This was evaluated by the GSH-Glo™ Glutathione Assay. Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

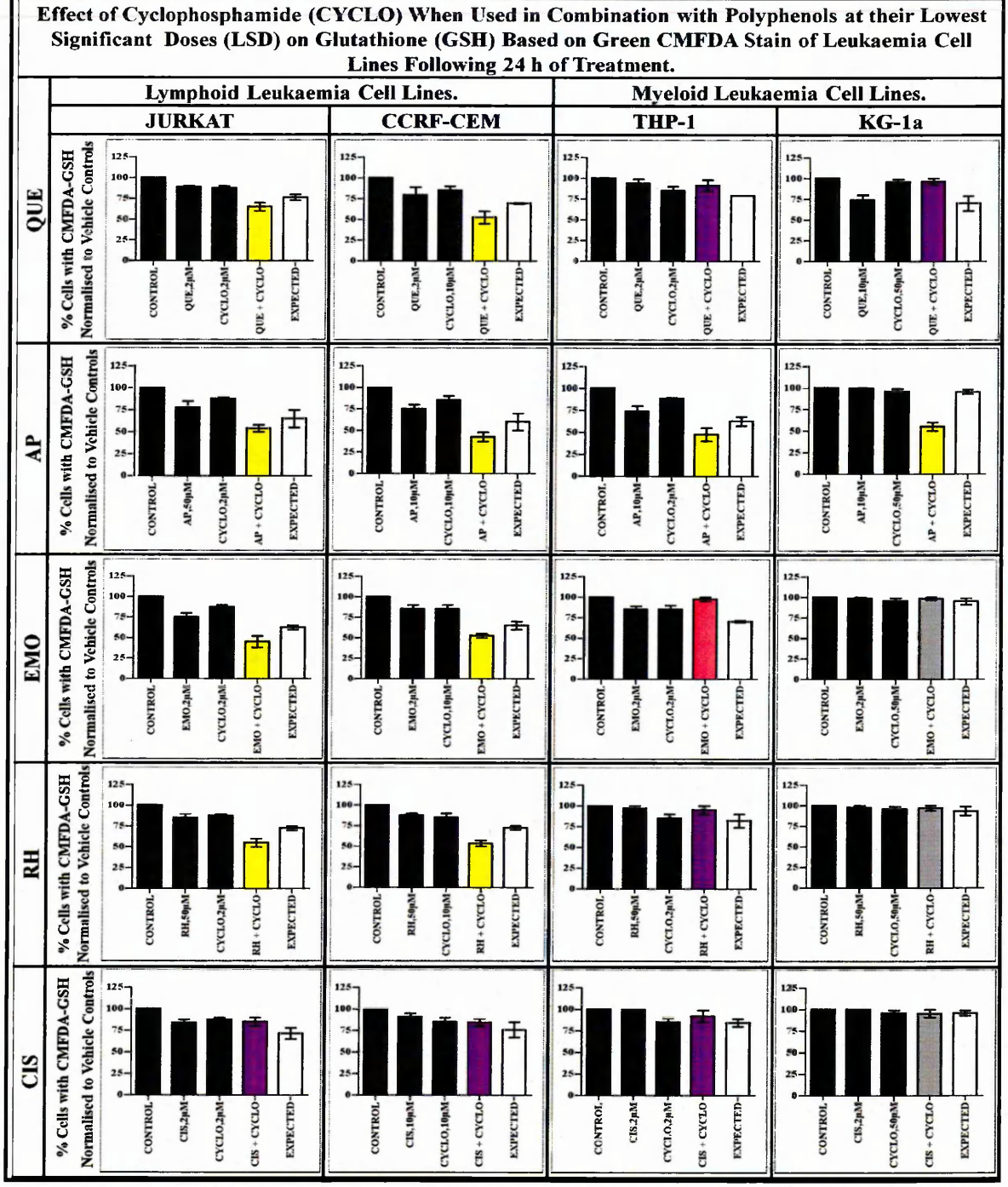


Figure 4.30: The effect of CYCLO used in combination with QUE, AP, EMO, RH or CIS on intracellular GSH levels of leukaemia cell lines. This was evaluated by double staining with the CellTracker GSH- Green CMFDA (5-chloromethylfluorescein diacetate) and blue Hoechst 33342 using fluorescence microscopy. Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. The gray bars indicate for no significant effect. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

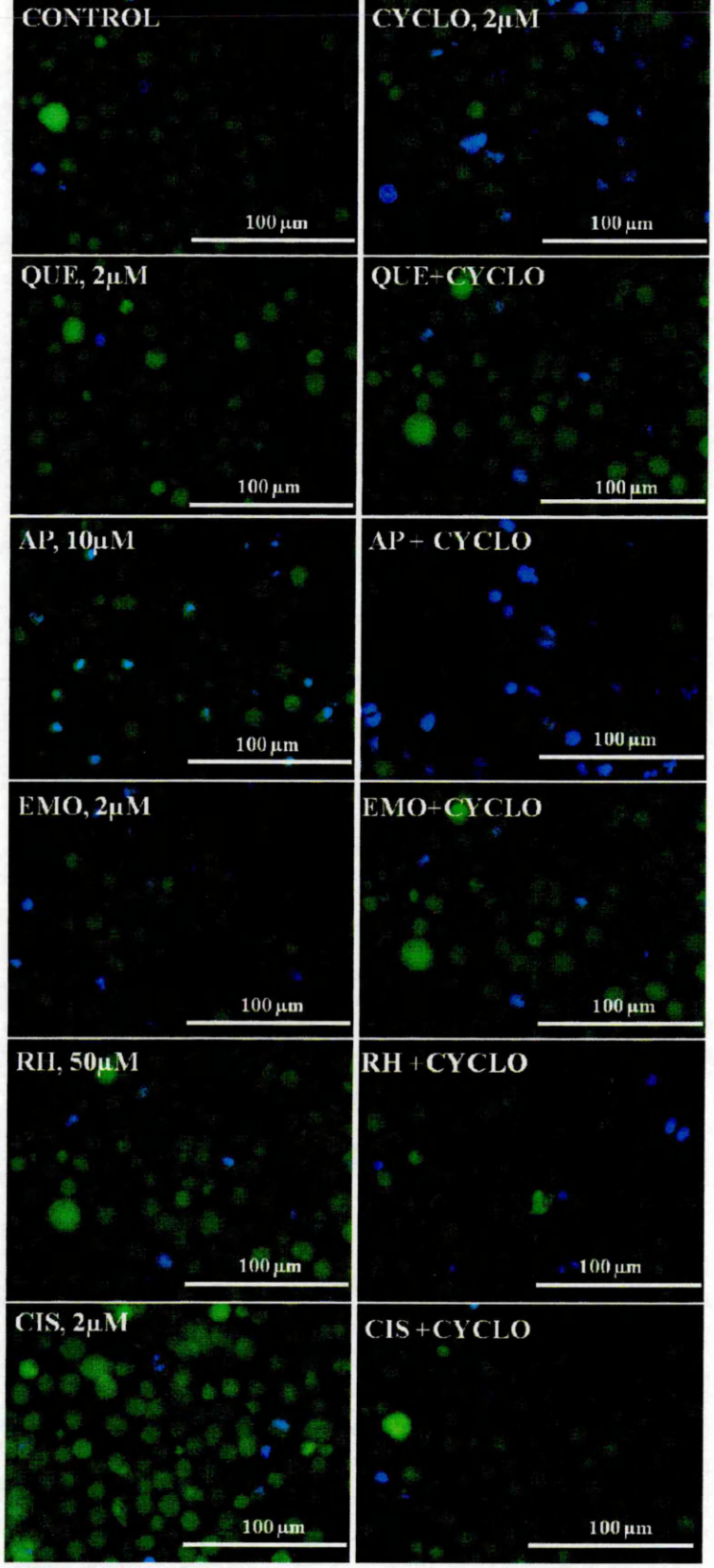


Figure 4.31: An example of fluorescent detection of cellular glutathione (GSH) for THP-1 myeloid leukaemia cells when treated with LSDs of CYCLO and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination for 24 h; using the CellTracker GSH- Green 5-chloromethylfluorescein diacetate (CMFDA) and blue Hoechst 33342 stains. Cells with green-CMFDA staining indicate the presence of GSH, while cells with blue Hoechst 33342 staining indicate a decrease in GSH. . Scale bar = 100 µm.

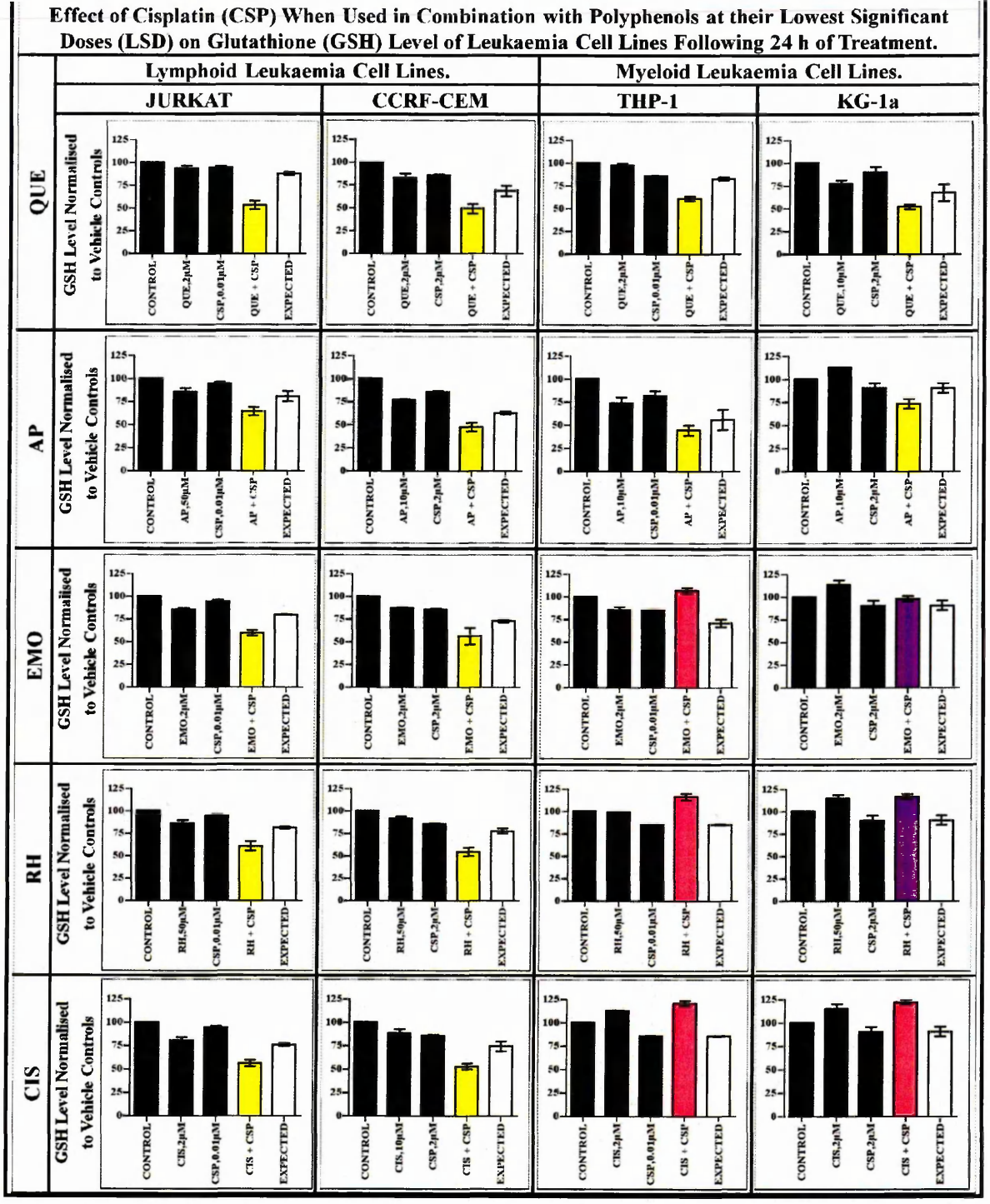


Figure 4.32: The effect of CSP used in combination with QUE, AP, EMO, RH or CIS on GSH levels of leukaemia cell lines. This was evaluated by the GSH-Glo™ Glutathione Assay. Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical signficant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

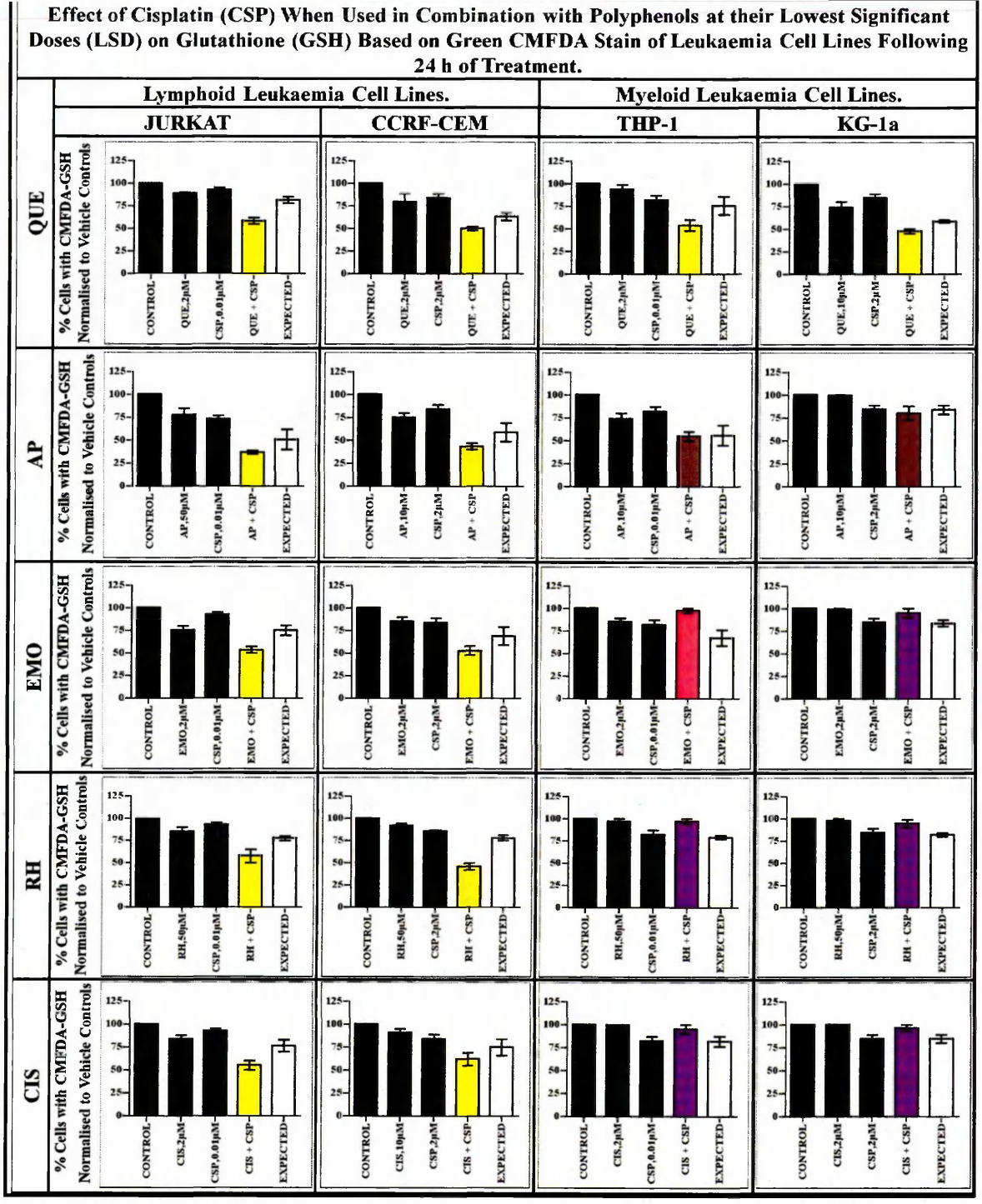


Figure 4.33: The effect of CSP used in combination with QUE, AP, EMO, RH or CIS on intracellular GSH levels of leukaemia cell lines. This was evaluated by double staining with the CellTracker GSH- Green CMFDA (5-chloromethylfluorescein diacetate) and blue Hoechst 33342 using fluorescence microscopy. Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

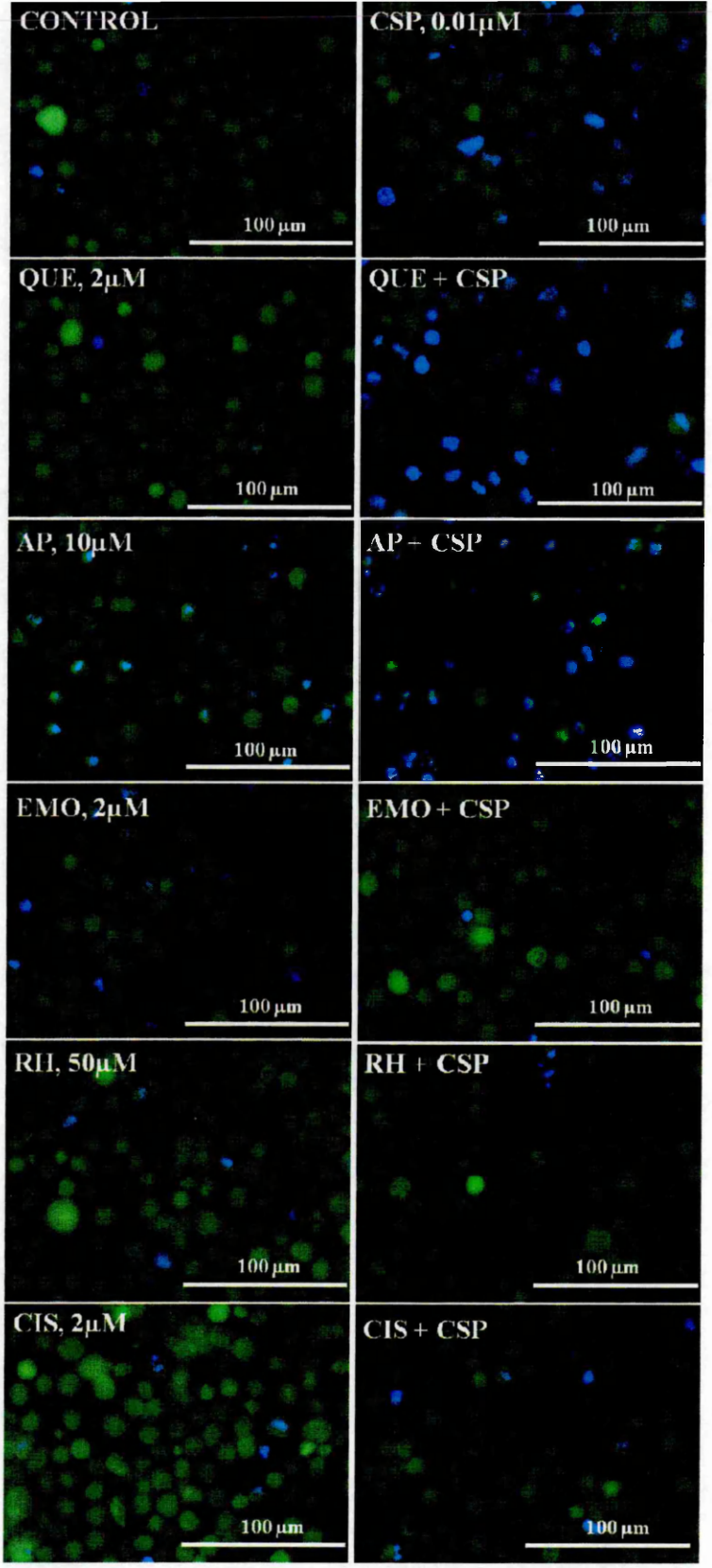


Figure 4.34: An example of fluorescent detection of cellular glutathione (GSH) for THP-1 myeloid leukaemia cells when treated with LSDs of CSP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination for 24 h; using the CellTracker GSH- Green 5-chloromethylfluorescein diacetate (CMFDA) and blue Hoechst 33342 stains. Cells with green-CMFDA staining indicate the presence of GSH, while cells with blue Hoechst 33342 staining indicate a decrease in GSH. . Scale bar = 100 µm.

4.3.2.5 Effects of Anti-Metabolites Chemotherapy Agents Alone and in Combination with Polyphenols on GSH Levels

(A) Effect of Methotrexate (MTX) Alone and in Combination with Polyphenols on Glutathione (GSH) Levels

MTX alone was significantly reduced the level of GSH in all the leukaemia cell lines ($p<0.05$) (Figure 4.35, 4.36, 4.37). MTX when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) caused a competitively antagonistic and/or antagonistic effects significantly increasing the GSH levels in all leukaemia cell lines ($p<0.05$) (Figure 4.35, 4.36 and Table 4.8).

(B) Effect of 6-Mercaptopurine (6-MP) Alone and in Combination with Polyphenols on Glutathione (GSH) Levels

6-MP alone significantly reduced the level of GSH in three leukaemia cell lines (JURKAT, THP-1 and KG-1a) ($p<0.05$), but had no significant effect on CCRF-CEM cells (Figure 4.38, 4.39, 4.40). 6-MP when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) caused competitive antagonistic and/or antagonistic effects significantly increasing the GSH levels in all leukaemia cell lines ($p<0.05$) (Figure 4.38, 4.39 and Table 4.9).

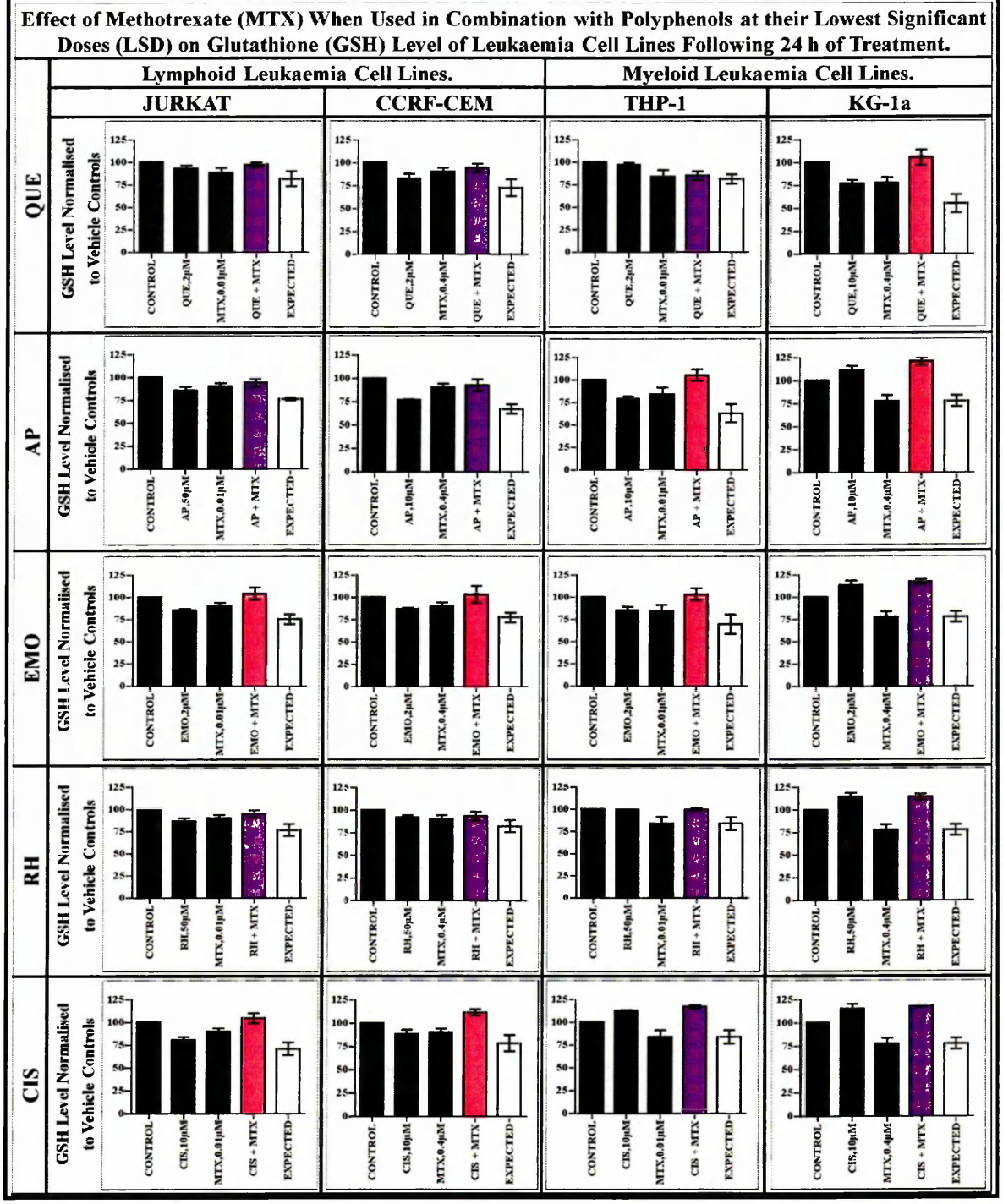


Figure 4.35: The effect of MTX used in combination with QUE, AP, EMO, RH or CIS on GSH levels of leukaemia cell lines. This was evaluated by the GSH-Glo™ Glutathione Assay. Cells were treated with MTX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

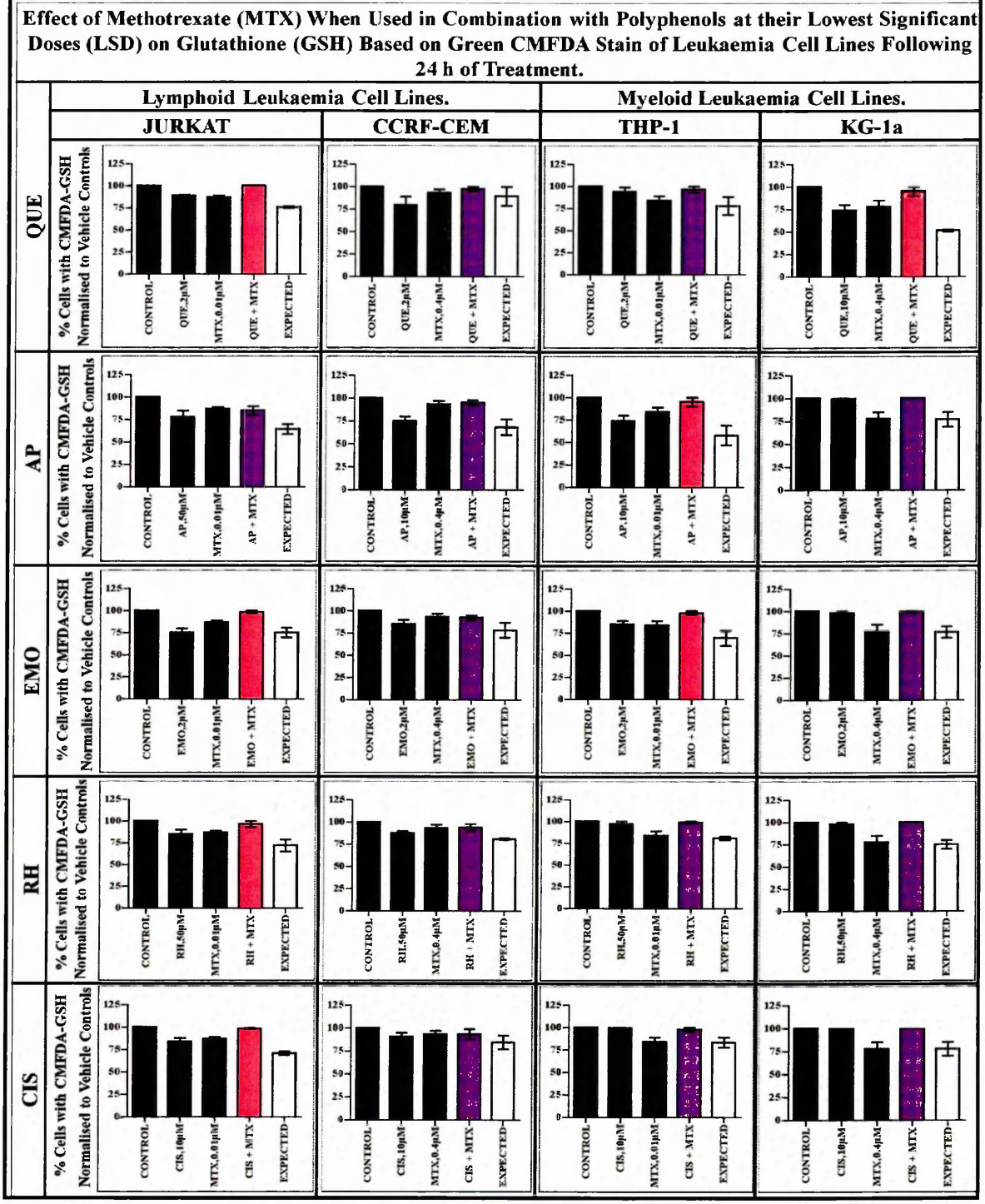


Figure 4.36: The effect of MTX used in combination with QUE, AP, EMO, RH or CIS on intracellular GSH levels of leukaemia cell lines. This was evaluated by double staining with the CellTracker GSH- Green CMFDA (5-chloromethylfluorescein diacetate) and blue Hoechst 33342 using fluorescence microscopy. Cells were treated with MTX and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

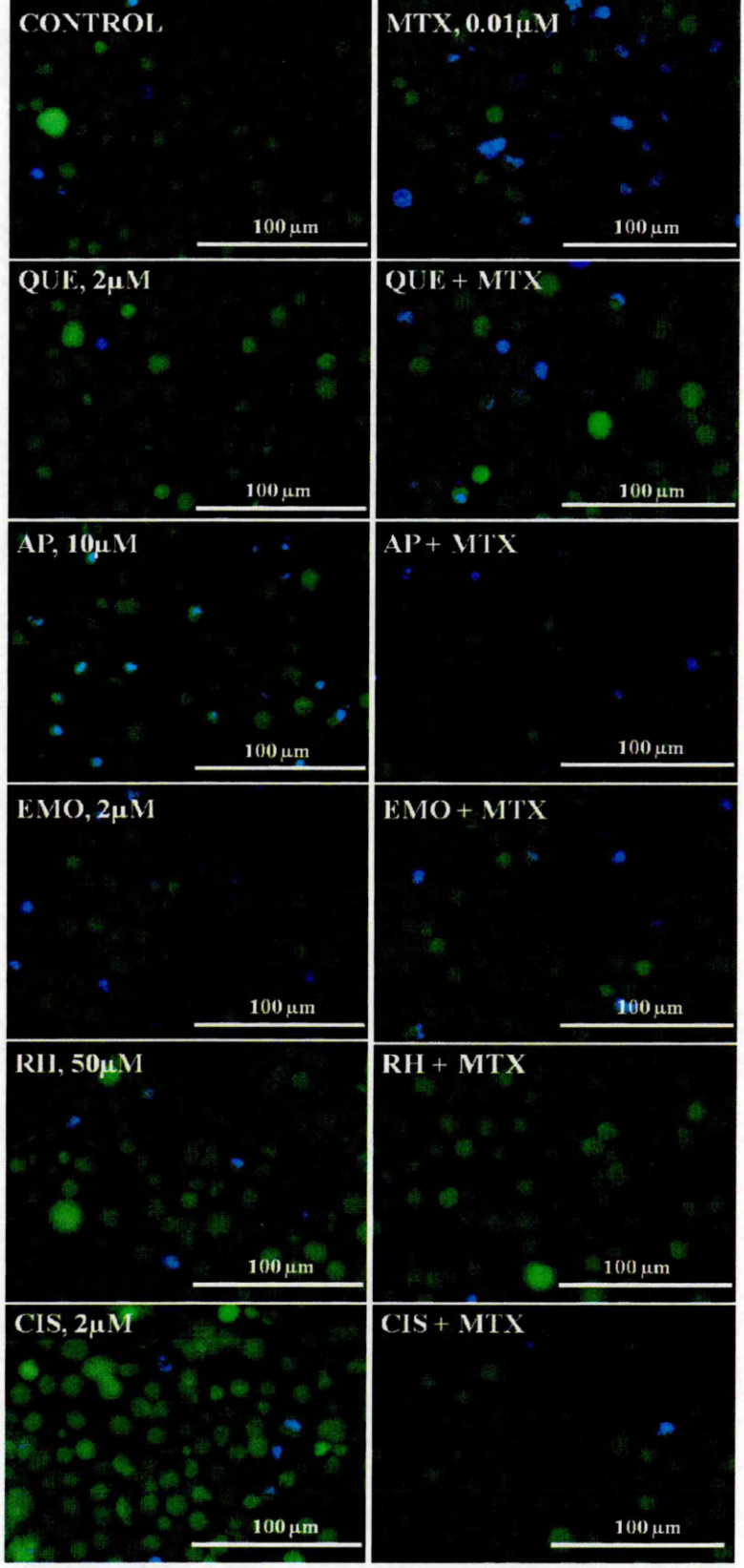


Figure 4.37: An example of fluorescent detection of cellular glutathione (GSH) for THP-1 myeloid leukaemia cells when treated with LSDs of MTX and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination for 24 h; using the CellTracker GSH- Green 5-chloromethylfluorescein diacetate (CMFDA) and blue Hoechst 33342 stains. Cells with green-CMFDA staining indicate the presence of GSH, while cells with blue Hoechst 33342 staining indicate a decrease in GSH. . Scale bar = 100 µm.

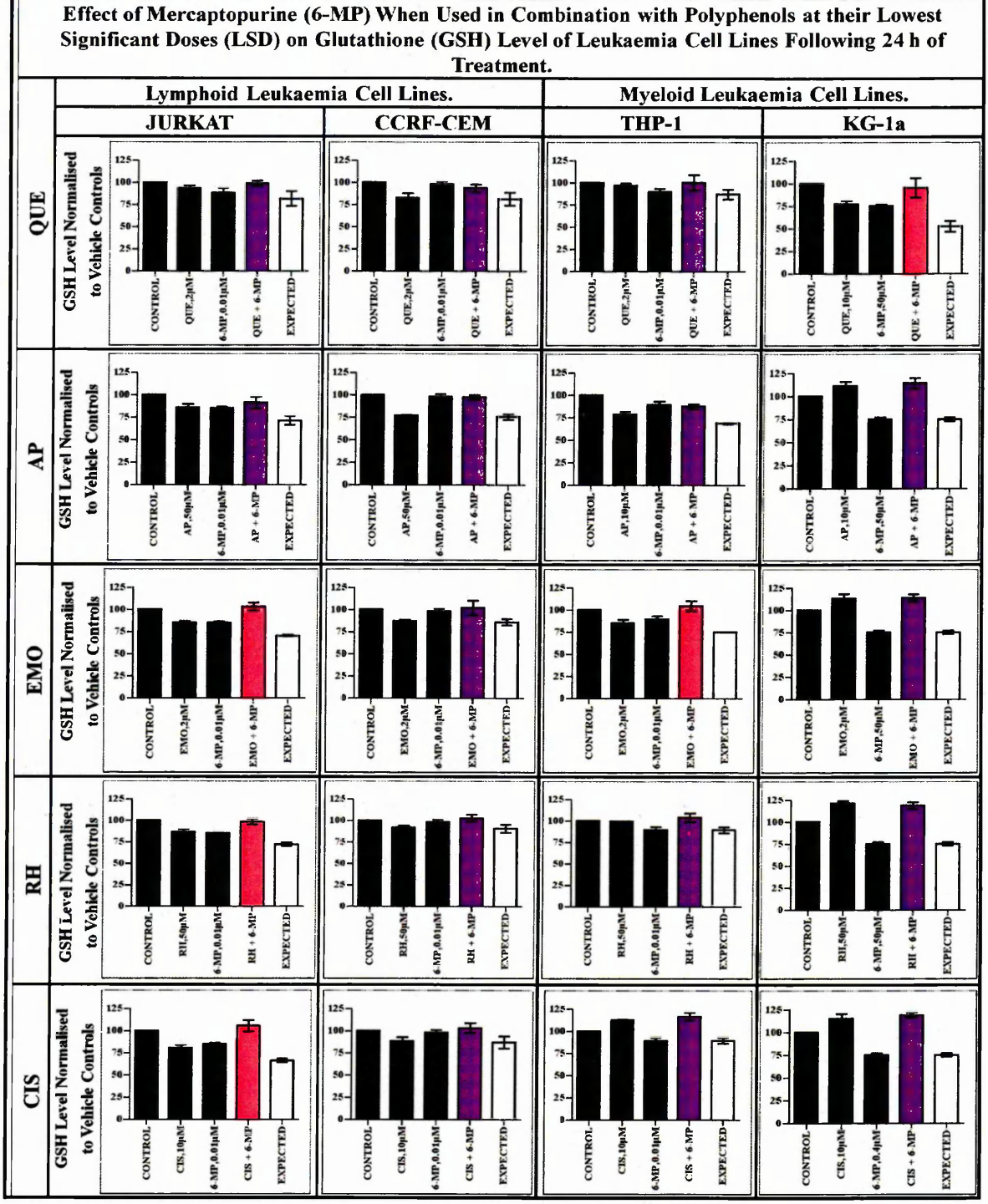


Figure 4.38: The effect of 6-MP used in combination with QUE, AP, EMO, RH or CIS on GSH levels of leukaemia cell lines. This was evaluated by the GSH-Glo™ Glutathione Assay. Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels ; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

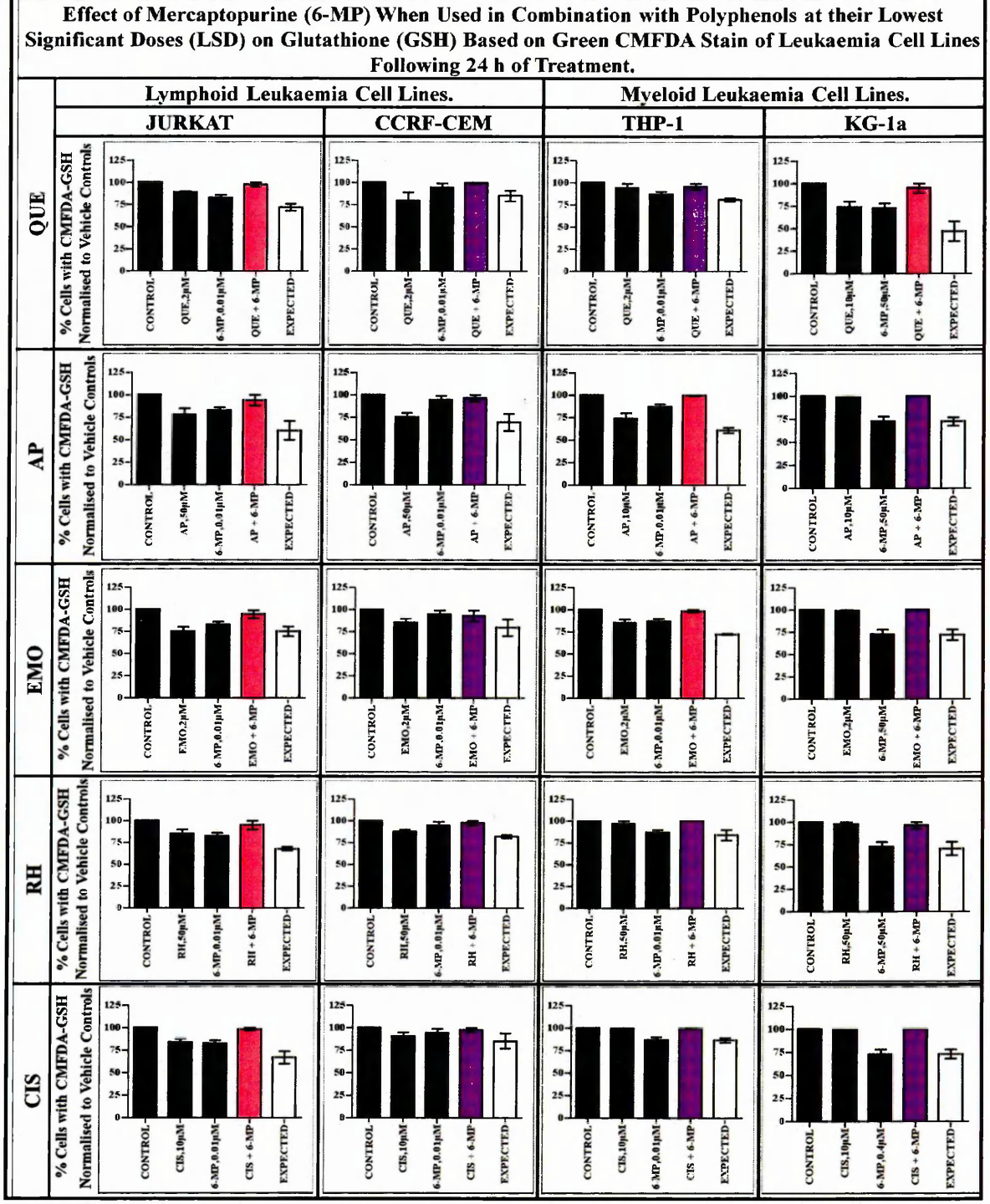


Figure 4.39: The effect of 6-MP used in combination with QUE, AP, EMO, RH or CIS on intracellular GSH levels of leukaemia cell lines. This was evaluated by double staining with the CellTracker GSH- Green CMFDA (5-chloromethylfluorescein diacetate) and blue Hoechst 33342 using fluorescence microscopy. Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using their LSD. Data was normalised to the vehicle control which was assigned 100% of GSH level. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on GSH levels; the coloured bars indicate a significant additive effects in brown, synergistic effects in yellow, competitive antagonistic effects in purple and an antagonistic effects in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

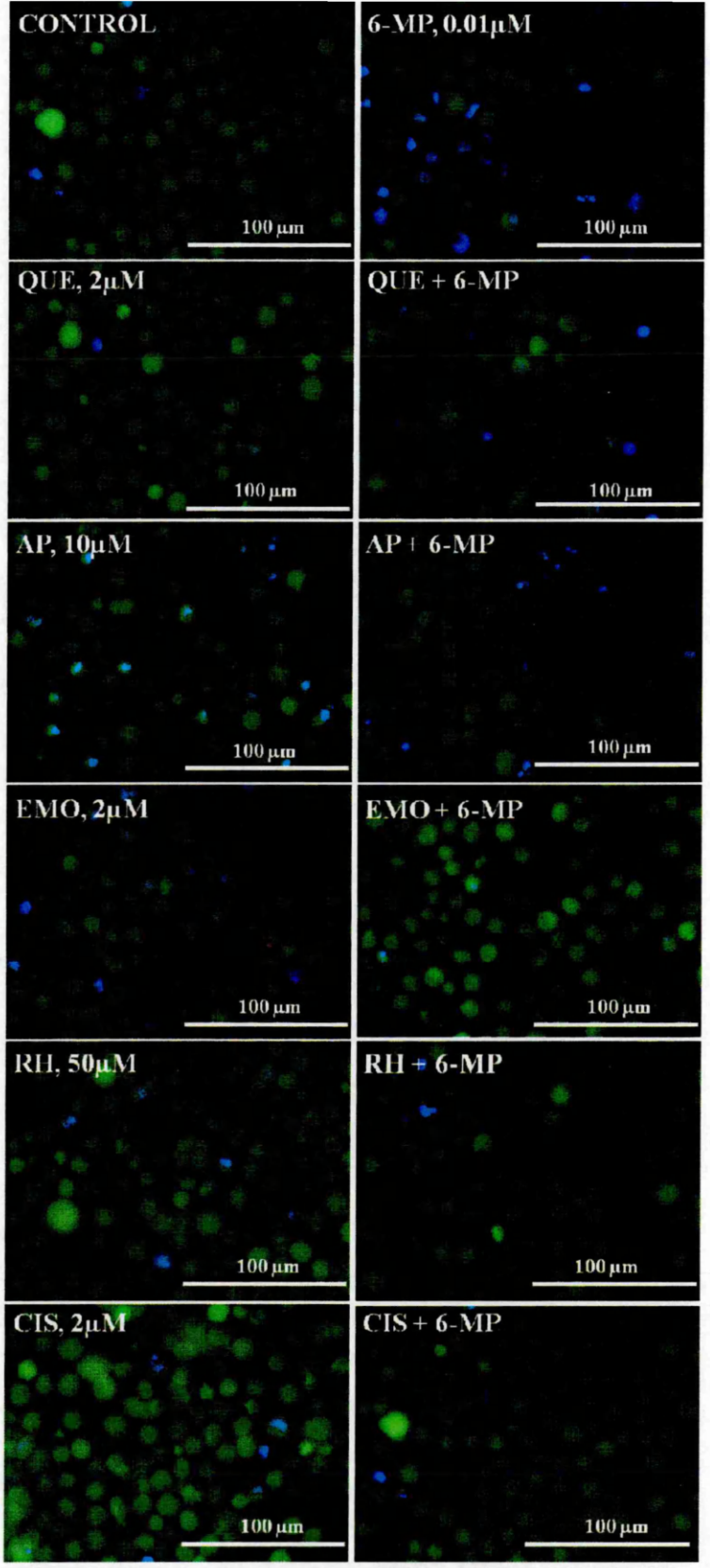


Figure 4.40: An example of fluorescent detection of cellular glutathione (GSH) for THP-1 myeloid leukaemia cells when treated with LSDs of 6-MP and polyphenols (QUE, AP, EMO, RH or CIS) alone and in combination for 24 h; using the CellTracker GSH- Green 5-chloromethylfluorescein diacetate (CMFDA) and blue Hoechst 33342 stains. Cells with green-CMFDA staining indicate the presence of GSH, while cells with blue Hoechst 33342 staining indicate a decrease in GSH. . Scale bar = 100 µm.

4.3.3 Gamma-H2AX (γ-H2AX) Foci Formation in Leukaemia Cells

4.3.3.1 Effect of Polyphenols and Chemotherapies Alone on γ-H2AX Foci Formation

Polyphenols alone caused different capacities to induce the formation of γ-H2AX foci within the leukaemia cell lines (Figure 4.41; 4.45). In particular at the LSD following 24 h treatment, QUE significantly increased the number of γ-H2AX foci in three out of four leukaemia cell lines (JURKAT, CCRF-CEM and THP-1) ($p<0.05$) (Figure 4.41; 4.45). However, AP significantly increased γ-H2AX foci formation in all the leukaemia cell lines ($p<0.05$) (Figure 4.41; 4.45). EMO and RH did cause a significant increase in γ-H2AX foci in the CCRF-CEM lymphoid cell line ($p<0.05$), but did not show any significant effect on the remaining three leukaemia cell lines (JURKAT, THP-1 and KG-1a) ($p<0.05$) (Figure 4.41; 4.45). Whilst, CIS did not cause any significant increase in γ-H2AX foci in any leukaemia cell lines ($p<0.05$) (Figure 4.41; 4.45). DOX, ETP, CSP, MTX and 6-MP when used alone at the LSD for 24 h caused a significant increase in γ-H2AX foci formation in all the leukaemia cell lines ($p<0.05$) (Figure 4.41; 4.45). In contrast, CYCLO when used alone only significant increased γ-H2AX foci formation in CCRF-CEM lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.41; 4.45), and did not significant affect the remaining leukaemia cell lines (JURKAT, THP-1 and KG1a) (Figure 4.41; 4.45).

4.3.3.3 Effects of Topoisomerase Inhibitors in Combination with Polyphenols on γ-H2AX Foci Formation

(A) Effects of Doxorubicin (DOX) in Combination with Polyphenols on γ-H2AX Foci Formation

DOX when used in combination with QUE or AP caused a synergistic increase in γ-H2AX foci ($p<0.05$) in both lymphoid and myeloid cell lines (Figure 4.41 and Table 4.4). Likewise, when DOX was used in combination with EMO, RH or CIS it also caused a synergistic formation of γ-H2AX foci ($p<0.05$), but only in the lymphoid leukaemia cell lines (Figure 4.41 and Table 4.4). In contrast, when DOX was combined with EMO or RH it produced a competitive antagonistic effect, which significantly decreased γ-H2AX foci formation in the myeloid leukaemia cell lines ($p<0.05$) (Figure 4.41 and Table 4.4). When DOX was used combination with CIS it produced an additive effect increasing γ-H2AX foci formation in the myeloid leukaemia cell lines ($p<0.05$) (Figure 4.41 and Table 4.4).

(B) Effects of Etoposide (ETP) in Combination with Polyphenols on γ-H2AX Foci Formation

ETP when used in combination with QUE or AP produced a synergistic increase in γ-H2AX foci formation in all leukaemia cell lines ($p<0.05$) (Figure 4.42 and Table 4.5). Likewise ETP when used in combination with EMO produced a synergistic increase in γ-H2AX foci in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.42 and Table 4.5); and an additive increase in

γ-H2AX foci in the myeloid leukaemia cell lines ($p<0.05$) (Figure 4.42 and Table 4.5). ETP when used in combined with RH or CIS produced a synergistic increase in the formation of γ-H2AX foci in the lymphoid leukaemia cell lines only (Figure 4.42 and Table 4.5). In the myeloid leukaemia cell lines, ETP when used in combination with RH or CIS produced a competitively antagonistic or antagonistic effect significantly decreasing the γ-H2AX foci formation of ($p<0.05$) (Figure 4.42 and Table 4.5).

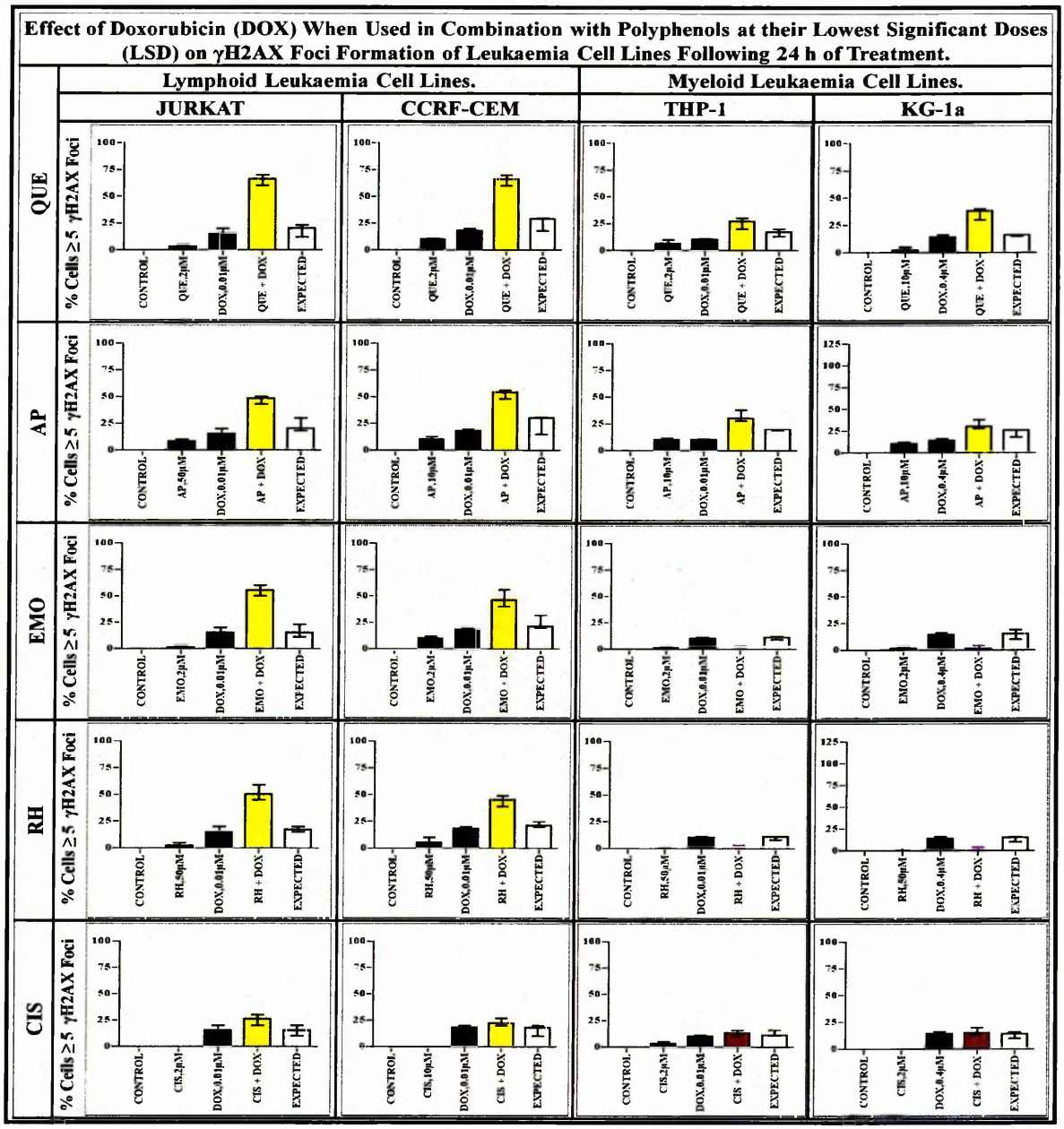


Figure 4.41: The effect of DOX when used in combination with QUE, AP, EMO, RH or CIS on γ-H2AX foci formation (DNA damage marker) in four leukaemia cell lines. This was evaluated by the immunofluorescent staining using Alexa Fluor® 647 Mouse anti-H2AX (pS139). Cells were treated with DOX and polyphenols alone and in combined for 24 h using their LSD. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on γ-H2AX; the coloured bars indicate a significant: additive increase in DNA damage in brown, synergistic increase in DNA damage in yellow, competitive antagonistic decrease in DNA damage in purple and an antagonistic decrease in DNA damage in pink. Statistical signficant was set at $p\leq0.05$ compared to vehicle control and drugs alone.

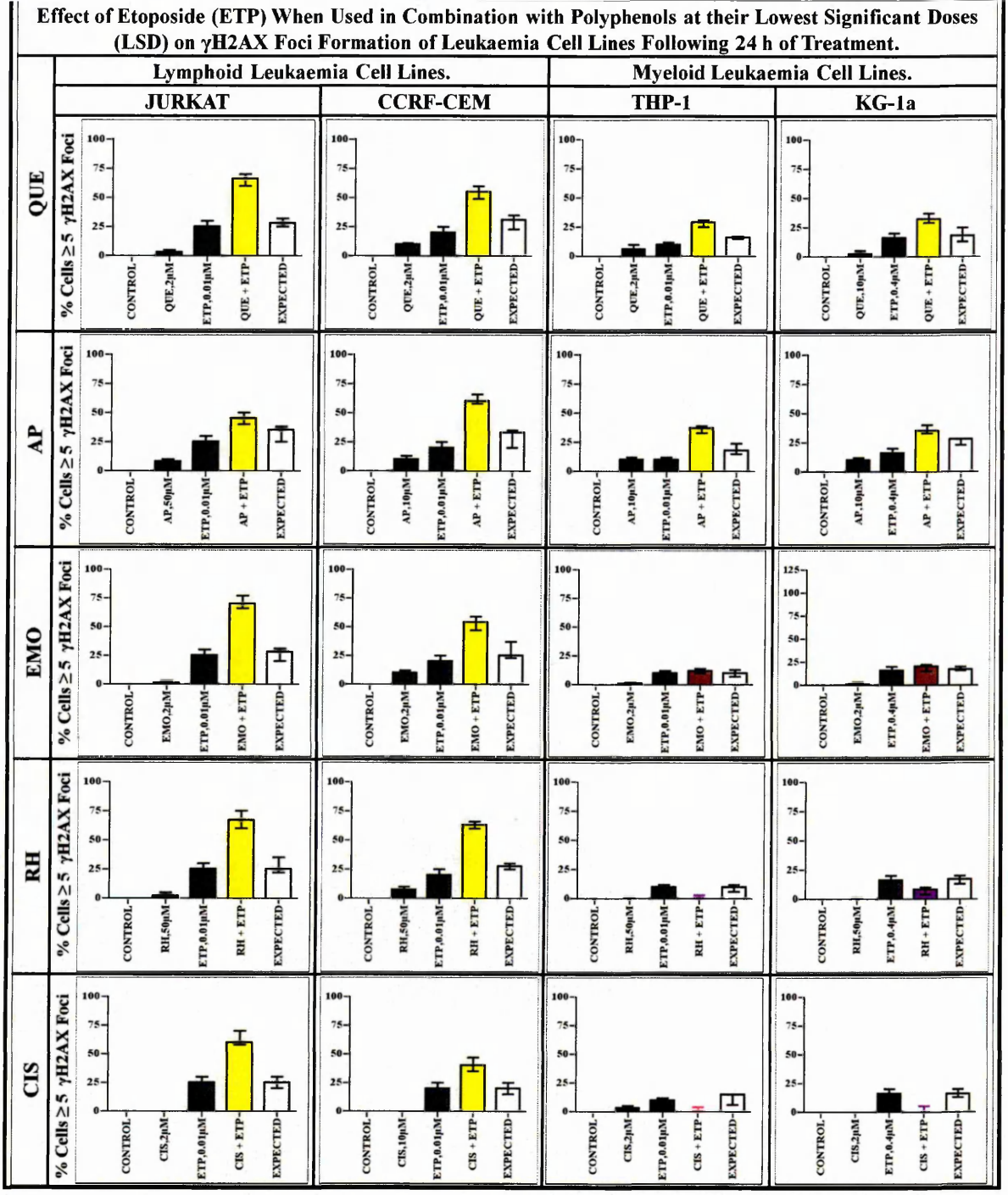


Figure 4.42: The effect of ETP when used in combination with QUE, AP, EMO, RH or CIS on γ-H2AX foci formation (DNA damage marker) in four leukaemia cell lines. This was evaluated by the immunofluorescent staining using Alexa Fluor® 647 Mouse anti-H2AX (pS139). Cells were treated with ETP and polyphenols alone and in combined for 24 h using their LSD. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on γ-H2AX; the coloured bars indicate a significant: additive increase in DNA damage in brown, synergistic increase in DNA damage in yellow, competitive antagonistic decrease in DNA damage in purple and an antagonistic decrease in DNA damage in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

4.3.3.4 Effects of Alkylating Chemotherapy Agents in Combination with Polyphenols on γ-H2AX Foci Formation

(A) Effects of Cyclophosphamide (CYCLO) in Combination with Polyphenols on γ-H2AX Foci Formation

CYCLO when used in combination with AP caused a synergistic increase in γ-H2AX foci formation ($p<0.05$) in all the leukaemia cell lines (Figure 4.43 and Table 4.6). Likewise CYCLO when used in combination with QUE, EMO or RH caused a synergistic increase in γ-H2AX foci formation in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.43 and Table 4.6). In contrast, in the myeloid leukaemia cell lines (THP-1, KG1a); when CYCLO was used in combination with QUE, EMO or RH if caused an competitively antagonistic effect, significantly decreasing the formation of γ-H2AX foci ($p<0.05$) (Figure 4.43 and Table 4.6). Additionally, when CYCLO used in combination with CIS caused a competitive antagonistic effect and significantly decreased the formation of γ-H2AX foci in all leukaemia cell lines ($p<0.05$) (Figure 4.43 and Table 4.6).

(B) Effects of Cisplatin (CSP) in Combination with Polyphenols on γ-H2AX Foci Formation

CSP when use in combination with QUE or AP, caused a synergistic increase in γ-H2AX foci formation in all the leukaemia cell lines ($p<0.05$) (Figure 4.4 and Table 4.7). Additionally, when CSP was used in combination with EMO, RH, or CIS, it caused a synergistic increase in γ-H2AX foci formation, but only in the lymphoid leukaemia cell lines ($p<0.05$) (Figure 4.4 and Table 4.7). In contrast, when CSP was used in combination with EMO, RH, or CIS, in myeloid leukaemia cell lines this caused a competitive antagonistic effect significantly decreasing γ-H2AX foci formation ($p<0.05$) (Figure 4.4 and Table 4.7).

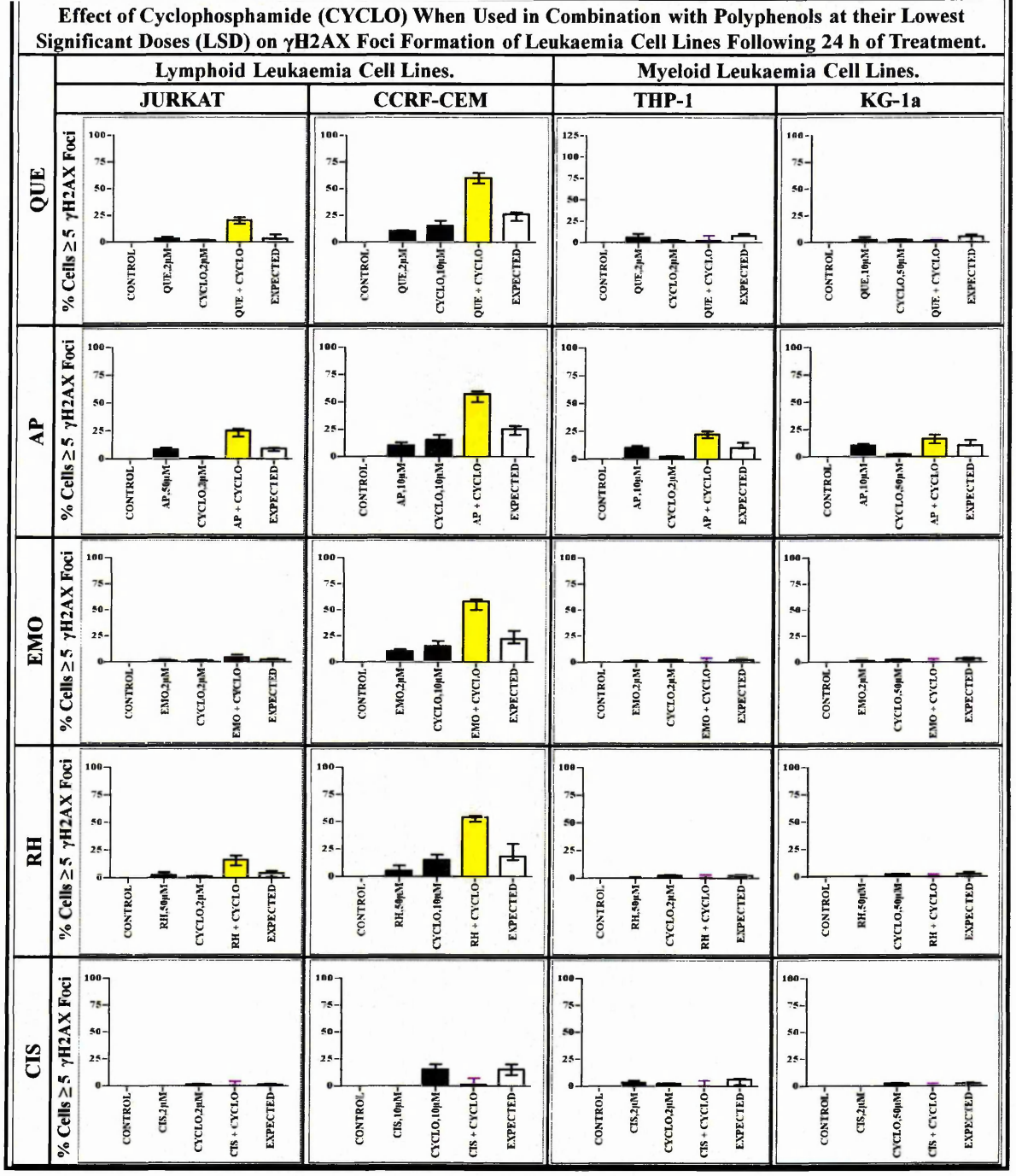


Figure 4.43: The effect of CYCLO when used in combination with QUE, AP, EMO, RH or CIS on γ-H2AX foci formation (DNA damage marker) in four leukaemia cell lines. This was evaluated by the immunofluorescent staining using Alexa Fluor® 647 Mouse anti-H2AX (pS139). Cells were treated with CYCLO and polyphenols alone and in combined for 24 h using their LSD. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on γ-H2AX; the coloured bars indicate a significant: additive increase in DNA damage in brown, synergistic increase in DNA damage in yellow, competitive antagonistic decrease in DNA damage in purple and an antagonistic decrease in DNA damage in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

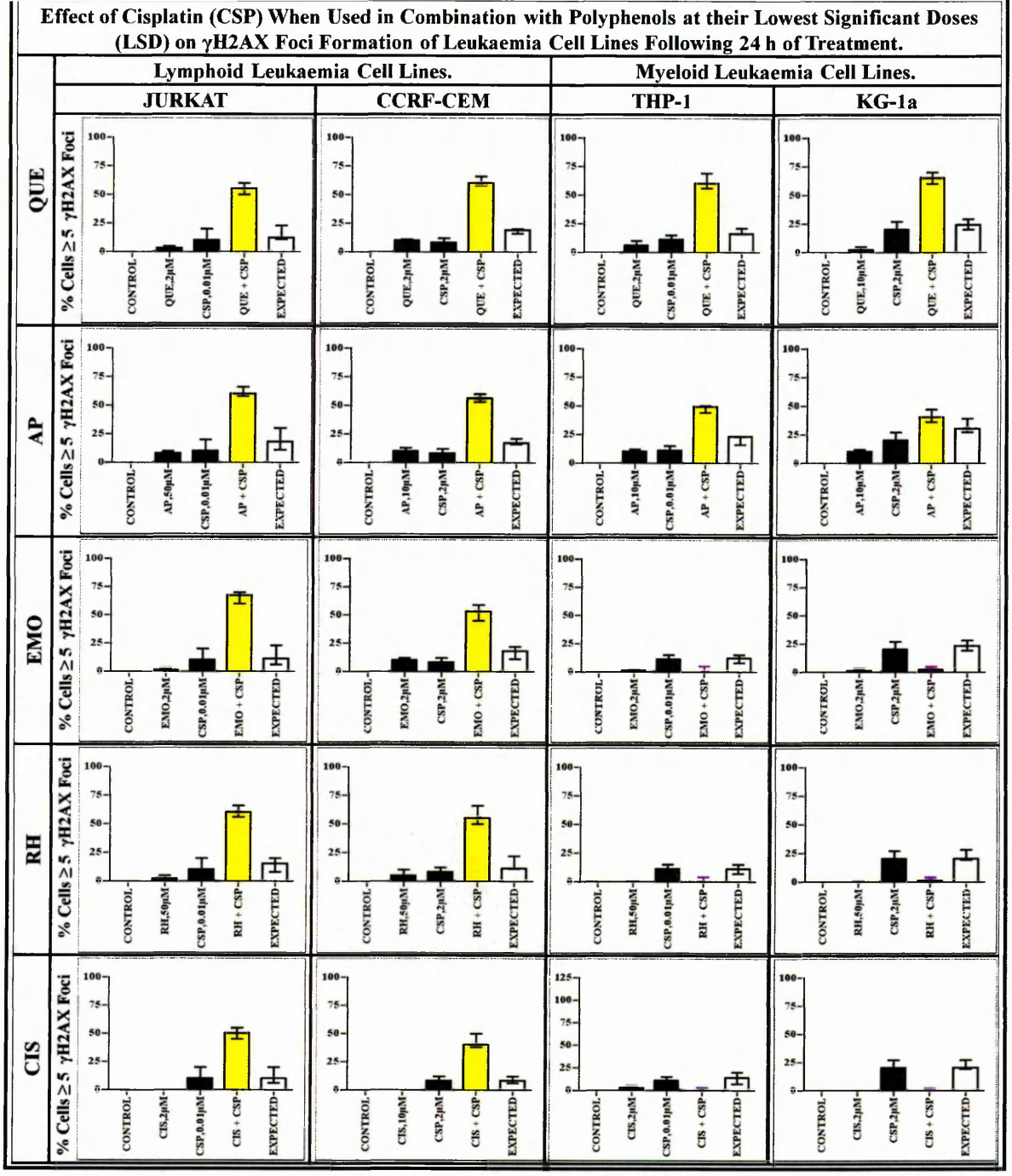


Figure 4.44: The effect of CSP when used in combination with QUE, AP, EMO, RH or CIS on γ-H2AX foci formation (DNA damage marker) in four leukaemia cell lines. This was evaluated by the immunofluorescent staining using Alexa Fluor® 647 Mouse anti-H2AX (pS139). Cells were treated with CSP and polyphenols alone and in combined for 24 h using their LSD. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on γ-H2AX; the coloured bars indicate a significant: additive increase in DNA damage in brown, synergistic increase in DNA damage in yellow, competitive antagonistic decrease in DNA damage in purple and an antagonistic decrease in DNA damage in pink. Statistical signficant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

4.3.3.5 Effects of Anti-Metabolites Chemotherapy Agents in Combination with Polyphenols on γ-H2AX Foci Formation

(A) Effect of Methotrexate (MTX) in Combination with Polyphenols on γ-H2AX Foci Formation

MTX when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) caused a competitively antagonistic and/or antagonistic effect significantly decreasing the formation of γ-H2AX foci in all the leukaemia cell lines ($p<0.05$) (Figure 4.45 and Table 4.8).

(B) Effect of 6-Mercaptopurine (6-MP) in Combination with Polyphenols on γ-H2AX Foci Formation

6-MP when used in combination with all polyphenols (QUE, AP, EMO, RH or CIS) caused a competitive antagonistic and/or antagonistic effects significantly decreasing the formation of γ-H2AX foci in all lymphoid and myeloid leukaemia cell lines ($p<0.05$) (Figure 4.46 and Table 4.9).

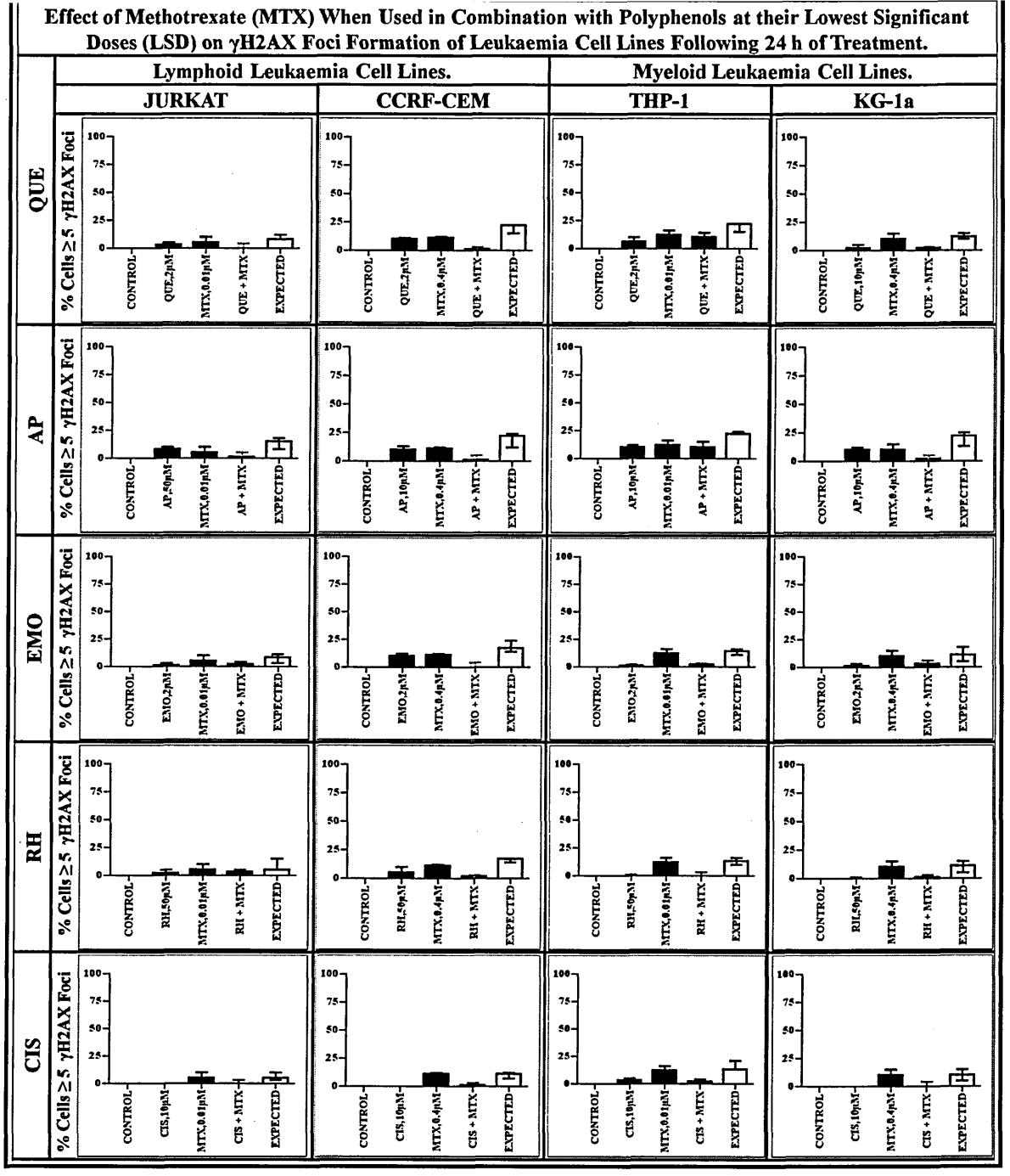


Figure 4.45: The effect of MTX when used in combination with QUE, AP, EMO, RH or CIS on γ-H2AX foci formation (DNA damage marker) in four leukaemia cell lines. This was evaluated by the immunofluorescent staining using Alexa Fluor® 647 Mouse anti-H2AX (pS139). Cells were treated with MTX and polyphenols alone and in combined for 24 h using their LSD. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on γ-H2AX; the coloured bars indicate a significant: additive increase in DNA damage in brown, synergistic increase in DNA damage in yellow, competitive antagonistic decrease in DNA damage in purple and an antagonistic decrease in DNA damage in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

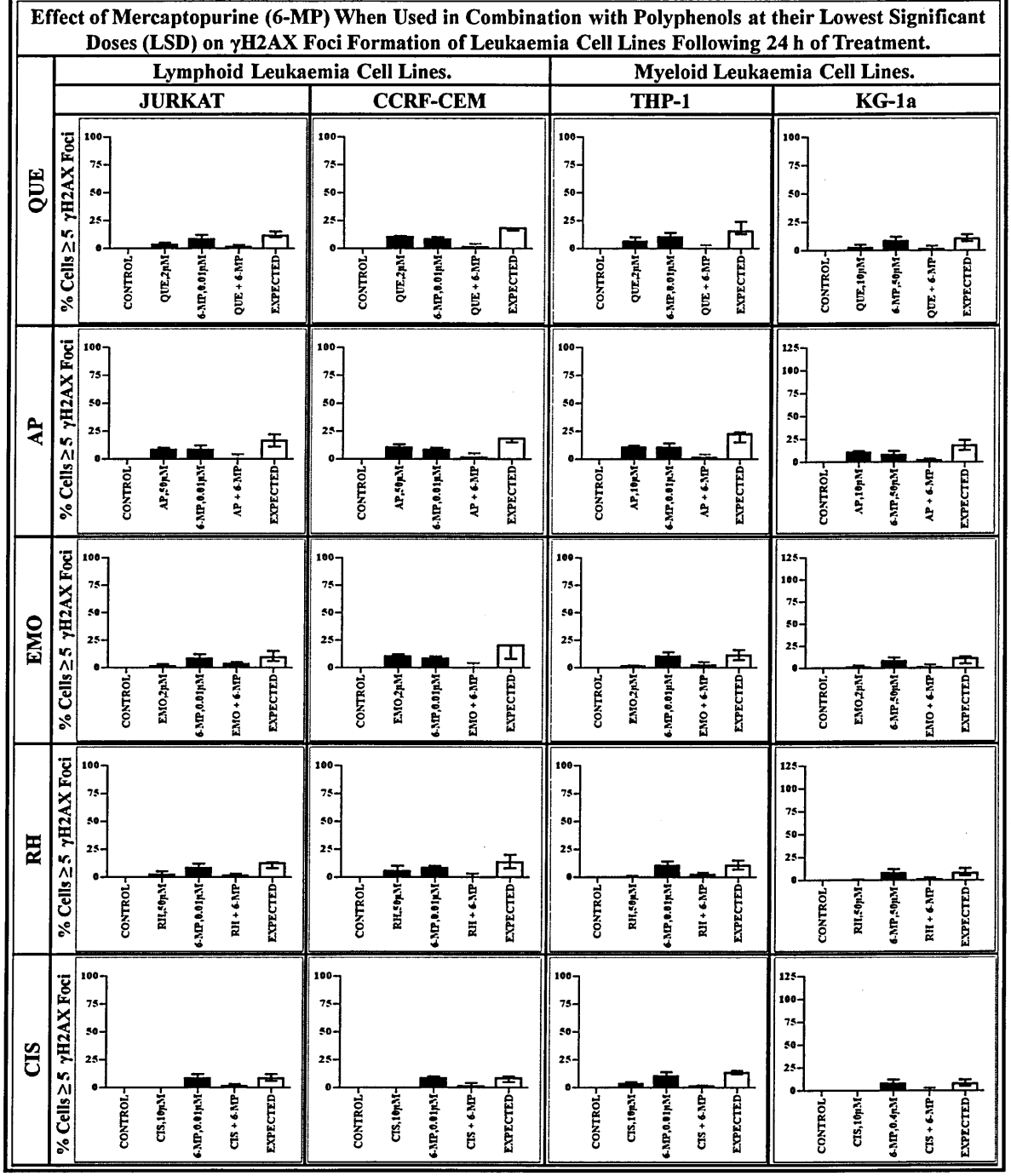


Figure 4.46: The effect of 6-MP when used in combination with QUE, AP, EMO, RH or CIS on γ-H2AX foci formation (DNA damage marker) in four leukaemia cell lines. This was evaluated by the immunofluorescent staining using Alexa Fluor® 647 Mouse anti-H2AX (pS139). Cells were treated with 6-MP and polyphenols alone and in combined for 24 h using their LSD. The data was expressed as medians and ranges (n=4). The black bars show the vehicle controls and treatments alone; the white bar show the expected effect of the combined treatments on γ-H2AX; the coloured bars indicate a significant: additive increase in DNA damage in brown, synergistic increase in DNA damage in yellow, competitive antagonistic decrease in DNA damage in purple and an antagonistic decrease in DNA damage in pink. Statistical significant was set at $p \leq 0.05$ compared to vehicle control and drugs alone.

Assays	Targets	Combination of Doxorubicin and Polyphenol	The Significant Effects of Doxorubicin (DOX) and Polyphenols Combination Treatment on Caspase 8 and 9 Activity, Glutathione (GSH) Levels, H2AX Foci Formation and Cell Cycle Arrest at 24 h in Leukaemia Cell Lines.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Caspase-Glo® 8 Assay	Caspase 8 Activity	DOX + QUE (LSD)	ADD	SYN	ADD	ADD
Caspase-Glo® 9 Assay	Caspase 9 Activity	DOX + QUE (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	DOX + QUE (LSD)	SYN	SYN	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	DOX + QUE (LSD)	SYN	SYN	SYN	SYN
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	DOX + QUE (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	DOX + QUE (LSD)	G_2/M phase	S phase	S & G2/M phases	S & G2/M phases
Caspase-Glo® 8 Assay	Caspase 8 Activity	DOX + AP (LSD)	ADD	ADD	ADD	ADD
Caspase-Glo® 9 Assay	Caspase 9 Activity	DOX + AP (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	DOX + AP (LSD)	SYN	SYN	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	DOX + AP (LSD)	SYN	SYN	SYN	SYN
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	DOX + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	DOX + AP (LSD)	G_2/M phase	S & G2/M phases	S & G2/M phases	S & G2/M phases
Caspase-Glo® 8 Assay	Caspase 8 Activity	DOX + EMO (LSD)	ADD	No Activity	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	DOX + EMO (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	DOX + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	DOX + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	DOX + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	DOX + EMO (LSD)	G_2/M phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	DOX + RH (LSD)	ADD	No Activity	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	DOX + RH (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	DOX + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	DOX + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	DOX + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	DOX + RH (LSD)	G_2/M phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	DOX + CIS (LSD)	ADD	ADD	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	DOX + CIS (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	DOX + CIS (LSD)	SYN	SYN	ADD	ADD
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	DOX + CIS (LSD)	SYN	SYN	ADD	ADD
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	DOX + CIS (LSD)	SYN	SYN	ADD	ADD
Cell Cycle/PI	Arresting Phases	DOX + CIS (LSD)	G_2/M phase	S phase	S phase	S & G2/M phases

Table 4.4: A summary of the effects of doxorubicin (DOX) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on caspase 8 and 9 activity, glutathione (GSH) levels, γ-H2AX foci formation and cell cycle progression (Chapter 3) in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1and KG1a) cell lines at 24 h. Combination treatments were investigated at their lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 4.2.5. SYN = Synergistic, ADD = Additive. NT= non-tested. Cell cycle data summarised to enable comparison to results of γ-H2AX formation.

Assays	Targets	Combination of Etoposide and Polyphenol	The Significant Effects of Etoposide (ETP) and Polyphenols Combination Treatment on Caspase 8 and 9 Activity, Glutathione (GSH) Levels, H2AX Foci Formation and Cell Cycle Arrest at 24 h in Leukaemia Cell Lines.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Caspase-Glo® 8 Assay	Caspase 8 Activity	ETP + QUE (LSD)	ADD	ADD	ADD	ADD
Caspase-Glo® 9 Assay	Caspase 9 Activity	ETP + QUE (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	ETP + QUE (LSD)	SYN	SYN	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	ETP + QUE (LSD)	SYN	SYN	SYN	SYN
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	ETP + QUE (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	ETP + QUE (LSD)	G_2/M phase	S & G2/M phases	S & G2/M phases	S & G2/M phases
Caspase-Glo® 8 Assay	Caspase 8 Activity	ETP + AP (LSD)	ADD	ADD	ADD	ADD
Caspase-Glo® 9 Assay	Caspase 9 Activity	ETP + AP (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	ETP + AP (LSD)	SYN	ADD	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	ETP + AP (LSD)	SYN	SYN	SYN	SYN
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	ETP + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	ETP + AP (LSD)	S phase	S & G2/M phases	S & G2/M phases	S & G2/M phases
Caspase-Glo® 8 Assay	Caspase 8 Activity	ETP + EMO (LSD)	ADD	SYN	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	ETP + EMO (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	ETP + EMO (LSD)	SYN	SYN	ADD	ADD
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	ETP + EMO (LSD)	SYN	SYN	ADD	ADD
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	ETP + EMO (LSD)	SYN	SYN	ADD	ADD
Cell Cycle/PI	Arresting Phases	ETP + EMO (LSD)	S & G2/M phases	S phase	S & G2/M phases	S & G2/M phases
Caspase-Glo® 8 Assay	Caspase 8 Activity	ETP + RH (LSD)	ADD	SYN	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	ETP + RH (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	ETP + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	ETP + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	ETP + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	ETP + RH (LSD)	G2/M phase	G2/M phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	ETP + CIS (LSD)	ADD	SYN	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	ETP + CIS (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	ETP + CIS (LSD)	SYN	SYN	C-ANTG	ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	ETP + CIS (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	ETP + CIS (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	ETP + CIS (LSD)	G2/M phase	S phase	No Arrest	No Arrest

Table 4.5: A summary of the effects of etoposide (ETP) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on caspase 8 and 9 activity, glutathione (GSH) levels, γ-H2AX foci formation and cell cycle progression (Chapter 3) in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1and KG1a) cell lines at 24 h. Combination treatments were investigated at their lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 4.2.5. SYN = Synergistic, ADD = Additive. NT= non-tested. Cell cycle data summarised to enable comparison to results of γ-H2AX formation.

Assays	Targets	Combination of Cyclophosphamide and Polyphenols	The Significant Effects of Cyclophosphamide and Polyphenols Combination Treatment on Caspase 8 and 9 Activity, Glutathione (GSH) Levels, H2AX Foci Formation and Cell Cycle Arrest at 24 h in Leukaemia Cell Lines.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Caspase-Glo® 8 Assay	Caspase 8 Activity	CYCLO + QUE (LSD)	SYN	SYN	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CYCLO + QUE (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CYCLO + QUE (LSD)	SYN	SYN	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CYCLO + QUE (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ-H2AX Foci FORMATION	CYCLO + QUE (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CYCLO + QUE (LSD)	S phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
Caspase-Glo® 9 Assay	Caspase 9 Activity	CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
Alexa Fluor® 647 Mouse anti-H2AX	γ-H2AX Foci FORMATION	CYCLO + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	CYCLO + AP (LSD)	S phase	S phase	S & G_2/M phases	G_0/G_1 phase
Caspase-Glo® 8 Assay	Caspase 8 Activity	CYCLO + EMO (LSD)	ADD	ADD	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CYCLO + EMO (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CYCLO + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CYCLO + EMO (LSD)	SYN	SYN	ANTG	NO EFFECT
Alexa Fluor® 647 Mouse anti-H2AX	γ-H2AX Foci FORMATION	CYCLO + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CYCLO + EMO (LSD)	G_0/G_1 phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	CYCLO + RH (LSD)	ADD	ADD	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CYCLO + RH (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CYCLO + RH (LSD)	SYN	SYN	ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CYCLO + RH (LSD)	SYN	SYN	C-ANTG	NO EFFECT
Alexa Fluor® 647 Mouse anti-H2AX	γ-H2AX Foci FORMATION	CYCLO + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CYCLO + RH (LSD)	G_0/G_1 phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	CYCLO + CIS (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CYCLO + CIS (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CYCLO + CIS (LSD)	ANTG	ANTG	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CYCLO + CIS (LSD)	C-ANTG	C-ANTG	C-ANTG	NO EFFECT
Alexa Fluor® 647 Mouse anti-H2AX	γ-H2AX Foci FORMATION	CYCLO + CIS (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CYCLO + CIS (LSD)	No Arrest	No Arrest	No Arrest	No Arrest

Table 4.6: A summary of the effects of cyclophosphamide (CYCLO) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on caspase 8 and 9 activity, glutathione (GSH) levels, γ-H2AX foci formation and cell cycle progression (Chapter 3) in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1and KG1a) cell lines at 24 h. Combination treatments were investigated at their lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 4.2.5. SYN = Synergistic, ADD = Additive. NT= non-tested. Cell cycle data summarised to enable comparison to results of γ-H2AX formation.

Assays	Targets	Combination of Cisplatin and Polyphenols	The Significant Effects of Cisplatin (CSP) and Polyphenols Combination Treatment on Caspase 8 and 9 Activity, Glutathione (GSH) Levels, H2AX Foci Formation and Cell Cycle Arrest at 24 h in Leukaemia Cell Lines.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Caspase-Glo® 8 Assay	Caspase 8 Activity	CSP + QUE (LSD)	SYN	SYN	SYN	SYN
Caspase-Glo® 9 Assay	Caspase 9 Activity	CSP + QUE (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	CSP + QUE (LSD)	SYN	SYN	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CSP + QUE (LSD)	SYN	SYN	SYN	SYN
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	CSP + QUE (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	CSP + QUE (LSD)	S phase	S phase	G_2/M phase	G_2/M phase
Caspase-Glo® 8 Assay	Caspase 8 Activity	CSP + AP (LSD)	SYN	SYN	SYN	SYN
Caspase-Glo® 9 Assay	Caspase 9 Activity	CSP + AP (LSD)	SYN	SYN	SYN	SYN
GSH-Glo™ Glutathione Assay	GSH Level	CSP + AP (LSD)	SYN	SYN	SYN	SYN
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CSP + AP (LSD)	SYN	SYN	ADD	ADD
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	CSP + AP (LSD)	SYN	SYN	SYN	SYN
Cell Cycle/PI	Arresting Phases	CSP + AP (LSD)	S phase	S phase	G_2/M phase	G_2/M phase
Caspase-Glo® 8 Assay	Caspase 8 Activity	CSP + EMO (LSD)	ADD	ADD	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CSP + EMO (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CSP + EMO (LSD)	SYN	SYN	ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CSP + EMO (LSD)	SYN	SYN	ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	CSP + EMO (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CSP + EMO (LSD)	S phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	CSP + RH (LSD)	ADD	ADD	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CSP + RH (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CSP + RH (LSD)	SYN	SYN	ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CSP + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	CSP + RH (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CSP + RH (LSD)	S phase	S phase	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	CSP + CIS (LSD)	SYN	SYN	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	CSP + CIS (LSD)	SYN	SYN	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	CSP + CIS (LSD)	SYN	SYN	ANTG	ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	CSP + CIS (LSD)	SYN	SYN	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	CSP + CIS (LSD)	SYN	SYN	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	CSP + CIS (LSD)	S phase	S phase	No Arrest	No Arrest

Table 4.7: A summary of the effects of cisplatin (CSP) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on caspase 8 and 9 activity, glutathione (GSH) levels, γ-H2AX foci formation and cell cycle progression (Chapter 3) in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1and KG1a) cell lines at 24 h. Combination treatments were investigated at their lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 4.2.5. SYN = Synergistic, ADD = Additive. NT= non-tested. Cell cycle data summarised to enable comparison to results of γ-H2AX formation.

Assays	Targets	Combination of Methotrexate and Polyphenols	The Significant Effects of Methotrexate (MTX) and Polyphenols Combination Treatment on Caspase 8 and 9 Activity, Glutathione (GSH) Levels, H2AX Foci Formation and Cell Cycle Arrest at 24 h in Leukaemia Cell Lines.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Caspase-Glo® 8 Assay	Caspase 8 Activity	MTX + QUE (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	MTX + QUE (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	MTX + QUE (LSD)	C-ANTG	C-ANTG	C-ANTG	ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	MTX + QUE (LSD)	ANTG	C-ANTG	C-ANTG	ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	MTX + QUE (LSD)	C-ANTG	ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	MTX + QUE (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	MTX + AP (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	MTX + AP (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	MTX + AP (LSD)	C-ANTG	C-ANTG	ANTG	ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	MTX + AP (LSD)	C-ANTG	C-ANTG	ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	MTX + AP (LSD)	ANTG	C-ANTG	ANTG	ANTG
Cell Cycle/PI	Arresting Phases	MTX + AP (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	MTX + EMO (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	MTX + EMO (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	MTX + EMO (LSD)	ANTG	ANTG	ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	MTX + EMO (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	MTX + EMO (LSD)	C-ANTG	ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	MTX + EMO (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	MTX + RH (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	MTX + RH (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	MTX + RH (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	MTX + RH (LSD)	ANTG	C-ANTG	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	MTX + RH (LSD)	C-ANTG	ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	MTX + RH (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	MTX + CIS (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	MTX + CIS (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	MTX + CIS (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	MTX + CIS (LSD)	ANTG	C-ANTG	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	MTX + CIS (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	MTX + CIS (LSD)	No Arrest	No Arrest	No Arrest	No Arrest

Table 4.8: A summary of the effects of methotrexate (MTX) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on caspase 8 and 9 activity, glutathione (GSH) levels, γ-H2AX foci formation and cell cycle progression (Chapter 3) in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1and KG1a) cell lines at 24 h. Combination treatments were investigated at their lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 4.2.5. SYN = Synergistic, ADD = Additive. NT= non-tested. Cell cycle data summarised to enable comparison to results of γ-H2AX formation.

Assays	Targets	Combination of 6-Mercaptopurine and Polyphenols	The Significant Effects of 6-Mercaptopurine (6-MP) and Polyphenols Combination Treatment on Caspase 8 and 9 Activity, Glutathione (GSH) Levels, H2AX Foci Formation and Cell Cycle Arrest at 24 h in Leukaemia Cell Lines.			
			Lymphoid Leukaemia		Myeloid Leukaemia	
			JURKAT	CCRF-CEM	THP-1	KG1a
Caspase-Glo® 8 Assay	Caspase 8 Activity	6-MP + QUE (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	6-MP + QUE (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	6-MP + QUE (LSD)	C-ANTG	C-ANTG	C-ANTG	ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	6-MP + QUE (LSD)	ANTG	C-ANTG	C-ANTG	ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	6-MP + QUE (LSD)	C-ANTG	ANTG	ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	6-MP + QUE (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	6-MP + AP (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	6-MP + AP (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	6-MP + AP (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	6-MP + AP (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	6-MP + AP (LSD)	ANTG	ANTG	ANTG	ANTG
Cell Cycle/PI	Arresting Phases	6-MP + AP (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	6-MP + EMO (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	6-MP + EMO (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	6-MP + EMO (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	6-MP + EMO (LSD)	ANTG	C-ANTG	ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	6-MP + EMO (LSD)	C-ANTG	ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	6-MP + EMO (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	6-MP + RH (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	6-MP + RH (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	6-MP + RH (LSD)	ANTG	C-ANTG	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	6-MP + RH (LSD)	ANTG	C-ANTG	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	6-MP + RH (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	6-MP + RH (LSD)	No Arrest	No Arrest	No Arrest	No Arrest
Caspase-Glo® 8 Assay	Caspase 8 Activity	6-MP + CIS (LSD)	NT	NT	NT	NT
Caspase-Glo® 9 Assay	Caspase 9 Activity	6-MP + CIS (LSD)	NT	NT	NT	NT
GSH-Glo™ Glutathione Assay	GSH Level	6-MP + CIS (LSD)	ANTG	C-ANTG	C-ANTG	C-ANTG
CellTracker GSH-Green CMFDA	% of Cells with GSH Stain	6-MP + CIS (LSD)	ANTG	C-ANTG	C-ANTG	C-ANTG
Alexa Fluor® 647 Mouse anti-H2AX	γ -H2AX Foci FORMATION	6-MP + CIS (LSD)	C-ANTG	C-ANTG	C-ANTG	C-ANTG
Cell Cycle/PI	Arresting Phases	6-MP + CIS (LSD)	No Arrest	No Arrest	No Arrest	No Arrest

Table 4.9: A summary of the effects of 6-mercaptopurine (6-MP) and polyphenols [quercetin (QUE), apigenin (AP), emodin (EMO), rhein, (RH) or cis-stilbene (CIS)] combination treatments on caspase 8 and 9 activity, glutathione (GSH) levels, γ-H2AX foci formation and cell cycle progression (Chapter 3) in two lymphoid leukaemia (JURKAT and CCRF-CEM) and two myeloid leukaemia (THP-1and KG1a) cell lines at 24 h. Combination treatments were investigated at their lowest significant dose (LSD). Note that the combination effects of drugs were determined statistically as described as in Section 4.2.5. SYN = Synergistic, ADD = Additive. NT= non-tested. Cell cycle data summarised to enable comparison to results of γ-H2AX formation.

4.4.1 The Role of Polyphenols on Extrinsic and Intrinsic Apoptosis Pathways: Caspases 8 and 9 Activity

4.4.1.1 Effect Flavonoids Compounds: Quercetin, Apigenin and Chrysin on Caspases Activity

(A) Quercetin

Quercetin (used at its NSD, LSD and IC_{50}) caused a significant increase in caspase 8, 9 and 3 activity in the lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) at 6 and 24 h, and the myeloid leukaemia cell lines (THP-1 and KG-1a) at 24h (Table 4.3) (Chapter 2; Mahbub *et al*, 2013); suggesting that quercetin directly activates both the extrinsic and intrinsic apoptotic pathways.

These finding are consistent with previous works in solid cancer tumour cells, which showed that quercetin treatment induce apoptosis in breast cancer (MDA-MB- 231 following 48 h treatment and MCF-7 following 24 h treatment) (Chien *et al*, 2009; Chou *et al*, 2010), and a recent study using K562 myeloid leukaemia cell line (Chen *et al*, 2015) via both the intrinsic and extrinsic pathways.

In contrast, quercetin treatment of human hepatoma cell lines (HepG2) for 18 h (Serrano *et al*, 2006) and cervical cancer (HeLa) cell lines for 24 h cuases apoptosis via the intrinsic pathway with the activation of caspase 9 and 3, in the absence of caspase 8 (Huang *et al*, 2009; Priyadarsini *et al,* 2010; Zhang *et al*, 2009).

These finding demonstrate that quercetin is capable of inducing apoptosis by the direct activation of caspases, however whether this leads on to the activation of the intrinsic and/or extrinsic pathways varies depending to the cancer cell lines; suggesting that the mechanism of action within the apoptotic pathway may be cell lineage- specific.

(B) Apigenin

Apigenin (used at its LSD, NSD and IC_{50}) caused a significant increase in caspases 8, 9 and 3 activity in both the lymphoid leukaemia (JURKAT and CCRF-CEM) at 6 and 24hrs, and myeloid leukaemia cell lines (THP-1 and KG-1a) at 24 h (Table 4.3) (Chapter 2; Mahbub *et al*, 2013); suggesting once again that apigenin was capable of inducing apoptotic via the extrinsic and intrinsic pathways.

These results seem to be compatible with two earlier studies which found that apigenin treatment for 24 h in breast (MDA-MB-453) and prostate (22Rv1) cancer cell lines causes an increase in caspases 8, 9 and 3, and induced apoptosis via both the intrinsic and extrinsic pathways (Choi and Kim, 2009; Shukla and Gupta, 2008).

There are however two contrasting reports; that show apigenin treatment for 24 h in six leukaemia cell lines (THP-1, U937, HL60, JURKAT, K562 and NIH3t3) (Vargo *et al,* 2006 and one lung cancer cell lines (H460) (Lu *et al*, 2010) causes the activation of intrinsic pathway only; through activation the caspase 9 and 3. There was however no reported increases in caspase 8 activity, or up-regulation of Bax, AIF or cytochrome c. Further to this it was reported that this was accompanied by a decreased in the mitochondrial membrane potential; and an increase in the level of reactive oxygen species (Vargo *et al,* 2006; Lu *et al*, 2010).

These findings demonstrate that apigenin can provide direct activity to promote caspase-dependent-apoptosis of cancer cells.

(C) Chrysin

Chrysin investigated at its IC_{50} doses; significantly increased both caspase 8 and 9 activity in three out of four leukaemia cell lines (JURKAT, CCRF-CEM and THP-1) at 24 h; suggesting that chrysin act via both the intrinsic and extrinsic pathways (Table 4.3) (Mahbub *et al*, 2013; Chapter 2). However chrysin did not affect caspase 8 or 9 activity in KG-1a myeloid cell lines at any studied time points (Table 4.3), consistent with the lack of apoptosis seen (Mahbub *et al*, 2013; Chapter 2). Since KG-1a undergoes apoptosis in other treatment regimes, this suggests that KG-1a specifically do not respond to crysin, unlike other lines tesed.

Only two published works has investigated the pro-apoptotic potential of chrysin in solid tumours cell lines. The first study by Li *et al,* 2011 showed that chrysin treatment for 24 and 48 h up-regulation of caspase 8 and 3 and an induces apoptosis through the extrinsic pathway in gastric adenocarcinoma (SGC-7901), colorectal adenocarcinoma (HT-29 and HCT-116), cervical cancer (HeLa) and hepatoma (HepG2) cells lines (Li *et al,* 2011).

In contrast, a recent study by Samarghandian *et al*, 2014; showed that chrysin treatment for 48 and 72 h induces apoptosis in lung adenocarcinoma epithelial cell lines (A549) through the activation of intrinsic pathways only, via an up-regulation the Bax:Bcl-2 ratio and an increase in caspase 9 and 3 activity (Samarghandian *et al*, 2014).

Based on these earlier studies and the results here it can be concluded that chrysin is capable of inducing apoptosis via caspase activation in cancer cells lines; although the mechanisms of induction is cell type specific cancer cell lines.

4.4.1.2 Effect of Anthraquinones: Emodin, Rhein and Aloe-Emodin on Caspases Activity

Emodin, rhein (when used at their NSD, LSD and IC_{50}) and aloe-emodin (when used at its IC_{50} doses) significantly increased caspase 9 activity; but did not affect caspase 8 activity in three

out of four leukaemia cell lines (Table 4.3); suggesting that the observed apoptosis induction and caspase 3 activation previously shown by these anthraquinones (Mahbub *et al*, 2013; Chapter 2) caused the activation of the intrinsic pathway only. The only exception was seen once again in the KG-1a myeloid cell lines; when treated with emodin failed to increase either caspase 8 or 9 activity at any treatment doses (Table 4.3). This would explain earlier findings in which emodin failed to induction of apoptosis in this cell line (Mahbub *et al*, 2013).

Previous work in cancer cell lines has shown that emodin treatment for 24 and 48 h in human oral squamous cell carcinoma cell lines (SCC-4) (Chen *et al,* 2010), T-lymphocytic leukaemia cell lines (JURKAT) (Wei *et al,* 2009) and murine leukaemia cell lines (WEHI-3) (Chang *et al,* 2011); were shown to induce apoptosis through the production of reactive oxygen species (ROS) and activation of mitochondria-dependent pathways, which subsequently was increased the activity of caspase 9, followed by the activation of caspase 3 and up-regulation the expression of PARP (Wei *et al,* 2009; Chen *et al,* 2010; Chang *et al,* 2011). Thus, suggesting activation of the intrinsic apoptotic pathway only.

A similar effect has been shown by Chen *et al* 2010, in which human oral squamous cell carcinoma cell lines (SCC-4) when treated with either aloe-emodin or rhein for 24 and 48 hr; were shown to induce apoptosis through the production of reactive oxygen species (ROS) and mitochondria-dependent pathway via up regulation the caspase 9 and 3 activity (Chen *et al* 2010). Thus again demonstrating that in this cell line only the intrinsic apoptotic pathway was activated.

This evidence taken together, suggests that anthraquinones specifically targeted the intrinsic apoptotic pathway and activated caspases 9 only in all studied solid tumour and leukaemia cell lines.

4.4.1.3 Effect of Stilbenoid Compounds: Cis-Stilbene and Trans-Stilbene on Caspases Activity

This has been shown here that cis-stilbene and trans-stilbene treatment for 24 h caused a significant increased in caspase 8, 9 and 3 activity within all the lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CE, THP-1 and KG-1a) (Table 4.3) (Mahbub *et al*, 2013; Chapter 2). This study is considered as first work investigating the effect of cis-stilbene and trans-stilbene on initiator caspases in leukaemia; concluding that stilbenoid compounds targeted both the extrinsic and intrinsic pathways to induce apoptosis in leukaemia.

In contrast, previous works in lungs (A549 and CH27) (Lee *et al*, 2004; Weng *et al*, 2009) and prostate (DU145 and PC3) cancer cell lines (Shankar *et al*, 2007) has shown that cis-stilbene and trans-stilbene treatment induce apoptosis via the intrinsic/mitochondrial pathway,

increasing the Bax: Bcl-2 ratio, caspase 9 and 3 activity, but not caspase 8 or subsequent PARP cleavage (Lee *et al*, 2004; Shankar *et al*, 2007; Weng *et al*, 2009). Thus once again it would seem that the mechanism of induction of apoptosis by the stilbenes is cell type specific.

4.4.2 The Role of Polyphenols Alone on Major Intracellular Antioxidant Molecule: Glutathione (GSH)

4.4.2.1 The Basal Levels of Intracellular Glutathione (GSH) Levels in Leukaemia Cell Lines

The lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) had a low basal GSH level when compared to myeloid (THP-1 and KG-1a) and non-tumour cell lines ($CD133^+$ HSC and $CD34^+$ HSC). As polyphenols are thought to reduce GSH levels and make cells more susceptible to apoptosis (Babich *et al*, 2011); this can explained why these cell lines were usually more sensitive to polyphenol treatment, when used either alone or in combined with chemotherapeutic agents.

In contrast, the myeloid leukaemia cell lines (THP-1 and KG-1a) had higher basal GSH levels compared to lymphoid and non-tumour cell lines. So, this can possible explain why these myeloid cell lines were commonly more resistant to polyphenol treatments, either when used alone or in combined with chemotherapy agents. From this it can be concluded that the basal level of glutathione (GSH) in leukaemia cell lines is significantly related to their sensitivity or resistance to polyphenols alone or in combined with chemotherapeutic agents.

These findings are consistent with earlier work by Morales *et al*, 2005 who reported that intracellular glutathione levels play an essential role in determine the cancer cell sensitivity to apoptosis induction by the anti-neoplastic agent such as retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) in melanoma (A375) and leukaemia (JURKAT and CCRF-CEM) cell lines (Morales et al, 2005). In fact, they showed that the higher the intracellular GSH levels in melanoma cell lines (A375) cells the greater the resistance 4-HPR induced apoptosis. Meanwhile the lower the intracellular GSH levels in leukaemia cell lines (JURKAT and CCRF-CEM) the greater the sensitivity these cells hade to 4-HPR induce apoptosis (Morales et al, 2005). Taken together, these results indicated the glutathione content in cancer cells is strongly related and contributed to determining the cancer cells sensitivity or resistance to induce apoptosis by anti-cancer agents; such as polyphenols.

4.4.2.2 Effect Flavonoids Compounds: Quercetin, Apigenin and Chrysin on GSH Levels

(A) Quercetin

Quercetin has been shown here to act as GSH depleting agent for all the leukaemia cell line, when used at both low doses (NSD and LSD) and high doses (IC_{50}) for 24 h (Table 4.8).

However, some researchers have reported that quercetin can exhibit both antioxidant and/or pro-oxidant effects in cancer cell lines depending on the: (1) basal GSH levels in cells (Tsao, 2010; Khan *et al*, 2012), (2) the treatment dose (Scharf *et al*, 2003; Ramos, 2008; Chang *et al*, 2009; Granado-Serrano *et al*, 2012) and/or (3) the treatment duration (Ferraresi *et al*, 2005). However these bimodal effects have observed in solid tumour cancer cells lines; with most work being carried out in hepatoma cell lines (Scharf *et al*, 2003; Ramos, 2008; Chang *et al*, 2009; Granado-Serrano *et al*, 2012); and with very little study being undertaken in leukaemia cells (Cipak *et al*, 2003; Ferraresi *et al*, 2005). In particular, quercetin when used at a low doses this caused an antioxidant effects, increasing GSH levels and reducing ROS generation. However at high doses quercetin causes a pro-oxidant effect, decreasing GSH levels and increasing ROS; which lead to a reduction in cell proliferation and an increase in apoptosis in cell lines of hepatoma (HepG2, Hepa1c1c7 and HA22T/VGH (Scharf *et al*, 2003; Ramos, 2008; Granado-Serrano *et al*, 2012; Ramos, 2008 and Chang *et al*, 2009), laryngeal (HEp2) (Durgo *et al*, 2007); and murine leukaemia cell lines (L1210) (Cipak *et al*, 2003).

Another study in U937 leukaemia cells reported that short term quercetin exposure (for 2, 4 and 6 h) causes an antioxidant effect and increases GSH levels. However, during a more prolonged exposure (for 12 and 24 h) to quercetin this caused a pro-oxidant effects: a depletion of GSH levels, an altered mitochondrial permeability and increased cytochrome c release, the increase production of caspase 9 and 3, S phase cell cycle arrest and apoptosis (Ferraresi *et al*, 2005). This study suggested also that depletion GSH levels can act specifically at an early event in the apoptotic process through the dissipation of the mitochondrial membrane potential (Ferraresi *et al*, 2005).

(B) Apigenin

Similar to quercetin result, apigenin significantly depleted GSH levels when used at all treatment doses both low and high in most of the leukaemia cell lines (JURKAT, CCRF-CEM and THP-1) ($p \leq 0.05$) (Table 4.8). However in the KG-1a myeloid cell lines, there was a differential effect on GSH levels dependent on dose (Table 4.8). In particular at the NSD and LSD for apigenin there was an elevation of GSH levels, while at its IC_{50} dose the GSH levels were depleted (Table 4.8). This effect is not surprising as the KG-1a cell line has the highest basal GSH level of all the studied leukaemia cell lines (Section 4.3.2.1). From this it can be concluded that apigenin can act as the anti- or pro-oxidant depending on basal GSH content and treatment doses used in the KG-1a myeloid cell lines.

Based on earlier works, the effect of apigenin on GSH has been only studied on solid tumour cell lines of the liver (HepG2), lungs (A549) and prostate (PC-3 and 22Rv1) (Ramos, 2008; Kachadourian and Day, 2006 and Shukla and Gupta, 2008). These studied has shown that

apigenin causes a rapid depletion in GSH levels, an increase in ROS generation and an activation of p53. This was followed by a disruption of the mitochondrial membrane potential via a decrease in Bcl-xl and Bcl-2, and an increase in Bax. This subsequent resulted in a cytosolic release of cytochrome c which triggers caspase 9 and 3 activation and apoptosis (Kachadourian and Day, 2006; Shukla and Gupta, 2008; Ramos, 2008).

(C) Chrysin

In this study chrysin was only tested at theIC_{50} which significantly decreased GSH levels all the leukaemia cell lines (Table 4.8). This finding is consistent with previous studies which demonstrated that chrysin could depleted GSH levels in solid tumours cell lines from non-small cell lung cancer (A549, H157, H1975 and H460), and prostate cancer cell lines (PC-3) (Kachadourian and Day, 2006; Kachadourian *et al*, 2007; Brechbuhl *et al*, 2012; Kachadourian and Day, 2006 and Brechbuhl *et al*, 2012). This work reported that chrysin causes a pro-oxidant effect, by directly depleting GSH and subsequently increasing caspase 8 and Bid activity (Kachadourian and Day, 2006; Kachadourian *et al*, 2007; Brechbuhl *et al*, 2012).

4.4.2.3 Effect of Anthraquinone Compounds: Emodin, Rhein and Aloe-Emodin on GSH Levels

In generally, each of the anthraquinone (emodin, rhein and aloe-emodin) caused a significant depletion in GSH levels at their highest treatment doses (IC_{50s}) in all the leukaemia cell lines (Table 4.8). However, emodin and rhein caused a dose-dependent effect on GSH levels (Table 4.8). When used at a low doses (NSD and LSD), emodin and rhein either caused no effect, or depletion in GSH levels (Table 4.8). However in the KG-1a myeloid cell line, which has the highest basal GSH levels, these polyphenols when used at a low dose caused an increase in GSH levels (Table 4.8). From this, it can be suggested that emodin and rhein can act as the anti- or pro-oxidant effects depending on the treatment dose used, the cell type and/or the basal GSH. However it would seem that if these anthraquinone are to be used therapeutically that need to be used a doses around those of the IC_{50} ranges.

This suggestion is confirmed by earlier work which showed that emodin at high dose causes a depletion of GSH levels, an increase ROS generation and apoptosis in gallbladder (SGC996) (Wang *et al*, 2010) and ovarian carcinoma cell lines (COC1) (Ma *et al*, 2014). Both studies specifically stated that emodin requires a high dose to achieve its growth inhibitory effect or induction of apoptosis in SGC996 and COC1 cell lines (Wang *et al*, 2010; Ma *et al*, 2014).

4.4.2.4 Effect of Stilbenoids: Cis-Stilbene and Trans-Stilbene on GSH Levels

Cis-stilbene and trans-stilbene significantly depleted GSH levels at their LSD and IC_{50} treatment doses and did not show any effect at NSD in lymphoid cell lines (JURKAT and CCRF-CEM) (Table 4.8). However, these stilbenoids caused dose-dependent differential effects in the myeloid leukaemia cell lines (THP-1 and KG-1a) (Table 4.8). Specifically, cis-stilbene at the lower doses (NSD and LSD) caused a significant elevation in GSH levels; while at higher doses (IC_{50}) it caused a significant depletion in GSH in both myeloid cell lines (Table 4.8); suggesting this that the action of cis-stilbene on GSH modulation is strongly dependent on doses. Trans-stilbene when used as it IC_{50} dose significantly depleted GSH levels in THP-1 cell line, but surprisingly it was caused an elevation on GSH levels in KG-1a cells (Table 4.8); concluding that the modulating effect of trans-stilbene could be dependent also on doses, cell type and/or the basal levels of GSH.

In summary it would seem that both stilbenoids compounds have a pro-oxidant activity in lymphoid cell lines, while they have both antioxidant and pro-oxidant activity in myeloid cell lines. The results shown that their modulation effects on GSH levels was strongly correlated to doses, cell type and/or basal GSH levels.

4.4.3 The Role of the Selected Polyphenols (Quercetin, Apigenin, Emodin, Rhein and Cis-Stilbene) Alone on DNA Damage

When investigating the effect of polyphenols of DNA damage only the most potent polyphenols were selected (quercetin, apigenin, emodin, rhein and cis-stilbene) and were used at their LSDs. Here it was shown polyphenols had differential capacities to induce DNA (shown as γ-H2AX foci) within the leukaemia cell lines. In particularly, quercetin increased the number of γ-H2AX foci in three out of four leukaemia cell lines; with only the KG1a cell line demonstrating some resistance to DNA damage. However, apigenin was shown to increase γ-H2AX foci formation in all the leukaemia cell lines. Emodin and rhein were less impressive as they only caused an increase in γ-H2AX foci in the CCRF-CEM lymphoid cell line; whilst cis-stilbene did not cause any increase in γ-H2AX foci in any leukaemia cell lines.

As a result, these polyphenols seem to cause different capacities to induce the formation of γ-H2AX foci within the leukaemia cell lines; with the most effective polyphenols being apigenin and quercetin and the less effective being emodin rhein and cis-stilbene.

A few earlier studies have reported that polyphenols such as quercetin, genistein, curcumin and resveratrol could directly induced DNA damage, with it associated increase of γ-H2AX phosphorylation through the activation of the ATM/ATR and CHK1/2 pathway-mediated S or G_2/M cell cycle arrest and apoptosis in solid tumours cell line of the lungs (A549) (Tan *et al*,

2009), ovaries (Ovcar-3 and HO-8910) (Tyagi *et al*, 2005 and Ouyang *et al*, 2009), and pancreas (BxPC-3) (Sahu *et al*, 2009).

Furthermore in a study by Singh *et al*, 2004) it has been reported that when DNA damage is induced by anti-cancer agents and is associated with the elevation in the expression of γ-H2AX levels it is causally linked to ROS generation and the depletion of GSH levels, followed by the activation of S or G_2/M checkpoints and apoptosis. This suggests that differentaial effects seen by polyphenols on DNA damage is strongly likned to GSH levels, and once the GSH level are depleted may there a progression to DNA damge.

4.4.4 The Mechanisms of Action of Toposiomerase Inhibitor Agents in Combination with Polyphenols

Previously, both DOX and ETP have been shown to act synergistically with polyphenols reducing ATP levels and inducing apoptosis with the all selected polyphenols (QUE, AP, EMO, RH or CIS) in lymphoid leukaemia cell lines (JURKAT and CCRF-CEM); and with QUE and AP only in the myeloid leukaemia cell lines (THP-1 and KG-1a) (Chapter 3).

A further study of caspase 8 and 9 activity in each of these toposiomerase inhibitor/ polyphenol combination treatments was caused by a synergetic activation of caspase 9 and additive activation of caspase 8 in all leukaemia cell lines (Table 4.4 and 4.5); suggesting that these combination agents were preferably induced apoptosis through the activation of intrinsic pathways; but can also activate the extrinsic pathway, most likely via p53-mediated death receptor expression. In addition, each of these synergetic combination treatments was associated with a synergetic depletion in GSH levels (Table 4.9 and 4.10), as well as, a synergetic formation of γ-H2AX foci (Table 4.15 and 4.16). In correlation to the previous cell cycle results, all those synergistic combination agents had also an interactive effect on cell cycle which significantly increased the cell accumulation in S phase and/or G_2/M phase; this results can be confirmed by the presence of formation of γ-H2AX foci as showed here which commonly indicated for the presence of DNA damage in particular DSBs (Table 4.15 and 4.16).

Alternatively, DOX and ETP topoisomerase inhibitor agents have shown either additive or antagonistic effects when combined with the EMO, RH or CIS in myeloid leukaemia cell lines (THP-1 and KG-1a) (Chapter 3). In particular, combination agents that had previously additive effects (DOX + CIS) and (ETP + EMO) can be associated also with additive effects on the depletion of GSH levels (Table 4.9 and 4.10) and an increase in γ-H2AX formation (Table 4.15 and 4.16). More importantly, when DOX was combined with EMO or RH, or when ETP was combined with RH or CIS an antagonistic and/or competitive antagonistic elevated in ATP levels and an inhibited the apoptosis was observed in the myeloid cell lines

(THP-1 and KG-1a) (Chapter 3). Interestingly, those antagonistic combination treatments were associated with an elevation in GSH levels (Table 4.9 and 4.10) and no γ-H2AX foci formation (Table 4.15 and 4.16). This may helps to also explain why there was no arrest of the cell cycle following the use of these treatment combinations in the myeloid cell lines.

As a result, the modulation on GSH level and γ-H2AX foci formation seem to play important role in synergetic and antagonistic effects topoisomerase inhibitor agents (DOX and ETP) polyphenols combination treatments in leukaemia cell lines. This suggestion is supported by the finding of a small study looking the effects of doxorubicin with quercetin in human breast cancer cell lines (MCF-7, MDA-MB-231 and MCF-10A). Here it was shown that synergistic inhibition of cell proliferation and induction of apoptosis was through the reduction of cellular GSH levels and its enzyme GST (Staedler *et al,* 2011).

In addition, the synergetic effects of DOX and ETP with polyphenols on apoptosis induction seem to be dependent on the activation of caspase cascades, in particular caspase 9 and 3 through the intrinsic pathway. This is very compatible with finding by Wang *et al,* 2012 which showed that doxorubicin when combined with quercetin causes synergistic induction of apoptosis through the accumulation of p53, and the activation of the intrinsic apoptotic pathway associated with the activation of caspase 9 and 3 in human hepatoma cell lines (SMMC7721 and QGY7701) (Wang *et al,* 2012).

Here for the first time we have shown the exact mechanisms of synergism and antagonism of topoisomerase inhibitor agents DOX and ETP when combined with QUE, AP, EMO, RH or CIS based on investigation the GSH levels, caspases cascades and γ-H2AX foci formation within the lymphoid and myeloid leukaemia cell lines.

4.4.5 The Mechanisms of Action of Alkylating Agents in Combination with Polyphenols

The alkylating agents CSP and CYCL have were the next most effective chemotherapeutics to interact with the polyphenols in leukaemia cell lines (Chapter 3). CSP and CYCLO demonstrated a potent synergistic effect when combined with 4 out of 5 polyphenols on ATP levels, apoptosis and cell cycle in the lymphoid cell lines (JURJAT and CCRF-CEM) (Chapter 3). However, they had a differential effects either synergistic or additive or antagonistic effects when combined with the 5 polyphenols (QUE, AP, EMO, RH, or CIS) in myeloid leukaemia cell lines (THP-1 and KG-1a) (Chapter 3).

4.4.5.1 Cisplatin (CSP) in Combination with Polyphenols

CSP was shown to synergistically reduced ATP levels and induced apoptosis when combined with all the selected polyphenols (QUE, EMO, RH or CIS); and additively interacted with AP

within the lymphoid leukaemia cell lines (Chapter 3). Similarly, CSP had a synergistic effect when combined with QUE and an additive effect when combined with AP in myeloid leukaemia cell lines (Chapter 3). Mechanistically CSP when combined with QUE, AP or CIS caused a synergistic activated both caspase 8 and 9 (Table 4.7); suggesting an activation of both the extrinsic and intrinsic apoptotic pathways all the leukaemia cell lines. While when CSP was combined with EMO or RH there was an additive activated the caspase 8 and a synergistic activated the caspase 9 in lymphoid leukaemia cell lines (Table 4.7); indicating that these combinations preferentially induced apoptosis by the intrinsic pathway, but could also activate the extrinsic pathway. Furthermore, all these synergetic combination agents were linked with a synergetic depletion in GSH levels (Table 4.12), as well as, with a synergetic formation of γ-H2AX foci in all the leukaemia cell lines (Table 4.18).

These results are supported by earlier work in which cisplatin was combined with quercetin (Li *et al,* 2014), apigenin (Chan *et al*, 2012) or emodin (Wang *et al*, 2010; Ma *et al*, 2014) and caused a synergistic inhibition of cells proliferation, an induction of apoptosis through the elevation of intracellular ROS and the reduction GSH levels and down regulation of MRP1 expression. This caused mitochondrial transmembrane potential dissipation and activated of the intrinsic apoptotic pathway via releasing of cytochrome c, down-regulation of Bcl-X(L) and up-regulation Bax, followed by caspase 9 and 3 activation and apoptosis in ovarian cancer cell lines (Li *et al,* 2014; Ma *et al*, 2014), gallbladder (SGC996) (Wang *et al*, 2010) and head and neck squamous carcinoma (SCC25) (Chan *et al*, 2012).

However in the myeloid cells when CSP was combined with EMO, RH or CIS this caused an antagonistic and/or completive antagonistic effects on ATP levels, apoptosis and cell cycle (Chapter 3). These antagonistic effects are caused by elevated GSH levels, which were significantly greater than those caused by the CSP or the polyphenols alone and/or the vehicle controls (Table 4.12). This lead to the suggestion the elevated GSH in the myeloid cells prevents a reduction of ATP levels and the induction of apoptosis. In addition, the combination of CSP with EMO, RH or CIS in the myeloid also had no γ-H2AX foci formation, and hence do DNA damage; which is also reflected in the lack of any effect of this treatment combination on progression of the cell cycle in the myeloid leukaemia cell lines (Table 4.18).

4.4.5.2 Cyclophosphamide (CYCLO) in Combination with Polyphenols

CYCLO demonstrated before a potent synergistic effect when combined with four out of five polyphenols (QUE, AP, EMO or RH) in the lymphoid leukaemia cell lines and with AP only in myeloid leukaemia cell lines by inhibition of ATP levels, induction of apoptosis via increased the caspase 3 activity and arrest the cell cycle (Chapter 3).

In the lymphoid leukaemia cell lines the synergetic induction of apoptosis by CYCLO when used in combined with QUE or AP was caused by the synergetic activation of both caspase 8 and 9; whilst when combined with EMO or RH this was caused by the synergetic activation of caspase 9 only. Meanwhile, in myeloid leukaemia cell lines the combination of CYCLO with AP caused the synergic induction of apoptosis via the activation of both caspase 8 and 9. These results show the combination of CYCLO with QUE or AP caused by the activation of both the extrinsic and intrinsic pathways; whilst the combination of CYCLO with EMO or RH is caused by the activation of the intrinsic pathway in leukaemia cells. Additionally, all those synergetic combination agents were associated with a synergetic depletion in GSH levels (Table 4.11), as well as, with a synergetic formation of γ-H2AX foci (Table 4.17), a reduction in ATP levels, induction of apoptosis and arresting the cell cycle (Chapter 3) in all the leukaemia cell lines.

Alternatively, CYCLO has been found to have an antagonistic and/or competitive antagonistic effect when combined with QUE, EMO, RH or CIS in myeloid leukaemia cell lines; which showed significant elevation on ATP levels, inhibition on apoptosis and no effect on cell cycle progression (Chapter 3). Here, it was found that antagonistically and/or competitive antagonistically effect was linked to a significant elevation in GSH levels, as well as, a lack of γ-H2AX foci in myeloid leukaemia cell lines. This may explain why there was no apoptosis or accumulation of cells at any phases of cell cycle in myeloid cell lines when treated with combination of CYCLO with QUE, EMO, RH or CIS (Chapter 3).

There are two previous studies that show that the efficacy of CYCLO can be differentially modulated by the natural agents such as curcumin and resveratrol in human breast cancer cell lines (MCF-7, MDA-MB-231, and BT-474) (Somasundaram et al, 2002; Singh et al, 2011). They concluded that the CYCLO activity was inhibited by curcumin, while it was enhanced by resveratrol although the actual mechanism of action was not reported. This study for the first time showed that the activity of CYCLO could be modulated by natural agents like polyphenols-dependent on their conjugation and interaction with the GSH, as well as, on the GSH content the basically present in cancer cells.

4.4.6 The Mechanisms of Action of Anti-Metabolites Agents in Combination with Polyphenols

Previously the anti-metabolites agents MTX and 6-MP when combined with all the selected polyphenols (QUE, AP, EMO, RH or CIS) have been caused antagonistic and/or competitive antagonistic effects on ATP, apoptosis and cell cycle in both the lymphoid and myeloid leukaemia cell lines (JURKAT, CCRF-CEM, THP-1, KG-1a) (Chapter 3). This study showed the antagonism of MTX and 6-MP by polyphenols was significantly linked with an elevation in GSH level when compared to drugs alone (Table 4.13 and 4.14) and absence the formation

of γ-H2AX (Table 4.19 and 4.20) in all the leukaemia cell lines. Actually, this is first work showed the effect of MTX and 6-MP in combined with polyphenols and their mechanisms of action within different type of leukaemia cell lines.

4.5 Conclusion

All eight polyphenols (quercetin, apigenin, chrysin, emodin, rhein, aloe-emodin, *cis*-stilbene and *trans*-stilbene) were previously shown to induce caspase 3 activity and morphological changes associated with apoptosis all leukaemia cell lines (Mahbub *et al*, 2013; Chapter 2). Here were have shown that the flavonoids (quercetin, apigenin, chrysin) and stilbenoids (cis-stilbene and trans-stilbene) (using the IC_{50} dose as for 24 h) increase both caspase 8 and 9 activity, thus causing activation both the intrinsic and extrinsic apoptotic pathways. The anthraquinones (emodin, rhein, aloe-emodin), however only up-regulated the caspase 9 activitythus working via the intrinsic pathway only.

When considering GSH levels it has been observed here that the basal level of GSH in leukaemia cell lines is related and contributed to their sensitivity or resistance to polyphenols treatment. The lymphoid leukaemia cell lines had the lowest basal GSH levels, whilst the myeloid leukaemia had higher levels of GSH comparable with or greater than that of non-tumour control cells. The KG-1a myeloid cell lines had the highest GSH levels, which may explain why this cell type was considered as the most resistant to polyphenol treatments. In this regard, our earlier work reported that myeloid leukaemia cell lines specifically the KG-1a cells were most resistant cell lines to the most of polyphenolic treatments (quercetin, chrysin, emodin, aloe-emodin, *cis*-stilbene treatments); while lymphoid leukaemia cell lines (JURKAT and CCRF-CEM) were the most sensitive leukaemia cell lines to polyphenol treatment (in particular to quercetin, chrysin, apigenin, emodin, aloe-emodin, *cis*-stilbene treatments) (Mahbub *et al*, 2013). When polyphenols were investigated at their high doses (IC_{50}) GSH levels were depleted. It is thought that this depletion in GSH it seems the major stimulus for caspase 8 and/or 9 activities and the induction of apoptosis in all leukaemia cell lines at these treatment doses at 24 h. The only exception was seen following stilbene treatment in the myeloid cell lines; in which elevated basal GSH levels could not be depleted by the IC_{50} treatment doses at 24h. When examining the effects of the most potent polyphenols on DNA damage (quercetin, apigenin, emodin, rhein and cis-stilbene) it has been shown that selected polyphenols have differential capacities to induce DNA damage in the leukaemia cell lines: apigenin and quercetin were the most effective, whilst emodin and rhein were least effective; whilst cis-stilbene had no effect.Futhermore, this study has concluded that the synergistic induction of apoptosis observed following the combination of chemotherapeutic agents with quercetin, apigenin or cis-stilbene was mainly caused by the activation of both intrinsic

apoptotic pathway through the up-regulation of caspase 9 and the extrinsic apoptotic pathway via the up regulation of caspase 8; while with emodin and rhein was specifically caused by the intrinsic apoptotic pathway via the activation of caspase 9 only within the lymphoid and myeloid leukaemia cell line.

Here it was found that the synergistic or antagonistic effects observed following the combination of chemotherapies agents and polyphenols were strongly dependent on the modulation of the glutathione levels in association with the formation of γ-H2AX nuclear foci in leukaemia cell lines. In conclusion, it was found the depletion of GSH levels and formation of γ-H2AX foci were significantly associated with the combination of chemotherapies and polyphenols that had previously synergetic inhibition on ATP levels, induction of apoptosis, and accumulation of cells at S and/or G_2/M phases. On the other hand, it was observed that the elevation of GSH levels and lack of γ-H2AX foci was linked with the combination of chemotherapies and polyphenols that had earlier antagonistic or competitive antagonistic effects on ATP, apoptosis and cell cycle. As a result the sensitivity or resistance of leukaemia cells to the combination of chemotherapeutic agents with polyphenols is modulated, either by increasing or decreasing the intracellular glutathione contents, respectively. Actually, our data provide new and interesting information on the role of intracellular GSH in leukaemia cells sensitively or resistance to the apoptosis by polyphenol and chemotherapeutic agent combinations. Firstly, GSH depletion could be useful to increase the therapeutic efficacy of cancer treatment combining the polyphenols with chemotherapeutic drugs. Secondly, a polyphenols/chemotherapy agent seems to be more effective to cells with low GSH content such as lymphoid cells, while less effective to cells with high GSH content such myeloid cells. Finally, cancer cell type and their basal level of GSH specificity should be taken into account when designing new clinical chemotherapy approaches using combinations of polyphenols.

Chapter 5
General Discussion.

5.1 Key Findings

5.1.1 The Effect of Polyphenols Treatment on Leukaemia Cell Lines

This study demonstrated for the first time the specific IC_{50} and AP_{50} values at 24 h for eight polyphenols on four lymphoid and four myeloid leukaemia cells lines, together with normal haematopoietic control cells. This enabled a direct comparison of the anti-proliferative and apoptotic effects of each polyphenol, and rank then in order of effectiveness from the most to least effect: emodin > quercetin > *cis*-stilbene > apigenin ≥ rhein > aloe-emodin ≥ *trans*-stilbene ≥ chrysin. This ranking did vary between individual cell lines. Polyphenols with similar molecular structures such as emodin and aloe-emodin had differential effects on leukaemia cells. The effectiveness of polyphenols was lineage dependent suggesting that polyphenols molecular mechanism of action may vary between cell lineages. Lymphoid cell lines were usually more sensitive to polyphenol treatment than myeloid cell lines. However, the most resistant myeloid (KG-1a and K562) cell lines, did still respond to emodin and quercetin treatment even when treated at low micromolar levels. These differential sensitive patterns seen within myeloid and lymphoid cell lines could be due to different cellular origin, genetic abnormalities and/or expression of differential proteins by different types of cells. However when considering cell-cycle arrest, the majority of polyphenols induce G_0/G_1 arrest, suggesting that they may inhibit CDK4 and/or CDK6 related pathways. This study also showed that the effect of the polyphenols was always greater in the leukaemia cells than the non-tumour blood progenitor cells.

The findings here together with earlier works on polyphenols in solid tumours suggested that polyphenols are promising anti-cancer agents, as they are capable of inhibiting cell proliferation, inducing cell cycle arrest and apoptosis; without causing any toxicity to normal cells (Dai *et al*, 2013; Han *et al*, 2007; Jaganathan *et al*, 2009; Patel *et al,* 2007; Sharif *et al*, 2010; Shukla *et al*, 2010). This study demonstrated that all eight polyphenols induced caspase 3 activity and caused apoptotic morphological changes in all leukaemia cell lines. Quercetin, apigenin, chrysin, cis-stilbene and trans-stilbene induced caspases 8 and 9 activity, whilst, emodin, rhein and aloe-emodin caused the activation of caspase 9 alone in all leukaemia cell lines. From the findings here in leukaemia cell lines, together with earlier studies on solid tumours cells, it can be demonstrated that polyphenols can induce apoptosis through the differential mechanisms (Dai *et al*, 2013; Han *et al*, 2007; Jaganathan *et al*, 2009; Patel *et al,* 2007; Sharif *et al*, 2010; Shukla *et al*, 2010).

Most polyphenols when used at their IC_{50} doses for 24 h caused a decrease of basal GSH levels. This depletion in GSH seems to be the major stimulus for caspase 8 and/or 9 activities and the induction of apoptosis in all leukaemia cell lines. The only exception was seen following

stilbene treatments (cis-stilbene and trans-stilbene) in the myeloid cell lines; in which there was an elevated basal GSH levels.

These findings are supported by studies in solid cancer cell lines in which GSH depletion was strongly correlated with the induction of apoptosis; and believed to regulate both the extrinsic and intrinsic apoptotic signalling cascades at distinct checkpoints (Franco *et al*, 2007; Franco and Cidlowski, 2009; Ortega *et al*, 2011; Traverso *et al*, 2013; Irwin *et al*, 2013; Rocha *et al*, 2014).

Polyphenols when used at their lower doses (NSD or LSD) caused differential effects on GSH levels. Earlier studies have reported that high basal GSH level in cancer cells is associated with an increased cell proliferation and antioxidant capacity, preventing oxidative stress, DNA repair and inhibiting cell death process; and as a result leads to resistance to cancer treatments (Abdalla, 2011; Traverso *et al*, 2013). Indeed, this study demonstrated that lymphoid cell lines (JURKAT and CCRF-CEM) had low basal GSH levels, compared to the myeloid cell lines (THP-1 and KG-1a); and as a result the lymphoid cell lines were more susceptible of polyphenol treatment; whilst the myeloid cell lines were more resistant. Thus it would seem that GSH levels contributed to the sensitivity or resistance to polyphenols treatment in leukaemia cell lines. Interestingly, KG-1a myeloid cell lines had the highest basal GSH levels, which may explain why this cell line was considered as the most resistant to most polyphenol treatments (quercetin, chrysin, emodin, aloe-emodin, and *cis*-stilbene treatments).

The most effective polyphenols (quercetin, apigenin, emodin, rhein and *cis*-stilbene) were also shown to induce DNA damage (measured as γ-H2AX foci); and thus drive apoptosis. Indeed many studies have reported that DNA damage induces cell cycle arrest and/or apoptosis and that DNA damaging agents make good anti-cancer therapies (Kuo and Yang, 2008; Rajendran *et al*, 2011). Here, apigenin and quercetin were the most effective polyphenols at inducing DNA damage, whilst emodin and rhein were least effective, with cis-stilbene having no effect at all. This suggests that the induction of apoptosis caused by these polyphenol is not always driven by DNA damage.

5.1.2 The Effect of Polyphenols and Chemotherapy Combination Treatments on Leukaemia Cell Lines

Here it was demonstrated that the combination of the five most effective polyphenols (quercetin, apigenin, emodin, rhein and *cis*-stilbene) differentially modulated the efficacy of nine chemotherapy agents; producing either synergistic, additive or competitive antagonistic/antagonistic effects on ATP levels, apoptosis and cell cycle arrest within the lymphoid and myeloid leukaemia cell lines. These differential effects were dependent on the type of chemotherapy or polyphenol and/or cell lineage being treated.

The most effective chemotherapeutics to interact with the polyphenols were doxorubicin, etoposide and cisplatin, followed by cyclophosphamide. In general it was shown that when these chemotherapeutic agents when combined with each polyphenols caused a synergistic affects in the lymphoid leukaemia cell lines (JURKAT and CCRF-CEM). The only exception was seen when cyclophosphamide was combined with cis-stilbene, this caused an antagonistic effects in the lymphoid cell lines. In contrast however, in the myeloid cell lines (THP-1 and KG-1a), the polyphenol/chemotherapy combination treatments caused a differential effects, producing either synergistic or additive or antagonistic effects.

The results of this study suggest that the use of polyphenols in combination with standard chemotherapy agents such as doxorubicin, etoposide, cisplatin, and cyclophosphamide is most effective in lymphoid cell lines. Most importantly among those polyphenols investigated, quercetin was the most interacted polyphenols with doxorubicin, etoposide and cisplatin; whilst apigenin was the most interacted polyphenols with cyclophosphamide; and this combination seem to be very promising for the treatment of both lymphoid and myeloid leukaemia cells.

These results are consistent with earlier work in which the activity of doxorubicin or cisplatin are strongly enhanced by quercetin in breast cancer (MCF-7, MDA-MB-231, and MCF-10A) (Staedler *et al,* 2011), hepatoma (SMMC7721 and QGY7701) (Wang *et al,* 2012), small cell lung cancer (SW1271), pulmonary adenocarcinoma (A549) (Borska *et al*, 2004), non-small cell lung carcinoma (NCL-H-520) (Kuhar *et* al, 2006), laryngeal carcinoma (Hep-2) (Kuhar *et* al, 2007), human malignant mesothelioma (SPC212 and SPC111) (Zergeroglu *et al*, 2010) and ovarian cancer (A2780) cell lines (Meher *et al,* 2011). Considering all these results together, it would seem that the activity of doxorubicin, etoposide, cisplatin and cyclophosphamide are influenced by the polyphenol type and the lineage of the cells being treated.

In contrast, in this study for the first time it has been shown that the combination of anti-metabolites chemotherapy agents such as methotrexate and 6-mercaptopurine when used in combination with the investigated polyphenols produced an competitive-antagonistic and/or antagonistic effects on ATP levels, apoptosis and cell cycle progression in both the lymphoid and myeloid leukaemia cell lines. This contradicts the finding of previous studies in which it has been suggest that the activity of chemotherapeutic agents is enhanced by polyphenols. There are only a few studies which demonstrated inhibitory actions of polyphenols on chemotherapeutic agents, which was attributed to the polyphenols chemical structure (Golden *et al,* 2009; Kim *et al,* 2009; Perrone *et al,* 2009), administration dose (Li *et al,* 2014) or the polyphenols anti-oxidant capacity (Somasundaram *et al,* 2002; Cipak *et al,* 2003; Kim *et al,* 2009; Davalli *et al,* 2012).

The exact mechanism by which polyphenols can enhance or inhibit the action of these chemotherapeutics is unknown. Here it has been shown however that is it is strongly correlated with modulation of the cellular GSH levels.

In particular, the synergistic effects from the combination of polyphenols and chemotherapy agents possibly occurred via a direct activation of extrinsic and/or intrinsic apoptotic pathways, which was associated with a depletion of GSH levels and elevation in levels of DNA damage.

Alternatively, the antagonistic effects produced by the combination of polyphenols and chemotherapy agents (e.g. QUE, AP, EMO, RH or CIS in combination with methotrexate and 6-mercaptopurine) were commonly associated with an elevation of GSH levels and a lack of γ-H2AX foci formation. This finding is importance as this suggests that polyphenols can inhibit the action of standard chemotherapy such as methotrexate and 6-mercaptopurine; and that patients taking these drugs should be careful about their consumption of polyphenol rich foods. Indeed this leads to the suggestion that the use of methotrexate and 6-mercaptopurine therapeutically should be taken alongside a polyphenol free diet.

5.2 Future Directions

In order to test the efficiency and safety of polyphenols alone and in combined with chemotherapeutic agents; further *in vitro* and *in vivo* research is required. These phases of study are necessary prior to clinical approval and the used of these combination treatments in leukaemia patients.

5.2.1 Elucidate the Molecular Mechanisms of Polyphenols

Experimental data obtained from this study has indicated that polyphenols induced apoptosis through caspase activation and arrested cell cycle at different phases in leukaemia cell lines. Further work is required to further elucidate the mechanism of action of polyphenols and chemotherapy agents at a molecular level in leukaemia cell lines. For this it would be necessary to: (1) Investigate whether polyphenols have ability to regulate the key pro-apoptotic proteins such as Bid, Bim, Noxa, Puma, Bad, Bax, Bak etc and anti-apoptotic proteins such as Bcl-2, Bcl-x, Bcl-x_L, Bcl-x, etc (Section 1.6.3). This could be investigated by western blot analysis using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, US). Work by Pesakhov *et al*, 2010 used western blot analysis to examine the effect of curcumin and carnosic acid alone and in combination on the levels of Bcl-2 family proteins, including the levels of anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic proteins (Bax and Bak) in two myeloid leukemia cell lines (HL-60 and KG-1a). They reported that treatments alone did not show any change in the expression of Bcl-2 family proteins, while in combination with carnosic acid caused a down regulation of Bcl-2 and Bcl-xL; and an

elevated the Bax levels; which they attributed to activation of mitochondrial caspase-9 (Pesakhov *et al*, 2010). (2) Investigate whether polyphenols have the ability to activate the production of vital apoptotic proteins in leukaemia cells. This would include a study of histone acetylation and the inhibition of HDAC and histone deacetylase (HDAC) activity, as these are involved in several cellular regulative processes including: transcription, cell cycle progressing, gene silencing, cell differentiation, DNA-replication and DNA-damage responses (Groh *et al*, 2013). This can be investigated using HDAC chemiluminescent drug discovery kit (Enzo, UK) which is designed to measure histone deacetylase (HDAC) activity in cells, nuclear extracts, immunoprecipitates or purified enzymes. Indeed recent work by Vargas *et a*l, 2014 showed that quercetin has the ability to inhibit HDAC activity and increase reactive oxygen species levels in glioma cell lines; (Vargas *et a*l, 2014). In fact, most evidence suggests that agents that inhibiting HDAC and/or protesome activity could be used as a potential strategy for cancer therapy and could be used in combination with standard chemotherapeutic agents to overcome the drug resistance (Almond and Cohen, 2002; Groh *et al*, 2013; Vargas *et a*l, 2014). (3) Investigate the direct effect of polyphenols on proteasome activity, as proteasomes plays a key role in cell cycle regulation, tumour growth and cell survival (Almond and Cohen, 2002). This can be investigated using proteasome fluorogenic assay kit for drug discovery (Enzo, UK) which is designed to measure chymotrypsin-like protease activity of purified 20S proteasome. The effects of polyphenols on proteasome activity have not been previously reported. However some preliminary examination using each of the polyphenols studied here; has shown each to act as proteasome inhibitor activity. This work however, required repeating. (4) Investigate the effect of polyphenols on protein kinases activity and cell cycle regulators. This would include assessment of cyclin and cyclin-dependent kinase activity which is crucial in the regulation of the cell cycle. This can be examined using western blot analysis and/or RT-PCR. Both have been previoulsy used by Weng *et al*, 2005; to identify protein kinases and cell cycle regulators that responsible for arresting the cell cycle at G_1 phase in C6 glioma cells, following treatment with chrysin. They demonstrated that chrysin enhanced $p21^{Waf1/\ Cip1}$ protein levels and inhibited CDK4- and CDK2-mediated phosphorylation of Rb (Weng *et al*, 2005); thus understanding the effects of chrysin progression of cell cycle. This is essential for the rational design of novel drugs to prevent abnormal cellular proliferation seen during cancer. (5) Investigate the effect of polyphenols alone and in combination with chemotherapeutic agents on reactive oxygen species (ROS) levels, which is considered to be an important protective biomarker and may be a crucial target for anti-cancer therapies by ameliorating oxidative stress and thus DNA damage (Babich *et al*, 2011). The intracellular ROS levels can be investigated and determined by the oxidation-sensitive fluorescent probe (DCFH-DA) using flow cytometer. Previousl work by Pesakhov *et al*, 2010 used this assay to measure the levels of ROS in two myeloid leukaemia cell lines (HL-60 and KG-1a) treated with curcumin, carnosic acid and

stilbene, alone and in combination with each other. They did not observed any significant elevation of the intracellular ROS levels in leukaemia cells; even when the treatment dose were increase to 50 μm (Pesakhov *et al*, 2010).

5.2.2 In Vivo Animal Studies

Earlier *in vivo* works using xenograft models has demonstrated the possible use of polyphenols alone or in combination with chemotherapeutic agents in the treatment of cancers (Dai and Mumper, 2010; Lewandowska *et al*, 2014). Genistein has been shown to significantly reduce tumour growth of pancreatic carcinoma (BxPC-3) xenografts; when used alone and in combination with cisplatin (Lewandowska *et al*, 2014). Similar Caltagirone *et al*, 2000 showed that quercetin and apigenin inhibited growth and metastasis of melanoma cell (B16-BL6) grown as xenograph on athymic mice. They concluded that quercetin and apigenin had a greater anti-cancer effect than the chemotherapy agent tamoxifen (Caltagirone *et al*, 2000). It is clear from these studies that it is important to investigate the anti-cancerous potential of polyphenols within an *in vivo* system; to date this has not been undertaken to study the effects of polyphenols alone or in combination with standard chemotherapy agents on leukaemia. Thus in order to confirm the current finding, an appropriate animal model or xenograft system is required for the further study the effect of polyphenols on leukaemia *in vivo*. Use of such models would be required to determine whether polyphenol treatment is feasible in the whole organisms, and if so, which are the most effective treatments, doses and routes of administration.

Some recent studies reported that polyphenols when administrated orally under *in vivo* conditions resulting in poor bioavailability, as a result of poor solubility and/or rapid metabolism (Bansal *et al*, 2011; Rodríguez *et al*, 2013). Rodríguez *et al*, 2013 reported that most polyphenols including quercetin, curcumin, resveratrol and epigallocatechin gallate (EGCG) when administrated orally are not absorbed by the intestine in their native form, instead they are conjugated before passing into the blood stream (Rodríguez *et al*, 2013). Furthermore, coexisting compounds in the gut lumen lead to inhibit the digestive enzyme activity and/or alteration of the intestinal transport system; and hence a reduced intestinal absorption (Rodríguez *et al*, 2013). Once absorbed, polyphenols are further metabolised in the liver and then eliminated through urine and bile (Rodríguez *et al*, 2013). It is known that polyphenols are normally subjected to 3 main types of conjugation: methylation, glucuronidation and sulphation (Rodríguez *et al*, 2013). So, this extensive conjugation followed by a rapid excretion of the conjugated metabolites is responsible of their poor bioavailability of polyphenols (Rodríguez *et al*, 2013). However, it has been shown that these limitations can be circumvented by intravenous administration of polyphenols (Rodríguez *et al*,

2013). Study by Bansal *et al*, 2011 showed that curcumin was rapidly metabolism in the intestine and liver; with approximately 60% to 70% of orally given curcumin was eliminated in faeces (Bansal *et al*, 2011). It was reported that when curcumin was administrated orally as an aqueous suspension (2 g/kg) the maximum plasma concentration reached was 1 mg/mL within 1 hour, and this then dropped rapidly to undetectable levels within 5 hours (Bansal *et al*, 2011). Likewise, when curcumin was administrated intraperitoneally in mice at 0.1 g/kg; only 2.25 mg/mL reached blood plasma within 15 mins; which rapidly dropped to 0.35 mg/mL after only 1 hour (Bansal *et al*, 2011). Similarly, intravenous administration of curcumin results in about 50% elimination in bile within 5 hours (Bansal *et al*, 2011). It is believed that this poor bioavailability of curcumin may be caused by the formation of sulphate and glucuronide conjugates which prevent its absorption and sped up its elimination (Bansal *et al*, 2011). Bansal *et al*, 2011 suggested that the bioavailability of polyphenols such as curcumin, ellagic acid, resveratrol and green tea polyphenols may be improved by the use of novel delivery systems such as liposomal formulations, nanoparticles, microemulsions and/ or polymeric implantable devices; which could deliver therapeutic concentrations directly into the systemic circulation (Bansal *et al*, 2011). These finding however suggest that more work is still needed to find the best administration route for polyphenols *in vivo* models and how these could be used in a clinical setting.

5.2.3 In Vitro Human Primary Leukaemia Studies

Once therapeutic doses of polyphenols and chemotherapy drug are determined, the next step is evaluate whether these two agents work with the same efficacy on human blood samples taken from patients with lymphoid and myeloid leukaemia (Kennedy and Barabé, 2008).

5.2.4 Human Clinical Trials

Following investigation of the effect of polyphenols *in vitro* and *in vivo*, the next step would be to examine the efficacy and safety in humans. Thus the expansion to clinical trials in patients with leukaemia would be an essential step towards developing the use of polyphenols in the treatment of leukaemia. In addition, it would be essential to evaluate whether combination treatments with polyphenols on a large group of patients to determine whether reduced side effects were observed compared to the treatment with chemotherapy alone. To date there are few clinical trials that have tested the effect polyphenols on cancer. One promising clinical study by Cruz-Correa *et al*, 2006, found that the combination of quercetin and curcumin supplements decreased the number and size of pre-cancerous rectal tumours in patients suffering from rectal adenomas with familialadenomatous polyposis (FAP). Here they supplemented five patients with 480 mg of curcumin and 20 mg of quercetin orally, three times a day, for six months. As a result, 5 patients had a remarkable decrease in polyp number and

size with an average reduction of 60% and 51% respectively, after 6 months; without appreciable toxicity (Cruz-Correa *et al*, 2006). Thus much work is required to further clarify the anti-cancer potential of polyphenols clinically in human.

5.3 Final Conclusion

This study has shown that polyphenols hold promise for the treatment of leukaemia. Lymphoid leukaemias were shown to be more sensitive than myeloid cell lines and limited effect were seen in non-tumour control cells. Selected polyphenols were shown to act differently either synergistically, additively or antagonistically with the standard chemotherapeutic agents, depending on the level of modulation of glutathione (GSH). Further *in vivo* investigations into polyphenols alone in the treatment of leukaemia are urgently needed to develop these potential agents as future treatments. Finally, this study also suggested a further *in vivo* study is urgently required to demonstrate whether diet-based polyphenols (QUE, AP, EMO, RH and CIS) affect chemotherapeutics activity.

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