Cytotoxic Potential of Some Plant Derived Compounds Targeting Lung Cancer

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CERTIFICATE

This is to certify that the thesis entitled **"Cytotoxic Potential of Some Plant Derived Compounds Targeting Lung Cancer"** and submitted by **V V S P C Rao Mathamsetti** ID No.**2011PHXF420H** for award of Ph.D. of the Institute embodies original work done by him under my supervision.

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ABSTRACT

Non-small cell lung cancer (NSCLC) accounting for about 85% of all lung cancer cases needs immediate attention because of the uncontrollable deaths of NSCLC patients in the world. Although, combination of chemotherapy and surgery has been identified as an optimal treatment for patients with early stage disease, no effective drug therapy is currently available. Literature claims hundreds of natural molecules screened for antiproliferative effects. However, only few among the several known cytotoxic natural compounds have been exploited for their mechanisms of action. Many potential molecules may emerge as chemotherapeutic drugs if relentless research is carried out on such underexploited natural compounds. In view of this, twenty phytocompounds belonging to withasteroids, alkaloids, iridoid glycosides, coumarins, anthraquinone and phenolics derivatives were screened against lung cancer cells (A549) to explore anti cancer agents. Results disclosed nine compounds exhibiting cell growth inhibition effect and significant cytotoxicity was exhibited by three compounds, withametelin, cedrelopsin and coptisine, against A549 cells with IC_{50} values of 6.1, 14.3 and 18.9 μM, respectively. Compounds withaphysalin E, 12-deoxy withastramonolide, Withaperuvin B, physalolactone and anthraquinones were also found to show cell growth inhibition effect against A549 cells. While coptisine and cedrelopsin were found to be absolutely non-toxic, withametelin exhibited moderate safety when tested against HEK-293 cells, suggesting their cancer cell specific activity. Isolation and purification of coptisine from the seeds of *Fumaria indica*, withametelin from *Datura innoxia* and cedrelopsin from *Hedyotis umbellate* were performed to gain more sample for extensive studies. The identity of the isolated compounds was established by spectral analysis and direct comparison studies with authentic samples. In view of their cytotoxicity against A549

cells and safety on normal cells, these three compounds were chosen for further investigation to decipher the molecular mechanisms underlying their cytotoxicity against A549 cells.

Experimental findings clearly demonstrated that coptisine inhibited the proliferation of A549 cells through cell cycle arrest and apoptosis. Coptisine-treated A549 cells were arrested at G2/M phase, accompanied by the reduction in the expression of cyclin B1, cdc2, and cdc25C and upregulation of p21. Treatment of A549 cells with coptisine resulted in the generation of reactive oxygen species (ROS), upregulation of Bax and downregulation of Bcl-2. The imbalance of Bax and Bcl-2 caused mitochondrial depolarization, which resulted in the cytochrome c release into the cytosol, followed by the activation of caspase-8, -9 and -3, and consequently cleavage of PARP, leading to apoptosis. To ascertain if coptisine-induced apoptosis is associated with ROS, coptisine-treated A549 cells were pre-exposed to the ROS scavenger N-acetyl cysteine (NAC). It effectively reduced the apoptotic activity, caspase 3 induction, and MMP loss by coptisine, indicating that coptisine-induced intrinsic mitochondrial apoptosis was unambiguously mediated by ROS.

The significant cytotoxic potential of withametelin against A549 cells was revealed to be mediated by G2/M arrest and induction of apoptosis. G2/M phase arrest was ascertained from the down-regulation of cyclin B1, cdc2 and cdc25C expression. Withametelin induced cell death in A549 cells through mitochondria dependent apoptotic pathway, causing the generation of ROS, up-regulation of Bax and parallel down-regulation of Bcl-2. Eventually, dissipation of MMP, translocation of cyt c and sequential activation of caspase-9 and -3 and cleavage of its downstream substrate PARP occurred, leading to apoptosis. However, it failed to modulate caspase-8 expression which is involved in extrinsic apoptotic pathway. To find if withametelin-induced apoptosis is associated with ROS, withametelin treated A549 cells were pre-treated with

NAC. NAC failed to abrogate the apoptotic induction by withametelin. Thus it was concluded that apoptosis was induced through intrinsic pathway but not mediated by ROS.

The study on cedrelopsin, a coumarin derivative demonstrated the inhibition of A549 cell proliferation mediated by G2/M arrest. Cedrelopsin induced G2/M arrest, as evidenced by downregulation of G2/M regulatory proteins such as Cyclin B1, cdc2 and cdc25C. However, cedrelopsin did not induce apoptosis in A549 cells.

In order to check the potentiality of the selected molecules in inhibiting other cancer cells, study was extended to test against human breast cancer (MDA-MB-231) and human colorectal cancer (HT-29) cell lines. Coptisine and withametelin displayed lower anti proliferative activity against MDA-MB-231 and HT-29 cells compared to A549 cells. Further, viability of both the cells were not affected by cedrelopsin, corroborating its cancer cell specific activity.

Thus the cytotoxic mechanisms of three potent natural compounds having entirely different basic nucleus were explored for the first time. In a nut shell, the study uncovered that withametelin, coptisine and cedrelopsin have the potential to be developed as drugs for treating NSCLC.

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ABBREVEATIONS

Akt	V-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of variance
AP-1	Activator protein 1
Apafs	Apoptotic protease factors
APO-1	Apoptosis antigen 1
Asp	Asparitic acid
ATP	Adenosine triphosphate
BAD	Bcl-2 associated death promoter
Bcl-2	B cell lymphoma
Bcl-Abr	Breakpoint cluster region-Abelson
BID	Bax like BH3 domain
BIK	Bcl-2 interacting killer
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CD120a	Cluster of differentiation 120
CD95	Cluster of differentiation 95
CD95 L	Cluster of differentiation 95 ligand
Cdc2	Cell division cycle protein 2
Cdc25c	Cell division cycle 25c
Cdk 4	Cyclin dependent kinase 4
Cdk 6	Cyclin dependent kinase 6

CDK2	Cyclin dependent kinase 2
CDLN	Cedrelopsin
CED	Caenorhabditiselegans domain
Chk1	Check point kinase 1
Cip	Cdk interacting proteins
COP	Coptisine
Cpp 32	Cysteine protease protein 32
Cyt c	Cytochrome c
d	Doublet
dATP	2-Deoxyadenosine 5’-triphosphate
DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
DD	Death domain
DED	Death effector domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DMSO-d6	Deuterated DMSO
EtoAC	Ethyl acetate
FADD	Fas-associated death domain
FAS L	FAS ligand
FITC	Fluorescent inhibitor isothiocynate
FLICA	Fluorescent inhibitor of caspases
G1 phase	Gap phase1
G2 phase	Gap phase2
h	Hour
HEK-293	Human embryonic kidney cells

HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration 50
ICE	Interleukin-1ß-converting enzyme
IL-3	Interleukin 3
kDa	Kilo Daltons
M phase	Mitosis phase
MAPK	Mitogen activated protein kinase
MeoH	Methanol
MMP	Mitochondrial membrane potential
MMP-9	Matrix metalloproteinase 9
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYC	V-myc avian myelocytomatosis viral oncogene homolog
NAC	N-acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCS	National Centre for Cell Science
NES	nuclear export signal
NF-*K*b	Nuclear factor *k*B
NMR	Nuclear magnetic resonance
NSCLC	Non small cell lung cancer
PARP	Poly ADP ribose polymerase
pH	Potential of hydrogen
PI	Propidium iodide
PI3K	Phosphoinositide-3-kinase
PPM	Parts per million

pRB	Phospho retinoblastoma
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
RAS	Rat sarcinoma
RH-123	Rhodamine 123
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
S	Singlet
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SOD	Superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
T	Triplet
Thr 14	Threonine 14
Thr160	Threonine 160
Thr161	Threonine 161
TIMP-1	Tissue inhibitor of metalloproteinase 1
TLC	Thin layer chromatography
TNFR1	Tumor necrosis factor receptor 1
TP53	Tumor protein53
TPA	12-O-tetradecanoylphorbol-13-acetate
TRADD	TNF receptor associated death domain
TRAIL	TNF related apoptosis inducing ligand

Tyr15	Tyrosine 15
WM	Withametelin
δ	Chemical shift
μM	micro molar

CHAPTER 1

INTRODUCTION

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1.1 Cancer

In order to maintain homeostasis in normal healthy tissue, balance between proliferation and cell death is absolutely necessary. Activation of appropriate pathways upon signalling by growth factor and elimination of potentially harmful cells by apoptosis results in cell proliferation (Plati *et al*., 2011). Disturbance of these process lead to the development of cancer. Carcinogenesis is a multifactorial and multistep process that requires several cellular genetic as well as epigenetic changes (Migliore *et al*., 2011) that disturb the balance between proliferation and apoptosis. These changes provide transforming cells the advantageous characteristics resulting in uncontrolled cell division, creating tumor mass and survival in the environment of growing tumor. Gain of function mutations in proto-oncogenes and loss-of-function mutations of tumor suppressor genes are the most common milestones on the way from healthy cell to cancer one.

Cancer is one of the leading causes of death worldwide. Approximately 14 million new cases and 8.2 million cancer related deaths occurred in 2012. Further, it has been estimated that the number of new cases may increase from 14 million in 2012 to 22 million in the next 2 decades. Among all, lung cancer has been identified as the leading cause of cancer deaths (1.59 million deaths), followed by liver (7,45,000), stomach (7,23,000), colorectal (6,94,000), breast (5,21,000) and oesophageal cancer (4,00,000) deaths. Every year more than 60% of the new cases and 70% of world's cancer deaths occur in low and middle-income countries (Stewart and Wild., 2014).

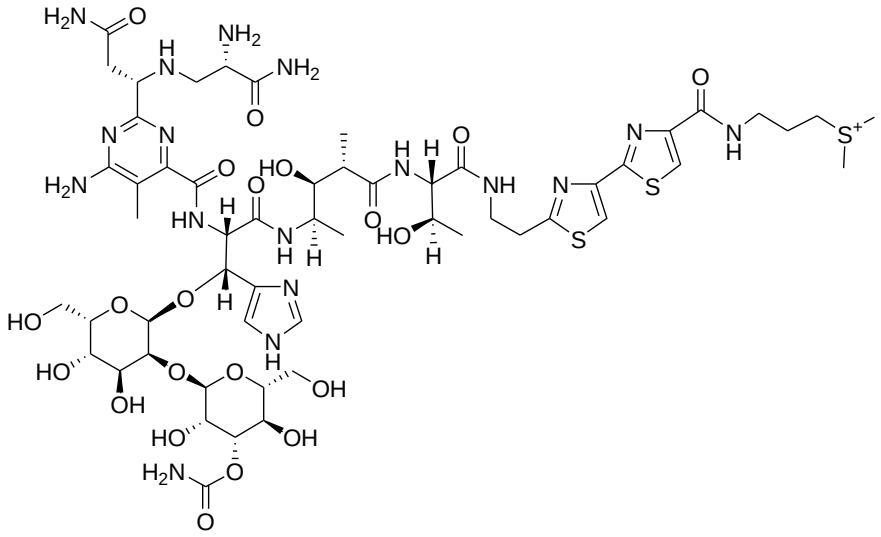
1.2. Cancer and plant derived anti cancer drugs

Nature has always been the source of structurally diverse and novel molecules. Based on the structural diversity, high selectivity and specific biological effects exhibited by natural molecules, anti-cancer research into natural products was focused. Also literature reveals the discovery of several new chemical entities based on the ethno-medicinal uses of the plants. The best examples are anti-malarial drugs, particularly artemisinin and quinine, which were isolated from *Artemisia annua* and *Cinchona* species, respectively that were used by indigenous people to treat fever (Editor., 2007).

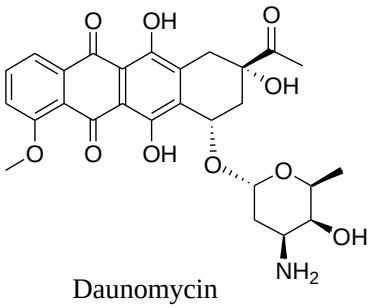
Cragg and Newman (2013) reported that, of the 1073 new chemical entities approved from 1981-2000, 64% were of natural product derived substances or nature inspired small molecules. Of 1073 small molecules, 69% of anti-infectives, 59% of anticancer agents were found to be naturally derived or inspired molecules. Similarly, out of 317 drugs approved from 2000 to 2013, natural products derived drugs were accounted for 17% (54 drugs). At the end of 2013 there were 100 natural product derived molecules that were undergoing clinical trials. Of these 100 molecules, majority were in oncology segment (38 molecules), followed by anti-infectives (26 molecules) and cardiovascular drugs (19 molecules) (Butler *et al*., 2014). Despite the intensive investigations on plants, it is estimated that out of 3,00,000 species of higher plants, only 6% were systematically studied for their pharmacologic effects and only 15% were subjected for phytochemical evaluations (Rates *et al*., 2001).

It is worthy to note that over 60% of first line therapeutic anticancer agents are derived from natural sources, including plants, marine organisms and microorganisms (Cragg *et al*., 2005; Newman *et al*., 2003). Several exciting anticancer agents have so far been derived from various

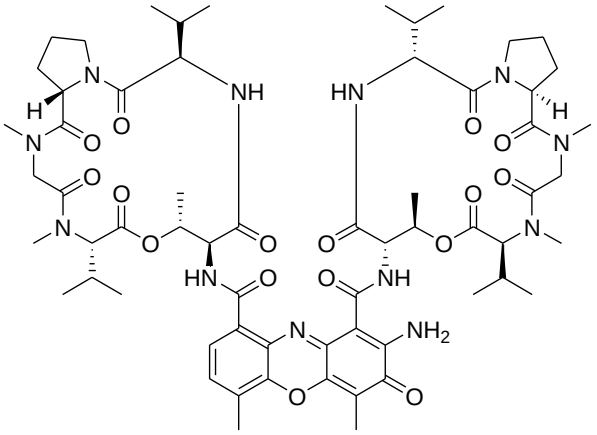
plant sources. Paclitaxel is one such drug, whose annual sale is over $ 1 billion. The other important plant derived anticancer agents are belotecan, irinotecan and topotecan all of them were derived from camptothecin, obtained from a Chinese plant called *Camptotheca acuminata* (Cragg and Newman., 2013). A few plant derived anticancer agents in clinical use are given in Figure 1.1. and Figure 1.2. These are primarily used in combination with other anti cancer therapies for variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers and Kaposi's sarcoma (Lynch *et al*., 2012; Cragg and Newman., 2005). The common side effects associated with these cancer treatments are severe pain, fatigue, bleeding, hair loss, reduction in blood cells, fertility problems and memory changes. Chemotherapy is a type of treatment that encompasses the use of drugs to cure or comparatively prolong the life of cancer patient. Though the existing drugs are effectively killing the cancer cells but they do kill fast growing normal cells too, causing severe side effects. Hence there is always a demand for the development of both effective and safer drugs to combat cancer.



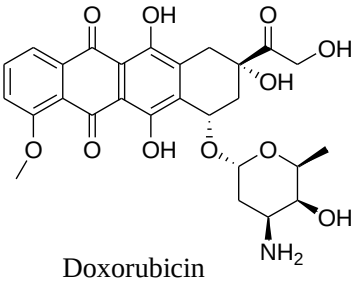
Bleomycin



Daunomycin



Actinomycin



Doxorubicin

Figure 1.1: Natural anti cancer agents in clinical use

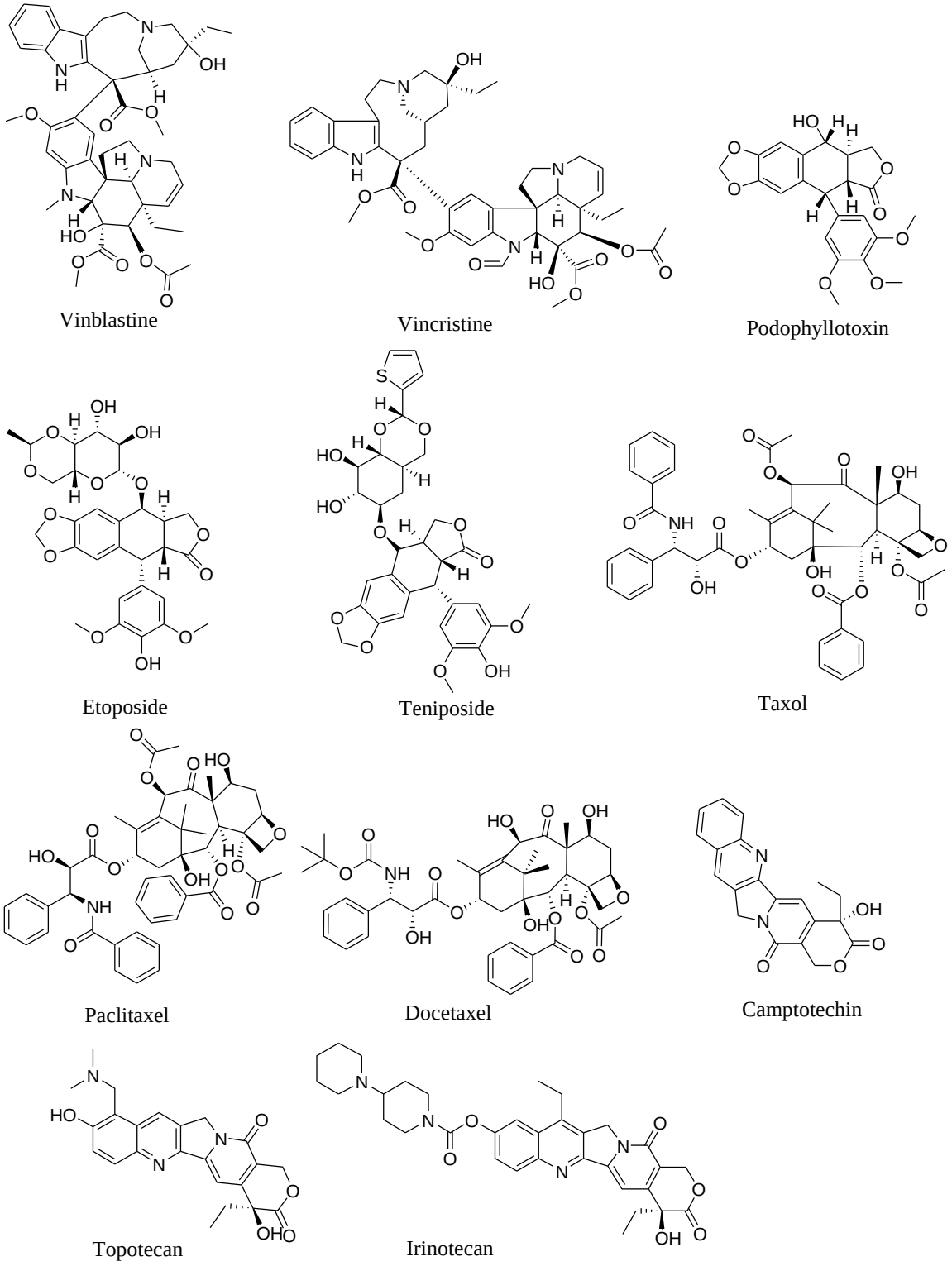


Figure 1.2: Structures of plant derived anti cancer agents in clinical use

CHAPTER 2

Literature review

CHAPTER 2

Literature review

2. Lung cancer

Lung cancer is the second most common cancer and one of the leading causes of tumor mortality associated with a poor survival rate even after complete surgical removal. Recent statistics evidenced that 224,390 adults were diagnosed with lung cancer (American Cancer Society., 2016). One year and 5 year survival rate have been reported as 44% and 17%, respectively in people suffering from lung cancer. Lung cancer is divided into three types, *viz*., Non small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and lung carcinoid tumor. SCLC accounts 10-15% of lung cancers and spreads very quickly. Usage of tobacco is the major cause of small cell lung cancer. However, people who do not smoke may also get cancer. Lung carcinoid tumors account only 5% of lung cancers and, grow slowly and spread rarely. Most common type of lung cancer is NSCLC accounting 85%, resulting from gene mutations triggered by environmental exposure (American Cancer Society., 2016). Over the past few years, research groups have investigated the possible involvement of several molecular mechanisms, such as cell-cycle and apoptosis regulators, oncogenes and tumor suppressor genes, cell adhesion molecules in the pathogenesis and progression of lung cancer. Similar to other tumors, a cascade of morphological changes is characterized during lung carcinogenesis. It is more than obvious that the malignant phenotype and its heterogeneity within a tumor subclass or even in a given tumor is caused by the interaction of many gene changes and pathways. Lung cancer needs immediate attention because of the uncontrollable deaths of NSCLC patients in the world. Although, combination of chemotherapy and surgery has been identified as an optimal treatment for patients with early stage disease, no effective single drug therapy is currently available (Van

Meerbeeck JP., 2007). Hence, discovery of specific and effective chemotherapeutic agents for NSCLC is a desperate need.

2.1 Cell cycle effects of chemotherapy

Chemotherapy causes DNA damage in cancer cells, which results in aborting cell normal activities till DNA gets repaired. Arresting cells in G1/S or G2/M phases by chemotherapy drugs affect cell cycle progression. Certain chemotherapeutics aim at specific checkpoints, for instance etoposide causes a G2/M arrest by downregulating the activity of cdc2, the main controller of mitotic entry (Lock and Ross., 1990). Doxorubicin causes G2/M arrest and also induces G1 arrest at high concentrations (Barlogie B *et al*., 1976).

2.2 Cell cycle

2.2.1 Introduction

The cell cycle is a regulated process consisting of a round of DNA replication called S phase, followed by cell division or mitosis (M) in which the replicated chromosomes are segregated to form two identical daughter nuclei. Both these events are closely monitored to ensure that the S and M phases will occur in the correct sequence, so that M will not occur before S has got completed and vice versa. Several control mechanisms have evolved to maintain the ordered sequence and prevent these events from occurring at inappropriate stages of the cell cycle. Gap phases termed as G1 and G2 are present between M and S, and between S and M, respectively. These phases act as preparatory periods during which the cell can prepare for entry to S or M phases. There is also a resting phase termed Go, in which cells can remain for an indefinite period of time, during which cells become quiescent, but can re-enter the cell cycle at any time when conditions become favourable for growth and division (Pardee., 1989).

2.2.2 Activation of Cyclin-Cdk complexes:

Cyclins and cyclin dependent kinases (Cdks) are necessary for the cells to progress through the different phases of cell cycle. Cdks are catalytic subunits and cyclins serve as regulatory subunits of these complexes and their expression fluctuates throughout the cell cycle. Cyclin levels change throughout the cell cycle, and the variations in cyclins regulate the activation of inactive Cdks that are present in constant levels in the cell. Once the cyclin-Cdk complex has formed, it becomes a substrate for a number of kinases which either positively or negatively regulate its activity as outlined in Figure 2.1. Activation of a Cdk occurs when cyclin-Cdk complex is phosphorylated on a conserved threonine residue, Thrl61 on Cdkl, Thrl60 on Cdk2 (Morgan DO and De Bondt HL., 1994; Russo AA *et al.*, 1996).

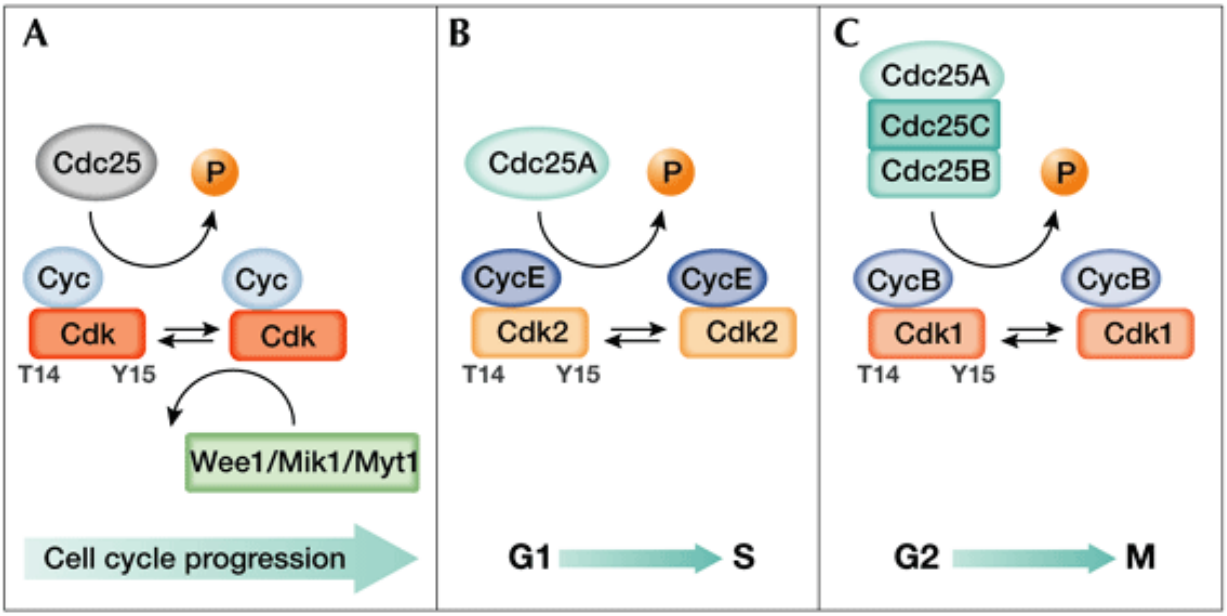


Figure 2.1: Regulation of cyclin-Cdk complexes (Lim S et al., 2013)

2.2.3 Inhibition of Cyclin-CDK complexes:

Inhibitory phosphorylations occur on the Cdk near the ATP binding site. Thrl4 and Tyrl5 are conserved phosphorylation sites in Cdkl and Cdk2, and the presence of phosphates on these residues inhibits kinase activity, perhaps through interference with ATP binding. Weel HU is responsible for phosphorylation on Tyrl5 (Parker LL *et al.,*1992; Blasina A *et al.,*1997) and the human Mytl can phosphorylate both Thr 14 and Tyr 15 (Liu X *et al.,* 1997; Booher RN *et al.,* 1997), although Booher RN *et al.* (1997) revealed that Mytl only phosphorylates Cdkl-containing complexes. The weel kinase plays an important role in mitotic control. The inhibitory phosphates are removed by a dual specificity phosphatase cdc25 (Lee MS *et al.,* 1992) resulting in dephosphorylation and activation of the cyclin-Cdk complex Three cdc25 homologues exist in humans, cdc25A, cdc25B and cdc25C. cdc25A is implicated in activation of cyclin E-Cdk2, cdc25C dephosphorylates and activates cyclin B1-Cdkl and cdc25B is thought to play a role in activating cyclinB1-Cdkl also. In order to activate of cdc25 phosphatases, they must be phosphorylated themselves and active Cyclin B1-Cdk1 does further activation of cdc25C through positive feedback mechanism.

2.2.4 Entry to cell cycle and G1 progression:

The main cyclin-Cdk complex implicated in entry to G1 is cyclin D-Cdk4. The rate limiting controllers of G1 phase progression are Cyclin D proteins, induced by mitogens in G1 and synthesis of Cyclin D proteins depends on persistent growth factor stimulation (Matsushime H *et al.,* 1991a, 1991b, 1992). Cyclin D binds mainly to Cdk4/Cdk6 and kinase activity increases during mid G1 phase, peaking as the cells approach Gl/S boundary (Matsushime H *et al.,* 1994) The retinoblastoma protein is one of the principle substrates of cyclin D-Cdk4 (Dowdy SF *et al.,*

1993).

The main role of the retinoblastoma protein (pRb) is to act as a signal transducer linking the cell cycle to the transcriptional controls in the cell. It is a key protein in the transition between cellular proliferation and growth inhibition, a molecular switch that can suppress expression of genes needed for the progression of the cell cycle (Weinberg RA, 1995). The phosphorylation state is the primary control mechanism of pRb, and phosphorylation levels vary during the cell cycle. When hypophosphorylated (mainly in Gl), pRb acts as a growth suppressor and binds to and negatively regulates the activity of transcription factors such as E2F, pi07, and pl30. These transcription factors play a critical role in progression into S phase and have consensus binding sites in the promoter regions of many genes required for S phase. When bound to E2F, pRb inhibits its function as a transactivator, thereby preventing induction of genes whose expression is required for cell cycle progression. However upon phosphorylation on specific sites, a conformational change occurs in pRb, and its growth suppressive effect on E2F is removed, releasing the transcription factor to activate genes required for Gl/S transition and S phase, such as c-myc, dihyrofolate reductase, cyclin E, cyclin A and Cdkl.

2.2.5 Entry to S phase:

After induction of cyclin D, expression of cyclin E increases in late G1 and binds to Cdk2, thereby resulting in an accumulation of cyclin E-Cdk2 complex levels, and cyclin E-Cdk2 activity peaks at the Gl/S transition (Koff A *et al*., 1992, 1993). Cells that fail to form active cyclin E-Cdk2 complexes cannot pass the Gl/S transition confirming its role in control of entry to S phase. Once cells enter S phase, cyclin E is rapidly degraded resulting in the destruction of the cyclin E-Cdk2 complex. However Cdk2 can now form a complex with cyclin A, therefore

Cdk2 has two periods of oscillating activity, and it acts on different substrates depending on which cyclin it binds to.

Cyclin A-Cdk2 activity is essential for the initiation of S phase. Cyclin A levels begin to rise at the end of G1 phase, and cyclin A-Cdk2 activity peaks at the onset of S phase. In early S-phase cyclin A-Cdk2 binds directly to, and phosphorylates E2F, and in doing so inhibits its DNA binding ability, thereby inactivating E2F and turning off the Gl/S phase genes that are no longer required once the DNA replication has begun. (Xu M *et al,* 1994). Cyclin A also binds to Cdkl during G2 phase (Pagano M *et al.,*1992), and is required during the G2/M transition, so cyclin A can play a role in two cell cycle phases depending on which Cdk it binds to.

2.2.6 Cell Cycle inhibitors

In addition to inhibition by phosphorylation, cyclin-Cdk complexes are inhibited and regulated by a group of proteins called Cdk inhibitors (CKI). The Cip family (Cdk interacting proteins) is comprised of p21/CIPl/WAFl (Xiong Y *et al.,* 1991), p27/KlPl and p57/KIP2, and they preferentially inhibit Cdks of the Gl/S phase (Harper JM *et al.,* 1995, Sherr CJ., 1995). p21is the most extensively studied of this family (Gartel AL *et al.,* 1996, review). It can bind Cdk2 and Cdk4 in complex with cyclins and inhibit the phosphorylation of pRb and prevent cell cycle progression (Harper JW *et al.,*1993). It is under the control of p53, and after DNA damage, p53 can induce p21 transcription, resulting in a cell cycle arrest. p21 exists in a quaternary complex with a cyclin, Cdk and proliferating cell nuclear antigen (PCNA), a DNA replication factor (Flores-Rozas F *et al.,* 1994). By binding to PCNA, p21 can inhibit DNA replication (Waga S *et al.,* 1994). p27 levels are elevated in terminally differentiated cells, and play a role in G1 checkpoint. It is responsible for blocking cells in G1 phase in response to TGF-β treatment by

potently inhibiting cyclinE-Cdk2. Even though p27 levels are high in quiescent cells, they decrease rapidly when the cells enter the cell cycle in the presence of mitogens. If mitogens are removed, p27 levels rise again to inhibit cell cycle progression in conditions that are unfavourable for growth (Kato J *et al.,* 1994).

2.2.7 Entry to Mitosis:

The key component of the mitotic pathway in cells is a mitotic regulator comprised of cyclin B (Pines J and Hunter T, 1989) and the kinase catalytic subunit cdc2/Cdkl (Draetta G and Beach D., 1988) which form mitosis-promoting factor (MPF) (Lee M and Nurse P., 1988).

MPF, once active can exert its effect on many cellular substrates to effectively and rapidly induce cytoskeletal rearrangement and cytokinesis. Indeed, the active kinase is intimately involved with reorganisation of the structural features of the cell during mitosis. Cyclin B-Cdkl causes dramatic changes in the microtubule network, the actin microfilaments and the nuclear lamina. The nuclear lamina is a structure that underlies the nuclear membrane during interphase, and is composed of lamin subunits. Lamins are hyper phosphorylated at mitosis, promoting their disassembly (Nigg E *et al.*,1992). Proteins in the intermediate filament network such as vimentin and desmin are also phosphorylated by Cdkl, promoting their dismantling at mitosis (Chou *et al.,* 1990). Another significant substrate is caldesmon, which is a component of cytoplasmic filaments and binds actin and calmodulin (Yamashiro S *et al.,* 1991). Histone HI when phosphorylated by cyclin B-Cdkl, possibly plays a role in chromosome condensation during mitosis (Langan TA *et al.,* 1989).

MPF is associated with a kinase activity that varies through the cell cycle, peaking at M phase and rapidly declining at the end of mitosis. Cyclin B levels accumulate gradually from Gl, and

upon reaching a threshold level it binds to Cdkl (Pines J and Hunter T, 1989). However the cyclin B-Cdkl complex is not active until modified by phosphorylations resulting in an abrupt increase in Cdkl kinase activity, promoting onset of mitosis. The switch that controls the transition between inactive cyclin B-Cdkl and active cyclin B-Cdkl is controlled by a number of phosphorylations as earlier described. Cdkl must be phosphorylated on Thrl61 by CAK for activity, and inhibitory phosphates occur on Thrl4 by Mytl and on Tyrl5 through the action of the mitotic inhibitor wee1 (Parker LL and Piwnica-Worms H, 1992).Weel was identified as a protein kinase that is required for delay in entry to mitosis (Russell P and Nurse P., 1987). Weel is itself negatively regulated through phosphorylation by niml kinase. Cdk activity occurs through dephosphorylation of Thrl4 and Tyrl5 by the cdc25C phosphatase (Strausfeld U *et al.,* 1991). These inhibitory phosphates play a critical role in timing of entry to mitosis, and cells that have mutated Cdkl which cannot be phosphorylated on Thrl4 and Tyrl5 fail to pause before entry to mitosis (Krek W and Nigg EA, 1991 & Norbury C *et al*., 1991).

2.2.8 Regulation of mitotic Cdk controllers

Regulation of weel kinase and cdc25 phosphatase activities is one of the key determining factors in the timing of mitotic progression. Cdc25 is regulated through several complex pathways. Both cdc25B and cdc25C are involved in entry to mitosis, and show different patterns of activation during the cell cycle indicating that they have different roles to play in the activation of cyclin B-Cdkl. Cdc25B activity appears in late S phase and peaks in G2 phase (Lammer C *et al.,* 1998). A rise in cdc25C activity was demonstrated by Izumi *et al,* 1992 as cells entered mitosis, and decreased upon exit from mitosis. The rise in cdc25C activity during mitosis is due to phosphorylation (Solomon M *et al.,* 1990) and active cyclin B-Cdkl is capable of phosphorylating and directly activating cdc25C (Hoffmann I *et al.,* 1993). This suggests that

there is a positive feedback mechanism in which a small amount of already active cyclin B-Cdkl can activate cdc25C, and this in turn dephosphorylates Cdkl resulting in a rapid activation of cyclin B-Cdkl. If phosphorylation sites in cdc25C are mutated, its mitosis promoting activity is reduced (Izumi T and Mailer JL., 1993) thus supporting the idea of a role for cdc25C activation in progression through mitosis. During G2 Cdc25B is translocated into the cytoplasm, and cytoplasmic cyclin B-Cdkl is an excellent substrate for cdc25B during late G2. Cdc25B could then dephosphorylate cyclin B-Cdkl in the cytoplasm, promoting translocation of the active cyclin-Cdk complex to the nucleus where it could phosphorylate and activate cdc25C thus initiating the feedback loop.

2.2.9 Chemotherapy Drugs and G2/M Arrest

Treatment of cells with agents that cause DNA damage often leads to a delay or arrest in G2 of the cell cycle. G2/M arrest has been reported in cells after treatment with different drugs such as doxorubicin (Barlogie B *et al.,* 1976; Krishan A *et al.,* 1976; Kimler BF *et al.,* 1978), rubidazone, etoposide and melphalan (Barlogie B *et al.,* 1978), also mitomycin C, bleomycin, hydroxyurea and lucanthone (Kimler BF *et al.,* 1978). This delay is enforced by altered regulation of the G2/M controllers, mainly cyclin B-Cdkl kinase activity. Alterations in the controllers of this kinase results in diminished activity and prevention of entry to mitosis, for example failure to dephosphorylate the inhibitory phosphates from Cdkl results in an inactive Cdkl which enforce a G2 delay. During a G2/M arrest there is altered regulation of cyclin B and Cdkl (Tsao YP *et al.*,1992). Preliminary studies by Lock RB and Ross WE (1990, 1991) demonstrated a decrease and inhibition of Cdkl kinase activity during a G2 arrest after etoposide treatment. They consequently demonstrated that the inhibition of kinase activity after etoposide treatment was due to a failure to dephosphorylate the activating tyrosine residue, even though

cyclin B-Cdkl complex forming ability is not affected. More recently Ling *et al.* (1996) studied the effect of doxorubicin on P388 cells and found inhibition of Cdkl activity due to absence of dephosphorylation on Tyrl5. Other chemotherapy drugs such as cisplatinum (Nishio K *et al.*,1993), camptothecin (Tsao YP *et al,* 1992), nitrogen mustard, 5- fluorouracil (Okamoto S *et al.,* 1996) and nucleoside analogues (Halloran PJ *et al.*, 1998) have been shown to induce a G2/M arrest, induced by a failure to dephosphorylate Tyrl5 of Cdkl resulting in a reduced Cdkl kinase activity. In addition, Weel activity is reportedly disrupted during a G2 arrest (Leach SD *et al.,* 1998).

2.2.10 DNA Damage checkpoints

The fact that cells have the ability to arrest after experiencing damage to their DNA led to the discovery of cell cycle checkpoints. Checkpoints exist in the cell cycle to act as regulatory surveillance mechanisms to control the order and timing of the critical events of DNA replication in S phase and chromosome segregation at mitosis. Therefore the checkpoint response plays a significant part in maintaining genomic stability throughout the cell cycle, by monitoring the state of the DNA and arresting cell cycle progression in response to DNA damage or inhibition of DNA replication (Elledge SJ., 1996).

2.2.11 G2/M Checkpoint pathway in response to DNA damage

A family of DNA damage sensor proteins, called Rad proteins, exist in fission yeast *(S. pombe*) which are required for checkpoints to occur (Carr AM., 1997). Human homologues of these DNA damage sensor proteins have been identified demonstrating an evolutionary conserved checkpoint pathway (Weinert T., 1997). When DNA damage occurs, checkpoint proteins such as Rad24 bind to damaged DNA (Garvik B *et al*., 1995) which results in phosphorylation of Rad9

(Emili A *et al.,* 1998). Rad9 is essential for a checkpoint to occur, and is thought to phosphorylate another Rad family member, Rad53, which is also essential for cell cycle arrest in response to DNA damage.

The link between the DNA damage sensor proteins and the cell cycle emerged from the discovery that human cdc25C is phosphorylated on a specific residue in response to DNA damage (Peng CY *et al.,* 1997). Phosphorylation is carried out by Chkl protein kinase, a homologue of the *S. pombe* Chkl which is required for the DNA damage checkpoint. In response to DNA damage Chkl is activated, presumably by the Rad 3 and other Rad proteins. When Chkl is active, it phosphorylates Cdc25C on ser216, and induces a conformational change which creates a binding site for 14-3-3 proteins resulting in the inhibition of cdc25C's ability to dephosphorylate and activate Cdkl. Therefore the key event in the pathway leading to G2/M arrest is the phosphorylation of cdc25C by Chkl.

However the phosphorylation of cdc25C by Chkl, and binding of 14-3-3 protein to cdc25C does not reduce the enzymatic activity of cdc25C, so another regulatory mechanism must exist, perhaps involving limiting substrate availability. Cdc25C is cytoplasmic during interphase, and enters the nucleus at G2 to initiate M. Chkl is nuclear, and when cdc25C is phosphorylated by Chkl in the nucleus in response to DNA damage, a nuclear14-3-3 protein binds (Lopez-Girona A *et al.,* 1999). Rad24 is a 14-3-3 protein important in the DNA damage checkpoint pathway in fission yeast *(S. pombe),* and it is a sufficiently small protein that can passively diffuse in and out of the nucleus. It contains a hydrophobic region called a nuclear export signal (NES) which exports it from the nucleus. Lopez-Girona A *et al.* (1999) recently demonstrated that Rad24 binds to phosphorylated cdc25C after DNA damage and escorts it from the nucleus, thus separating cdc25 from its substrate cyclinB-Cdkl. Taken together, these results suggest that

maintenance of Thrl4 and Tyrl5 phosphorylation of Cdkl and nuclear export of Cdkl-cyclin B are the two primary mechanisms through which eukaryotic cells achieve a G2 arrest following DNA damage.

2.2.12 Cell Cycle and Cancer

To maintain the stability of their genome, proliferating cells normally arrest in either Gl/S or G2/M in response to cellular damage, and the presence of these checkpoints provides the cell with additional time for repair processes to remove any potentially lethal damage before DNA is replicated or segregated. A wealth of evidence supports the idea that loss of checkpoint control can lead to genomic instability, inappropriate survival of genetically damaged cells and the evolution of cells to a malignant state (Kaufmann SH *et al.*,1995; Paules RS *et al*, 1995).

Dysregulation of cell proliferation is a frequent event in tumorigenesis, and this unconstrained proliferation can be a result of gain or loss of function of the proteins that control the cell cycle. Many of the cell cycle proteins associated with Gl-S transition in the cell cycle are altered in some way leading to a destabilisation of the normal growth control pathway and unrestrained proliferation. Moreover the majority of tumours have mutations in one or another of the genes involved in controlling progression throughout the Gl phase of the cell cycle. Cells lacking p53 fail to arrest in Gl or G2 after DNA damage, demonstrating that lack of p53 can result in defective checkpoint control and enhance the genomic instability and genomic rearrangements (Kastan MB *et al.*,1991; Hartwell L., 1992).

Cyclin D1 acts as a positive regulator of Gl by removing the growth suppressive function of Rb. Any defect in the action of cyclin D such as overexpression will lead to Rb being constantly hyperphosphorylated thus allowing free E2F to induce transcription of S phase genes.

Overexpression of cyclin D1 is associated with poor prognosis of patients with breast cancer (Borg *et al.,* 1992; Michalides R *et al.,* 1996; Van Diest *et al.,* 1997) and human pancreatic carcinoma (Gansauge S *et al.,* 1997). pl6 is frequently mutated, deleted or inactivated through methylation in many different types of human cancers, suggesting its role as a tumour suppressor. Deletions and mutations of chromosome 9 p21, the location of p16, occurs very frequently in pancreatic, head and neck, non-small cell lung, bladder and ovarian carcinomas (Hall M., 1996), and perhaps is the most common genetic alteration in human lymphoblastic leukemia.

The G2 checkpoint is also implicated in tumorigenesis. Attenuation or inactivation of the G2 checkpoint can result in cells with increased genomic instability, and also promotes immortalisation in normal human fibroblasts (Kaufmann WK *et al.,* 1995) investigated the G2 checkpoint control in familial cancer syndromes and concluded that these cells have a defective G2 checkpoint which may have contributed to development of tumorigenesis.

2.3 Apoptosis

Apoptosis is a genetically regulated biological process that is fundamental to the development of organisms and to homeostasis of tissues (Kerr JF *el al.,* 1972). Various stimuli can trigger diverse regulatory pathways which activate conserved execution machinery. This results in dramatic structural changes such as cytoplasmic shrinkage and distortion, membrane blebbing and formation of membrane-bound cell fragments called apoptotic bodies. These changes are accompanied by chromatin condensation, a cytosolic increase in Ca^{2+}, decrease in cellular pH and DNA cleavage and fragmentation (Cobb JP *et al*., 1996).

It is accepted that caspases play a key role in the regulation of this biochemical process.

Caspases comprise a family of cysteine proteases which have similar cleavage specificity in that they cleave their substrates after an aspartate residue. They are synthesized as inactive precursor, which must be proteolytically cleaved at specific aspartate residues to become active. They can be cleaved themselves (auto processing) or by other enzymes.

Caspases are expressed as inactive proenzymes and range in size from 20 to 50 kDa. The proenzyme is cleaved after a specific Asp residue to form two active subunits of 20 kDa and 10 kDa, although this can vary in size depending on the caspase in question. Caspases are composed of three domains, the prodomain at the NH_2- terminal, the large p20 subunit and the small p l0 subunit.

Activation of caspases involves proteolytic processing of the procaspase resulting in cleavage of the prodomain and two subunits. These subunits then heterodimerise and bind to another heterodimer of the same caspase to form a tetramer. A tetrameric formation is essential for catalytic activity of the complex and substrate binding (Walker N *et al.,* 1994; Wilson KP *et al.,* 1994).

2.3.1 Proteolysis and caspase substrates

When apoptosis occurs in a cell it is accompanied by many ultrastructural changes that are manifested as the prominent hallmarks of apoptosis - cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. The contribution of caspases to this process is not completely clear but there is a coordinated regulated hierarchical sequence of protein cleavage that leads to the eventual dismantling of the cell. Caspases promote disassembly in a number of ways such as nuclear structural protein cleavage, cytoskeletal protein cleavage, cleavage of membrane transport proteins, and inactivation of protective proteins.

2.3.2 Lamin cleavage

Lamins are intermediate filament proteins responsible for the structural rigidity of the nuclear membrane. During apoptosis nuclear lamina collapse when lamins are cleaved allowing chromatin condensation to take place. Caspase-6 is responsible for cleavage of lamina and is the main laminase in the cell (Takahashi A *et al.*,1996).

2.3.3 PARP cleavage

Poly ADP ribose polymerase (PARP) is a 116kDa nuclear DNA repair enzyme, that is cleaved during apoptosis into a 98 kDa and 25 kDa fragment, and is a widely used marker for onset of apoptosis. This cleavage may interfere with its role as a DNA repair enzyme. Tewari M *et al*. (1995) and Nicholson DW *et al*. (1995) confirmed that PARP is specifically cleaved *in vitro* by caspase-3, and this along with caspase-7 are the main caspases responsible for PARP cleavage (Hu Y et al., 1998). PARP is now a readily accepted indicator of apoptosis, by monitoring the appearance of one or both of the cleavage products (Hu Y *et al*., 1998).

2.3.4 Fodrin cleavage

Fodrin is an abundant membrane-associated cytoskeletal protein that is cleaved early during apoptosis, possibly allowing membrane blebbing and cell shrinkage to take place. Janicke RU *et al*. (1998) reported an absolute requirement for caspase-3 in fodrin cleavage during apoptosis in MCF-7 cells.

2.3.5 Apoptotic execution pathway

Since all caspases cleave after an Asp residue, the notion that caspases could sequentially

activate others arose, and was confirmed with evidence of the existence of a caspase cascade. Enari M *et al*. (1996) demonstrated that Fas sequentially activates ICE-like and caspase-like proteases, and cpp32-like proteases act downstream together with a cytosolic component to induce apoptosis.

2.3.6 Initiator and effector caspases

When a pro-apoptotic stimulus "damages" the cell, the signal is transduced through the membrane-associated receptors and promotes activation of the initiator pro-caspases (such as caspase-8, -10, -9). These in turn activate the effector pro-caspases downstream, and the effector caspases in conjunction with certain cofactors implement the execution phase of apoptosis - namely the morphological and biochemical features of apoptosis. Caspase-8 has been shown to activate many of the other caspases indicating that it must be at the apex of the cascade. Recently procaspase-3 was confirmed as the major cellular target of caspase-8 activity (Stennicke HR *et al*., 1998). Different stimuli activate different sets of initiator caspases, but these distinct signals are thought to converge into one common pathway involving the effector caspases. An outline of this proposed system can be seen in Figure 2.2 and will be discussed in the following sections.

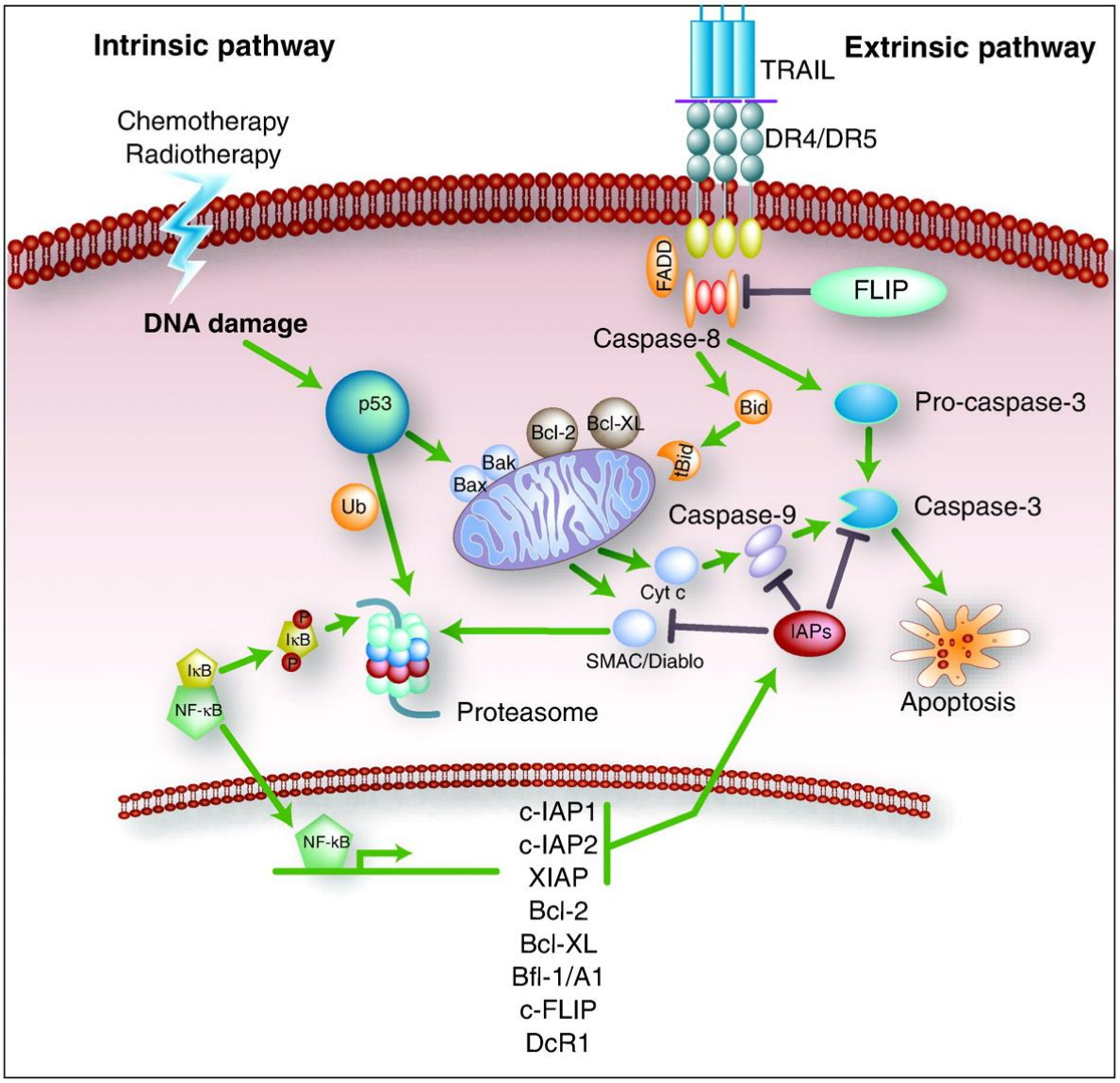


Figure 2.2: Schematic representation of apoptotic pathways. Apoptosis induction via the death receptor can result in activation of the extrinsic and intrinsic pathways. Chemotherapy and radiotherapy-induced apoptosis is executed via the intrinsic pathway. ⟶, activation; ⊣, inhibition (Fulda S *et al*., 2006).

2.3.7 CD95 Pathway

A growing number of cell surface receptors have been identified that play an important role in signal transduction in the apoptotic pathway. The best characterized receptors are CD95 (Fas/APO-1) and tumor necrosis factor receptor TNFR1 (p55/CD120a), which belong to the TNF superfamily of type I transmembrane receptors.

These receptors share a homologous region in their intracellular domain called a death domain (DD) (Tartaglia LA *et al*., 1993; Itoh N *et al.*, 1993) that is capable of transmitting a cytotoxic signal to induce apoptosis. A family of ligands has also been identified that activate their corresponding receptor of the TNF superfamily, including CD95-L (Fas-L), TNF and TRAIL/Apo-2L (Pitti RM *et al*., 1996). CD95-L binds to CD95 and TNF binds to TNFR1 (Beutler *et al*., 1994). The CD95 and CD95L interaction transduce an apoptotic signal by the binding of CD95 to adaptor molecules via a homologous region celled a death domain (DD). The first of these adaptor molecules to be identified was FADD (Fas-associated death domain) by Chinnaiyan and co-workers (1995). FADD preferentially binds to CD95 through the homologous DD, whereas TRADD binds to TNFR. TRADD lacks a DED so must first bind to FADD in order to transduce signals downstream.

The initiator caspases, caspase-8 and -10 differ from other caspases in one respect, that they possess two distinct sequences in their prodomains with homology to a region in FADD called the DED domain. The remainder of the proteins are similar to the other caspases. FADD plays a critical role as an double-adaptor protein in the CD95 pathway of apoptosis. When CD95-L binds to its transmembrane receptor CD95, the signal is transduced and FADD binds to Fas through homologous DD domains. Procaspase-8 can then bind to FADD through its DED

domain, and consequently procaspase-8 is activated by autoprocessing.

Once active, caspase-8 can process other downstream caspases such as caspase-3 in response to CD95-mediated apoptosis (Armstrong RC *et al*., 1996; Schlegel J *et al*., 1996). Stennicke HR *et al*. (1998) confirmed that caspase-3 can be directly activated by caspase-8 and caspase-10, indicating that activation of caspase-3 is the central event in the further transmission of the apoptotic signal. Chemotherapeutic drugs however can induce apoptosis independently of caspase-8. In caspase-8 mutant cells, apoptosis still occurred when induced by adriamycin, etoposide, staurosporine or UV irradiation, although there was a 30-40% reduction in apoptosis. This indicates that here must be another parallel pathway present in which another initiator caspase can transduce pro-apoptotic signals to the downstream caspases.

2.3.8 Activation of caspase-9

Liu X *et al*. (1996) investigated the apoptotic pathway in a cell-free system and concluded that dATP and three cytosolic apoptotic protease factors (apafs) are required for apoptosis to occur. They identified apaf-2 as cytochrome c, this protein was released to the cytosol upon apoptotic stimulation, and in combination with apafs was responsible for the cleavage of procaspase-3 to its active form.

Cytochrome c (cyt c) is an essential component of the mitochondrial respiratory chain, and is normally located in the inner mitochondrial membrane and in the intermembrane space. Numerous groups including Kluck RM *et al*. (1997) demonstrated cytochrome c release into the cytosol during the early stages of apoptosis thus supporting a central role for cytochrome c in the induction of apoptosis. Another essential protein for apoptosis to take place was identified as apaf-3 (Zou H *et al.*, 1997) and Li *et al*. (1998) revealed this to be human caspase-9.

Further studies revealed apaf-1 to be a 130 kDa cytosolic protein with a region in its NH_2-terminus homologous to the prodomain of caspase-9. This region is called a caspase recruitment domain or CARD. The presence of a CARD in apaf-1 implicates it as a recruitment protein for caspases. CARD regions have been found in other caspases with long prodomains, such as caspase-1, -2, and -4, but not in caspase-3 or -6, implying that caspases with a CARD may have roles as upstream initiators. Li *et al*. (1998) reinforced this by demonstrating that apaf-1 can interact with caspase-4, -8 and -9.

Apaf-1 is thought to bring about caspase-9 proteolytic processing by first binding to cytochrome c to become active, then self-associating to form apaf-1 oligomers in the presence of dATP, thus a large multimeric apaf-1-cytochrome c complex is formed (Zou H *et al.*, 1999). Caspase-9 precursor molecules are then induced to bind through the homologous CARD domains (Li *et al*., 1998; Pan G *et al*., 1998), which results in autocatalysis to form active caspase-9. Upon activation, caspase-9 can then process procaspase-3, thus triggering the substrate cleavage events of apoptosis. Reports confirm that caspase-9 interaction with apaf-1 and cytochrome c is a critical pathway involved in apoptosis (Seol DW and Billiar TR., 1999).

2.3.9 Regulation of apoptosis by Bcl-2 family

Bcl-2 plays an important role in apoptosis regulation, and overexpression of bcl-2 provides cells with a survival advantage. Bcl-2 is the mammalian homologue of ced- 9, which was shown in *C. elegans* to prevent pro-apoptotic actions of ced-3 and ced-4. Bcl-2 blocks apoptosis after a wide variety of stimuli and in doing so, allows the cells that experience DNA damage to avoid death and enables the acquisition of genetic aberrations and the emergence of neoplasia. Bcl-2 is located in the outer mitochondrial membrane, endoplasmic reticulum and nuclear envelope,

however some Bcl-2 family members are largely cytosolic, such as Bax. Numerous Bcl-2 family members have been identified in mammalian cells, and have been divided into pro-apoptotic members (e.g. Bcl-xs, Bax, Bad) and pro-survival members (e.g. Bcl-2, Bcl-XL).

Pro- and anti-apoptotic proteins can hetero- or homo-dimerise, and depending on the ratio of pro- to anti-apoptotic proteins, the susceptibility of a cell to apoptosis can be determined. For example, overexpression of Bax enhances apoptosis, but if Bcl-2 is overexpressed it can heterodimerise with Bax and apoptosis is prevented. Overexpression of anti-apoptotic proteins such as Bcl-2 or Bc1-Xl (Ibrado AM *et al.*, 1996; Schmitt E *et al.*, 1998) can inhibit caspase-3 activation (Ibrado AM *et al*., 1996), indicating that the Bcl-2 family interferes with one of the central events in apoptosis, namely caspase activation.

2.3.10 Functions of Bcl-2 family-Regulation of apoptosis

Bcl-2 proteins can regulate apoptosis in two ways, the first involving cytochrome c release from mitochondria and the second involving interaction with apaf-1. Pro- apoptotic proteins like Bax and Bid can directly induce cytochrome c release from mitochondria (Luo X *et al*., 1998) perhaps via a pore forming ability. Bcl-XL and Bcl-2 can prevent cytochrome c release from mitochondria (Kluck RM *et al*., 1997 and Yang J *et al*., 1997).

However the Bcl-2 family proteins can regulate apoptosis independently of dimerisation. Bax can antagonize Bcl-XL without hetero-dimerising with Bcl-XL (Simonian PL *et al*., 1997). Luo X *et al* (1998) and Li *et al*. (1998) reported Bid to be a direct link between activated caspase-8 and the downstream caspases. Possessing only a BH_3 domain, full length Bid usually exists as an inactive form in the cytosol of cells, but when caspase-8 becomes activated during the apoptotic signaling pathway, Bid is cleaved and activated by caspase-8. The truncated part translocates to

the mitochondria, thus unleashing its pro-apoptotic activity and inducing cytochrome c release into the cytosol, which in turn initiates the downstream caspase activation and subsequent cell shrinkage and nuclear condensation. The counter effect of the anti-apoptotic protein Bcl-XL inhibits all the apoptotic changes induced by the active Bid.

The second method of regulation is through binding to apaf-1, and consequently governing activation of procaspase-9. It is thought that Bcl-XL can bind to apaf-1 and prevent caspase-9 from binding to it. If a pro-apoptotic member such as Bik is present, heterodimers can form, allowing free apaf-1 to bind to procaspase-9. Procaspase-9 is then activated through autocatalysis.

Bcl-2 family members act downstream of caspase-8 activation. Srinivasan A *et al*. (1998) demonstrated that Bc1-Xl can block apoptosis in cells with a catalytically active caspase-8. This may occur through the ability of Bc1-Xl to interact with apaf-1 and inhibit its association with caspase-9 (Hu Y *et al*., 1998). Bcl-XL shares a CED-4 domain with apaf-1. So through binding at this region Bcl-XL may prevent caspase-9 from binding to the CARD region in the N-terminal of apaf-1. This finding indicates that Bc1-Xl can regulate apoptosis independently of its association with intracellular membranes.

2.3.11 Regulation of Bcl-2 family

Playing such a critical role in apoptosis regulation suggests that Bcl-2 family members must be tightly regulated to maintain an environment, which can switch on or prevent cell death at the introduction of a stimulus. Regulation occurs at different levels, both transcriptionally and post-translationally. In particular, the BH-3 domain- containing proteins seem to be regulated by being restricted to the cytosol of living cells, but in response to a death signal they can be

activated as a result of increased transcription or translation or post-translational modification such as phosphorylation or cleavage, and then transported to the mitochondria. Bax is upregulated in response to DNA damage (Kozopas KM *et al*., 1993) and translocates to the mitochondria (Wolter KG *et al.*, 1997). When survival signals exist in the cell, Bad is phosphorylated in response to the survival factor IL-3, binds to a 14-3-3 protein and becomes sequestered in the cytosol. The phosphorylation of Bad prevents it from heterodimerising with Bc1-Xl, thus allowing Bcl-XL to promote cell survival. However, on removal of IL-3 Bad is dephosphorylated, and then can move to the mitochondria to induce apoptosis (Zha J *et al*., 1996). Bid is also activated in response to apoptotic stimuli, is cleaved and translocated to the mitochondria (Luo X *et al*., 1998; Li H *et al*., 1998).

2.4 Apoptosis and DNA damaging drugs

Since there are a number of possible apoptotic signaling pathways present, each converging on the activation of effector caspases, the pathway resulting from chemotherapeutic drug treatment has very important implications for effective tumor cell kill to undergo chemotherapeutic drug-induced apoptosis. Any defects in this pathway would result in defective apoptosis and ensuing resistance.

Numerous groups have verified the role of caspases in apoptosis induced by various DNA damaging agents with diverse chemical structures and mechanisms of action. DNA damaging agents such as Ara-C, cisplatinum, etoposide, camptothecin (Datta R *et al*., 1996) have been shown to activate caspase-3 activity. Faleiro L *et al*. (1997) have reported that the major active caspases in cells induced to undergo apoptosis by etoposide are caspase- 3 and caspase-6, and these caspases are present as multiple species which can vary in activity between cell type

(Martins *et al*., 1997b). Simizu *et al*. (1998) demonstrated the requirement of caspase-3 for apoptosis (mediated by hydrogen peroxide production) induced by camptothecin, vinblastine, inostamycin and adriamycin. Keane RW *et al*. (1997) have also shown that caspase-3 activity is essential for apoptosis to occur after staurosporine treatment.

All of the reports confirm the role of caspase-3 in apoptosis induced by chemotherapeutic drugs, but caspase-3 is an effector caspase. So the question remains as to what are the initiator complexes involved in the chemotherapeutic drug signaling pathway? The main contenders are obviously CD95 or a caspase containing a CARD domain, namely caspase-9. A body of evidence exists for each of these initiators at present. Originally CD95 was thought to be the main signaling protein involved, but with the recent discovery of caspase-9, some very convincing reports prove the involvement of caspase-9 in cytotoxic induced apoptosis.

2.4.1 FAS-involvement

Fulda S et al. (1998) showed that CD95 and CD95-L levels were upregulated on neuroblastoma cells after treatment with doxorubicin, VP-16 and cisplatinum, and they proposed that this upregulation of CD95/Fas was responsible for apoptotic induction in the cells. Furthermore when CD95 and CD95-L production was inhibited by cyclosporin A, there was a significant (up to 50%) reduction in drug-induced apoptosis. They also demonstrated that CD95-resistant cells were resistant to doxorubicin and cisplatinum suggesting a common signaling pathway. These findings indicate that the activation of caspases was most probably through the activation of the CD95/CD95-L pathway. These results are reinforced by a recent publication by Fulda S *et al*. (1998) in which they report cleavage of the apical caspase-8, the downstream caspase-3 and PARP cleavage in doxorubicin treated neuroblastoma cells**.**

In contrast to these findings, a number of groups report that CD95-independent drug- induced apoptosis can also occur. Eischen CM *et al*. (1997) concluded that even though CD95-and chemotherapy-induced pathways converge on downstream apoptotic pathways, the mechanism of drug-induced apoptosis can occur in a CD95-induced manner. Using leukaemia cells which were resistant to CD95-induced apoptosis they demonstrated that these resistant cells were sensitive to apoptosis induced by a wide variety of chemotherapeutic agents such as etoposide, doxorubicin, cisplatin, staurosporine and methotrexate. Cells then treated with the ZB4 Fas-blocking antibody were still susceptible to apoptosis when cells were treated with etoposide or doxorubicin, suggesting a CD95-independent apoptotic pathway for these drugs.

2.4.2 Caspase-9 Involvement

The finding that drug-induced apoptosis could occur independently of the CD95/CD95-L pathway led researchers to search for another upstream component of the apoptosis pathway which could act in an independent and parallel fashion, and yet converge on the similar pathway in which caspase-3 is activated and apoptotic events commence. The pathway involving caspase-9, apaf-1 and cytochrome c soon became apparent. When cytochrome c is released from mitochondria, apoptosis is induced. Apaf1is the human homologue of the *C.elegans* CED4 protein, and this along with the cytochrome c (apaf-2) and caspase-9 have been shown to activate caspase-3. Kuida K *et al*. (1998) recently showed that caspase-9 deficient mice had reduced apoptosis, a result of inhibition of procaspase-3 activation. Caspase-9 is necessary *in vivo* for cytochrome c-mediated caspase-3 activation and apoptosis to occur. These findings indicate that caspase-9 is a key activator of the apoptotic cascade.

2.5 Oxidative stress

Recent studies suggest that targeting the unique biochemical alterations in cancer cells might be a feasible approach to achieve therapeutic activity and selectivity, and perhaps prevent the development of drug resistance. Most cancer cells exhibit increased aerobic glycolysis (the Warburg effect) and oxidative stress, features that could be important in the development of new anticancer strategies.

Oxidative stress has been defined as an imbalance between oxidants and antioxidants in favor of the former, resulting in an overall increase in cellular levels of reactive oxygen species. Mounting evidence suggests that, compared with their normal counterparts, many types of cancer cell have increased levels of reactive oxygen species (ROS) (Szatrowski TP., 1991; Kawanishi S *et al*., 2006; Toyokuni *et al*., 1995). Reactive oxygen species have essential functions in living organisms. A moderate increase in ROS can promote cell proliferation and differentiation (Boonstra, J *et al*., 2004; Schafer F *et al*., 2001) whereas excessive amounts of ROS can cause oxidative damage to lipids, proteins and DNA (Perry G *et al.*, 2000). Therefore, maintaining ROS homeostasis is crucial for normal cell growth and survival. As illustrated in Figure 2.3, cells control ROS levels by balancing ROS generation with their elimination by ROS-scavenging systems such as superoxide dismutases (SOD1, SOD2 and SOD3), glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin and catalase. Increased ROS may play a role in the development of cancer. However, as excessive levels of ROS cause toxicity in cells, exogenous agents triggering ROS above toxic threshold cause damage to cancer cells.

ROS can be found either in the environment, for example, as pollutants, tobacco smoke, iron salts and radiation, or it can be generated inside cells. ROS is produced in cells through multiple

mechanisms. A major source of ROS is produced in the mitochondria. Electron leakage from the mitochondrial respiratory chain may react with molecular oxygen, resulting in the formation of superoxide, which can subsequently be converted to other ROS. In phagocytes and some cancer cells, ROS can be produced through a reaction catalyzed by NADPH oxidase complexes. ROS can also be produced as a byproduct of certain biochemical reactions, such as β- oxidation in peroxisomes, prostaglandin synthesis and detoxification reactions by cytochrome P450.

Although importance has been placed on the role of oxidative nuclear DNA damage in neoplasia, other evidence has demonstrated the involvement of the mitochondrial oxidative DNA damage in the carcinogenesis process (Schumacher HR., 1973; Cavalli LR *et al*., 1998). The sustained oxidative burden in the mitochondria has been linked to the induction of mutation. Mitochondrial DNA mutations and alterations in mitochondrial genomic function appear to be causally related to the development of neoplasia. Furthermore, the mutation rate in mitochondrial DNA has been reported to be at least two orders of magnitude higher than that of nuclear DNA (Wang E *et al*., 1997). At least three factors for the increased susceptibility of the mitochondrial genome should be considered. (*a*) Mitochondrial DNA is in close proximity to the electron transport system, a major source of reactive oxygen species. Under physiological conditions, the mitochondria convert 4% to 5% of oxygen consumed into superoxide anion and subsequently hydrogen peroxide (Barber DA and Harris SR., 1994). (b) Mitochondrial DNA is not protected by histones. (*c*) DNA repair capacity is limited in the mitochondria, which completely lack nucleotide excision repair (Bohr VA and Dianov GL., 1999). Collectively, these finding may partially explain the increased frequency of mitochondrial mutations seen in tumor cells.

In spite of intrinsic oxidative stress, ROS can make cell survive by mobilizing the adaptive mechanisms. Such mechanisms not only trigger activation of ROS scavenging systems but also

inhibit apoptosis. Evidences suggest that such adaptation leads to malignant transformation, metastasis and anti cancer drug resistance. Increased ROS generation in cancer cells is owing to loss of p53 function, activation of oncogenes, mitochondria dysfunction and aberrant metabolism. The proto-oncogenes like Ras, Bcr-Abl and c-myc associated with tumor transformation were found to trigger the ROS production (Behrend L *et al*., 2003).

In advanced stages of cancer, cancer cells usually show genetic instability and exhibit significant elevation of ROS production. The excessive ROS generation induces gene mutations in mitochondrial DNA resulting in further metabolic malfunction and ROS generation (Pelicano H *et al*., 2004; Van houten *et al*., 2006). P53, guardian of genome, abolish the oxidative damage to mitochondrial DNA and nuclear genome thereby it prevents oxidative gene mutations and genetic instability (Achanta G *et al*., 2004; Zurer *et al*., 2004; Achanta G *et al*., 2005). When loss of functional p53 is associated with redox imbalance it leads to increased oxidative stress, eventually resulting aggressive tumor growth (Attardi L D *et al.,* 2005).

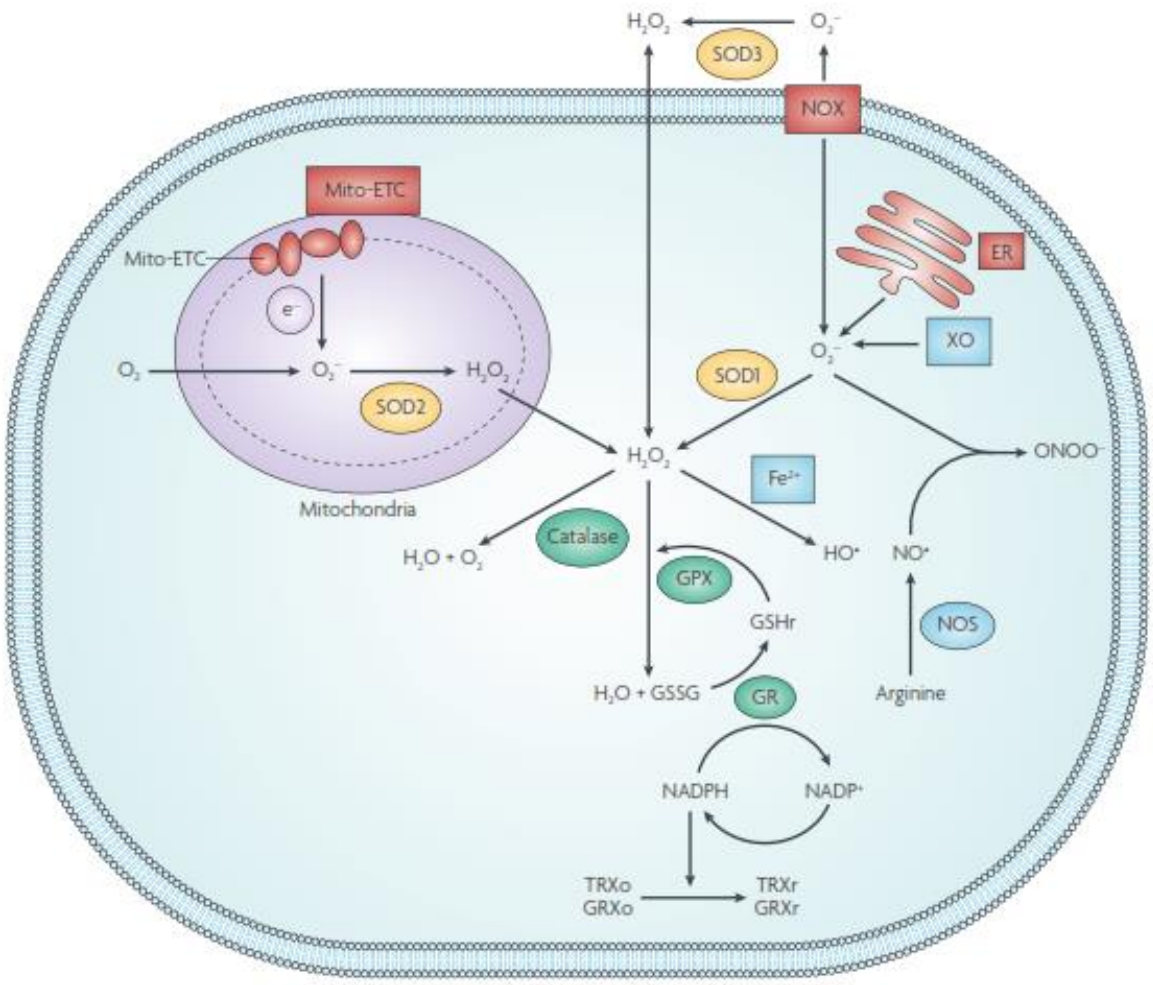


Figure 2.3: Schematic illustration of cellular redox homeostasis (Trachootham D *et al*., 2009)

2.5.1 Adaptation to ROS stress in cancer cells

Severe accumulation of cellular reactive oxygen species (ROS) under various endogenous and exogenous stress stimuli may induce lethal damage in cells that have inadequate stress responses or adaptation. In certain cancer cells, persistent ROS stress may induce adaptive stress responses including activation of redox-sensitive transcription factors, such as nuclear factor κB (NF-κB) and Nrf2, leading to an increase in the expression of ROS-scavenging enzymes, such as superoxide dismutase and glutathione, elevation of survival factors such as BCL2 and MCL1,

and inhibition of cell death factors, such as caspases. ROS-mediated DNA mutations or deletions promote genomic instability and thus provide an additional mechanism for stress adaptation. All these events enable cells to survive with the high level of ROS and maintain cellular viability. As these transcription factors also have roles in regulating the expression of genes that are responsible for proliferation, senescence evasion, angiogenesis and metastasis, the redox adaptation processes may promote cancer development. Furthermore, the increase in glutathione during adaptation can enhance the export of certain anticancer drugs and their inactivation. This altered drug metabolism together with enhanced cell survival may render cancer cells more resistant to chemotherapeutic agents (Figure 2.4) (Trachootham D *et al*., 2009).

2.5.2 Targeting redox alterations in cancer

In cancer cells, the increase in ROS generation from metabolic abnormalities and oncogenic signalling may trigger a redox adaptation response, leading to an upregulation of antioxidant capacity and a shift of redox dynamics with high ROS generation and elimination to maintain the ROS levels below the toxic threshold. As such, cancer cells would be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant system. A further increase of ROS stress in cancer cells using exogenous ROS-modulating agents is likely to cause elevation of ROS above the threshold level, leading to cell death. This might constitute a biochemical basis to design therapeutic strategies to selectively kill cancer cells using ROS-mediated mechanisms (Figure 2.5). ROS modulating agents are given in Table 2.1.

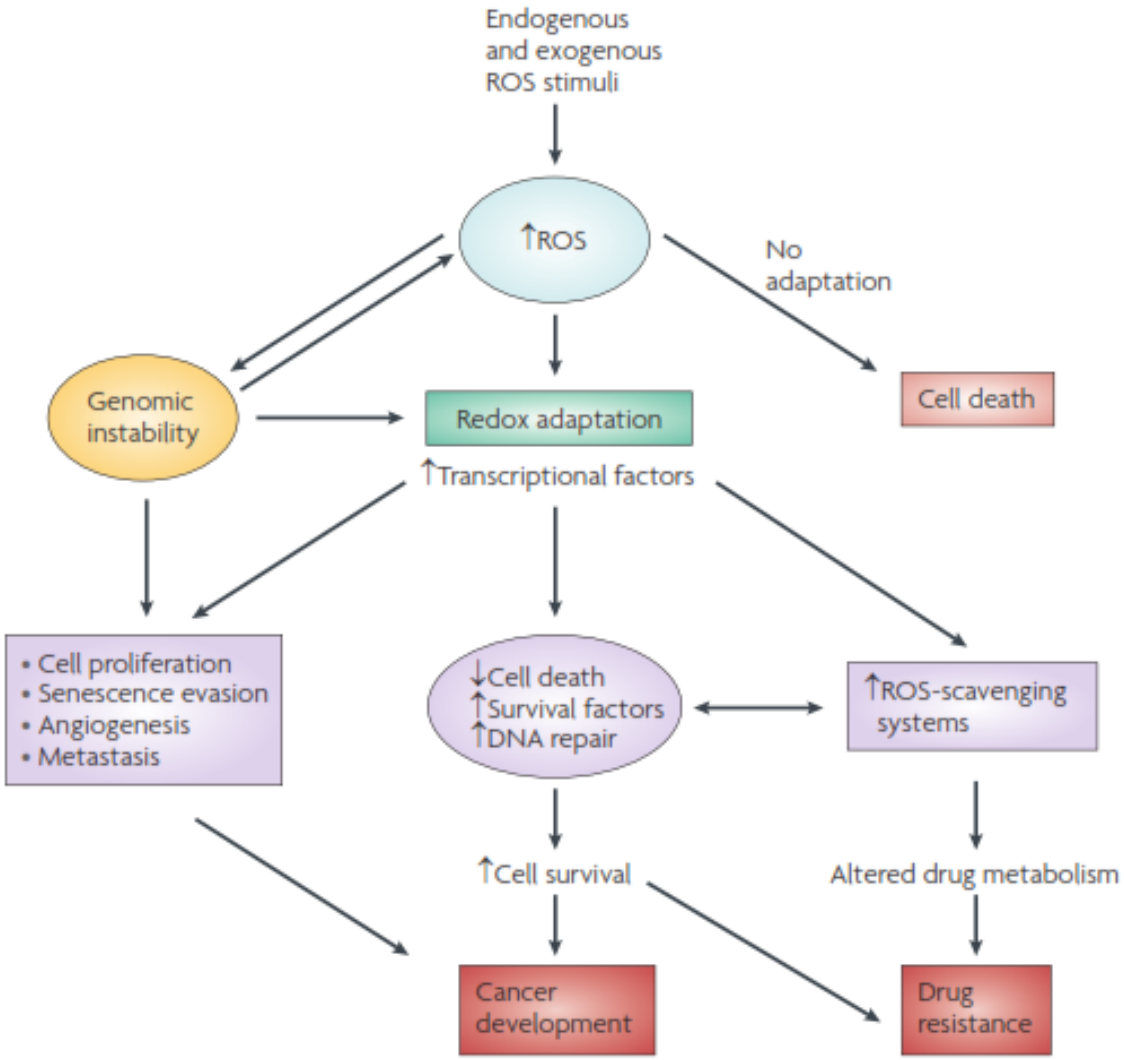


Figure 2.4: Redox adaptation in cancer development and drug resistance (Trachootham D et al., 2009).

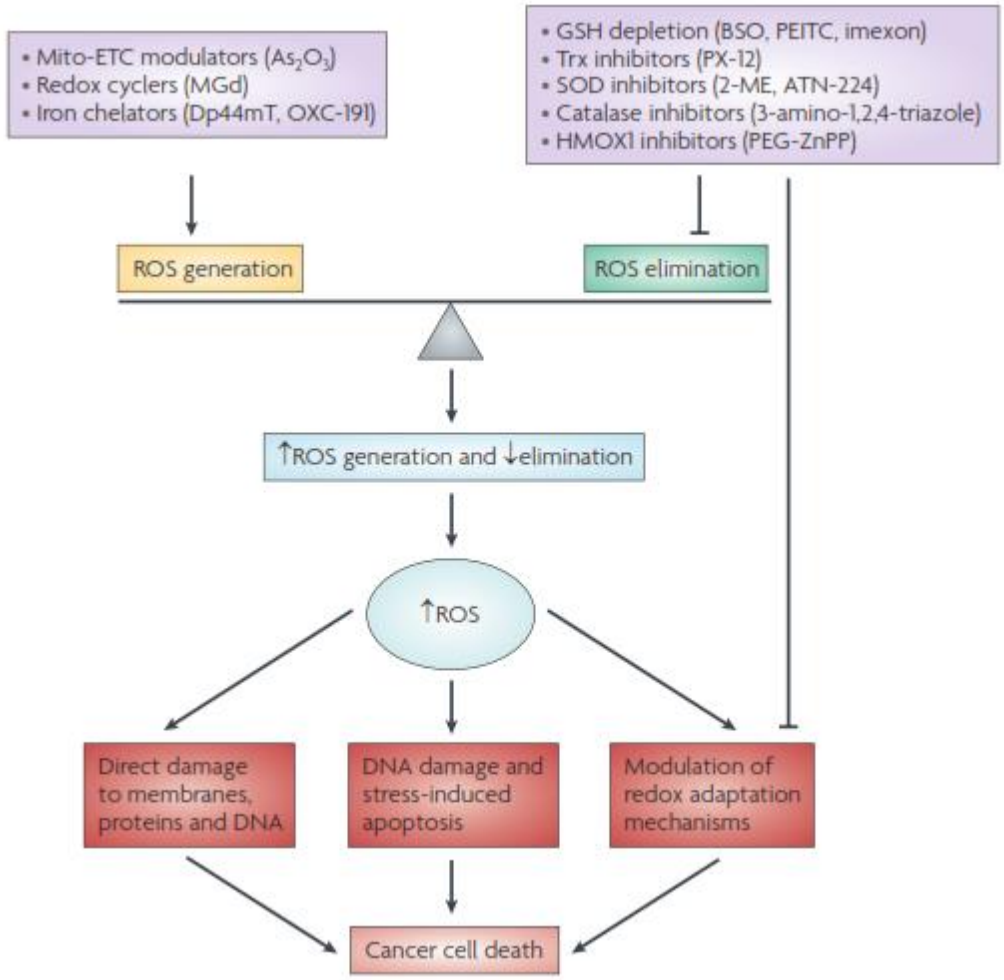


Figure 2.5: Targeting cancer cells through ROS-mediated mechanisms (Trachootham D *et al*., 2009)**.**

Table 2.1: ROS modulating agents (Trachootham D *et al*., 2009)

ROS modulating agents	**Mechanism of action**	**References**
Exogenous ROS generating agents		
Motexafin gadolinium (gadoliniumtexaphyrin)	Pro-oxidant catalyst that induces intracellular superoxide formation and inhibits TrxR, and preferentially accumulates in tumour cells	Magda D *et al*., 2006
β-Lapachone (ARQ 501)	Undergoes futile redox cycles catalysed by intracellular NQO1	Bey EA *et al*., 2007

Inhibitors of the antioxidant system		
Buthionine sulphoximine	• Inhibits GSH synthesis • Enhances As203 activity	Maeda H *et al*., 2004
Imexon	Depletes the GSH pool by binding tothiols	DragovichT *et al*., 2007
Phenylethyl Isothiocyanate	• Conjugates and exports GSH outside cancer cells. • Inhibits GPx and NF-B	Trachootham D *et al*., 2006
Mangafodipir	SOD, catalase and GSH reductase mimetic — increases H202levels in cancer cells but acts as antioxidant in normal cells	Alexandre J. *et al., 2006*
Tetra thiomolybdate	Inhibits cytosolic SOD1	Juarez JC *et al.*, 2008
Multiple mechanisms of action		
As203	• Inhibits GPx and TrxR • Inhibits the mitochondrial respiratory chain	Lu J *et al*., 2007; Pelicano H *et al*., 2003
Unknown mechanism of action		
Elesclomol (STA-4783)	• Induces rapid ROS accumulatiin cancer cells leading to apoptosis • Enhances paclitaxel activity	Tuma RS., 2008; Kirshner, JR. *et al.*, 2008

2.6 Review on anti cancer activity of selected phytocompounds

2.6.1 Withasteroids

Withasteroids are a group of naturally occurring C_{28}-steroidal lactones built on intact or rearranged ergostane framework. Withanolides yielded from familiar medicinal plants used in traditional systems of medicine possess wide spectrum of biological effects including antimicrobial, anti-inflammatory, hepatoprotective, immunomodulatory along with anti tumor activities (Budhiraja *et al*., 2000; Glotter *et al*., 1978., Ray AB., 1994).

2.6.1.1 Withametelin

Withametelin (WM), belong to the withasteroids family, was found to have cytotoxic effects against cancer cells including brain, colon and lung (Zhang H., 2012). Bellila A *et al* documented that WM arrested cell cycle at S phase and induced apoptosis in DLD-1 cells (Bellila A *et al*., 2011).

2.6.1.2 Withaphysalin E

Withaphysalin E was isolated from fruits and other aerial parts like leaves and shoots of *Physalis minima* Linn. and pharmacological activities of withaphysalin E included antimalarial, antigonorrheal, anti-inflammatory, analgesic and antipyretic, antibacterial, anti-ulcer, cytotoxic, hypoglycemic, anti-fertility activities (Chothani *et al*., 2012).

2.6.1.3 Withaphysalin D

Withaphysalin D was isolated from the leaves and shoots of *Physalis minima Linn* and pharmacological activities of withaphysalin D included antimalarial, anti-inflammatory, antipyretic, antibacterial, anti-ulcer, cytotoxic, hypoglycemic activities (Sahai M *et al.,* 1984).

2.6.1.4 Withaperuvin B

Withaperuvin B was isolated from the fruits of *Physalis peruviana* and *Nicandra physaloides* (Sahai M *et al.,* 1982). This compound has not been explored for any pharmacological effect.

2.6.1.5 Physalolactone

Sahai *et al* reported that physalolactone was isolated from *Physalis peruvania* (Ray AB., 1978). No literature reports on cytotoxic effects of physalolactone.

2.6.1.6 12-Deoxy withastramonolide

Roots of *Aswagandha* were found to be a source of 12-deoxy withastramonolide and *Aswagandha* was reported to have anti-stress, anti inflammatory, antioxidant and anti tumor agent activities.

2.6.2 Alkaloids:

2.6.2.1 Coptisine:

Coptisine (COP), broadly belonging to isoquinoline group of alkaloids was first reported by Awe W (1951) by processing *Chelidonium majus* tincture (Awe W., 1951). Subsequently presence of COP as a major constituent in plants, *Corydalis ternata* (Lee Hyang-Yi *et al*., 1999), *Fumaria indica* (Pandey V *et al*., 1976), *Chelidonii herba* (Colombo ML *et al*., 2001), *Coptic japonica* (Lim, SY *et al*., 2002), *Corydalis adunca* (Zhao DB *et al*., 2005), *Corydalis yanhusuo* (Chen YU

et al., 2006) and *Coptis chinensis* (Ye X *et al*., 2015; Pandey V *et al*., 1976) were identified. Previous pharmacological findings on COP have reported it to possess antibiotic (Jung HA *et al*., 2008), antibacterial (Yan D *et al*., 2008), antiviral (Li H *et al*., 2008) and cardiovascular protection (Gong L *et al*., 2012) properties.

Combo *et al* made an attempt for the first time to test cytotoxicity of coptisine against a panel of human cancer cells belonging to colorectal and leukemia. Coptisine displayed cytotoxicity against the Lovo cell line (IC_{50}=0.87 μg/ ml) and L1210 (IC_{50} = 0.87 μg/ml), its cytotoxicity nonetheless was twice higher on HT 29 cell line (IC_{50}=0.49 μg/ml). (Colombo ML *et al*., 2001)

Chun-Ching Lin *et al* evaluated cytotoxicity of coptisine further against eight different human cancer cells pertaining to hepatoma and leukemia. Coptisine showed more potent cytotoxic activity against hepatoma cell proliferation with IC_{50} values ranging between 1.4 to 6.6 μM and proliferation of leukaemia cell with IC_{50} values ranging from 0.6 to 10.9 μM. Results revealed that important role of COP in the cytotoxic effect against hepatoma and leukaemia cell growth. But the possible mechanism(s) of the pharmacological actions of these compounds remain unknown (Lin C *et al*., 2004).

Tanabe H *et al* reported that coptisine arrests cells of VSMCs at G_0/G_1 phase by accelerating proteasome-mediated degradation of cyclin D1, and at G_2/M phase by inhibiting tubulin polymerization. These findings may provide critical insights for the development of novel therapeutic agents against progressive atherosclerotic diseases (Tanabe H *et al*., 2005).

Jing *et al* demonstrated that coptisine efficiently suppressed the adhesion, migration and invasion of human breast cancer MDA-MB-231 cells by interfering in the expression of MMP-9 and its specific inhibitor TIMP-1. These findings provide insight for the possibility of coptisine as a

potential therapeutic candidate against tumor invasion (Li J *et al*., 2014).

2.6.2.2 Eleocarpine

Source of Eleocarpine is *elaeocarpus sphaericus*. The reported biological activities of *Elaeocarpus sphaericus* were anti depressant, anti-inflammatory, anti oxidant, anti microbial, anti diabetic, anti malarial and cytotoxic activities (Garg K *et al*., 2013). However, there are no cytotoxic reports on eleaocarpine.

2.6.2.3 Tigloidine

It was originally believed to be pharmacologically inactive compound. However, later it was found to be effective in treating Parkinsonism. (Sanghvi *et al*., 1968). Anti cancer activity of tigloidine has not been demonstrated so far.

2.6.3 Coumarins

2.6.3.1 Cedrelopsin

Cedrelopsin was isolated from *Oldenlandia umbellata* L. In Indian System of Medicines, leaves and roots of *O. umbellata* L. are used as an expectorant, given in asthma, bronchitis and consumption. A decoction of the leaves is used as a wash for poisonous bites (Rekha S *et al*., 2006; Yoganarasimhan S., 2000). Despite, their wide distribution and extensive clinical use, there were very limited amount of scientific data available on *O. umbellata*. The crude methanolic extract of *O. umbellata* at 250 and 500 mg/kg, had shown significant hepatoprotective and antioxidant effect against CCl_4 induced rat model (Gupta M *et al*., 2007). Similarly, the in-vitro studies carried out with aqueous extract have explored the anthelmintic effect of *O. umbellata* (somanth D *et al*., 2014). Antitussive activity on mice was explored by

Hema V *et al*., (2007) wherein the ethanolic extract of the plant at 500 mg/kg body weight showed significant activity when compared with control group. Further investigations on this plant have demonstrated the antibacterial (Rekha S *et al*., 2006), anti-inflammatory and antipyretic effects of *O. umbellata* (Padhy I and Endale A., 2014). Cytotoxic activity of Cedrelopsin has not been documented so far.

2.6.3.2 Cleomiscosin A

Cleomiscosin A was isolated from defatted seed of *Cleome viscosa* and was also found to be isolated from *Samba multiflora*, *Matayba arborescens* (Tanaka H *et al.*, 1985) and *Hyoscyamus niger* (Sajeli B *et al*., 2006). Anti tumor activity of cleomiscosin A was demonstrated in P-388 lymphatic leukemia test system and it also possessed hepatoprotective activity (Chattopadhyay S *et al.*, 2009). However, no cytotoxicity reports have been documented on selected cell lines for this work.

2.6.4 Iridoid glycosides

2.6.4.1 Feretoside

The bark of *Eucommia ulmoides* has been used to isolate feretoside and this plant has been used as traditional medicines in china to treat hypertension and liver and kidney damage. Feretoside was found to induce the different heat shock proteins including Hsp 1, 27 and 70 (Nam J *et al*., 2013). No scientific studies related to anti cancer activity of feretoside were reported.

2.6.4.2 Deacetyl asperuloside

Calis *et al* reported the isolation of Deacetyl asperuloside from *Globularia trichosantha* and additional sources for the same included *Coprosma pyrifolium*, *Hedyotis hedyotidea*,

Morinda citrifolia L. *and Oldenlandia corymbosa* (Calis *et al*., 2001). Anti cancer reports for deacetyl asperuloside has not been documented.

2.6.4.3 Asperulosidic acid

Source of asperulosidic acid was found to be herbs of *Hedyotis diffusa Willd* (Zhang HJ *et al*., 2006). Studies related to anti cancer activity on asperulosidic acid has not been reported.

2.6.5 Anthraquinones

Sources of both 1,2-dimethyl-3-hydroxy-9,10 anthracenedione and 1,3-di methyl-3 hydroxy-9,10 anthracenedione was reported to be *oldenlandia umbellata*. There were no scientific reports on anti cancer activity of 1,2-dimethyl-3-hydroxy-9,10 anthracenedione and 1,3-di methyl-3 hydroxy-9,10 anthracenedione.

2.6.6 Terpenoids

Jahromi MF *et al* reported that pedunculoside was isolated from *Ilex doniana*. It exhibited significant hypocholesterolemic activity in hyperlidemic albino rats (Jahromi MF *et al.*, 1999). Anti cancer activity of pedunculoside has not been documented so far.

2.6.7 Phenolics

2.6.7.1 Bergenin

Bergenin demonstrated potent inhibitory effect against skin tumor promotion in an *in vivo* two-stage mouse skin carcinogenesis test based on 7,12-dimethylbenz[a]anthracene (DMBA) as initiator, and with TPA (12-O-tetradecanoylphorbol-13-acetate) as promoter (Zhang J *et al*., 2013).

2.6.7.2 Norbergenin

Taneyama *et al* documented the isolation of norbergenin from *Saxifraga stolonifera* (Taneyama M *et al*., 1983). Anti ulcer activity and immunomodulatory activity of norbergenin have been demonstrated (Nazir N *et al*., 2007; Goel RK *et al*., 1997). Anti cancer activity of norbergenin has not been reported.

2.6.7.3 Methyl caffeate

Methyl caffeate is increasingly regarded as a potential anti cancer agent that could inhibit progression of cancer. Results suggested that it induced cytotoxicity in MCF-7 cells through activation of intrinsic apoptotic pathway (Bailly F *et al.*, 2013).

Lee *et al* reported that methyl caffeate inhibited the proliferation of PC14 and MKN45 human cancer cells (Lee IR and Yang MY., 1994).

CHAPTER 3

OBJECTIVES AND PLAN OF WORK

CHAPTER 3

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3.1 Objectives:

Lung cancer is one of the leading causes of death of men and women worldwide and its incidence and mortality is increasing day by day. Inspite of major developments in lung cancer therapy, mortality of lung cancer patients still stands at high level. For treating lung cancer, chemotherapy is one of the most common treatments in addition to surgery and radiation. Anti cancer drugs from medicinal plants account for significant proportion of first-line anti cancer therapies. Though surgery together with chemotherapy offers hope for cure, survival rate of patients remains poor. Also, current therapies have limitations, it is necessary to improve the treatment efficacy for combating this deadly disease. Therefore, our main objective was to explore effective cytotoxic agents and to comprehend their mechanism of action at molecular and cellular level.

1. Selection and Screening of cytotoxicity of some phytocompounds against A549 cells.
2. Isolation of potent molecules from specific herbal sources and followed by their authentication through spectral characterization.
3. Investigation of mechanism underlying the cytotoxicity of potential molecules.

3.2 Plan of work:

The plan of work for this project was drafted as follows:

1. **Selection and screening of plant derived compounds for their anti proliferative activity against A549 cell line:**

   Twenty compounds belonging to different groups of plant secondary metabolites *viz*., withasteroids, alkaloids, coumarins, iridoid glycosides, terpenoids, anthraquinones and phenolics were selected based on literature survey. These compounds were screened against A549 cells and HEK-293 cells.

2. **Isolation and characterization of potential cytotoxic molecules from specific herbal sources:**

   A. Isolation of coptisine from seeds of *Fumaria indica* (Papaveraceae)

   B. Isolation of withametelin from *Datura innoxia* (Solanaceae)

   C. Isolation of cedrelopsin from *Oldenlandia umbellata* (Rubiaceae)

   D. Characterization of isolated molecules using spectral analysis

3. Exploration of mechanisms underlying the cytotoxic effect of potent molecules:

A. Effect of potent cytotoxic compounds on cell cycle distribution

B. Effect of potent cytotoxic compounds on G2/M regulatory proteins

C. Effect of potent cytotoxic compounds on apoptosis of A549 cells and effect of NAC on compounds-induced apoptosis

D. Effect of potent cytotoxic compounds on ROS generation and effect of NAC on compounds triggered ROS generation

E. Effect of potent cytotoxic compounds on mitochondrial membrane potential and mitochondria related proteins and Effect of NAC on compound induced MMP loss

F. Effect of potent cytotoxic compounds on activity of caspases and effect of NAC on compounds induced caspase-3 induction

4. Testing of anti proliferative activity of phytocompounds against other cancer cells like breast and colorectal cancer cell lines

A. Effect of anti proliferative activity of selected phytocompounds against MDA-MB-231 cells

B. Effect of anti proliferative activity of selected phytocompounds against HT-29 cells

CHAPTER 4

MATERIALS AND METHODS

CHAPTER 4

MATERIALS AND METHODS

4.1 Compounds for evaluation

The compounds used for the evaluation were obtained from the repository of Natural Product Laboratory at Bits-Pilani Hyderabad Campus. The list of selected molecules is provided in Table 4.1.1. The authenticity of the compounds was established based on spectral analysis and purity was ascertained based on TLC studies using various solvent systems.

Table 4.1.1 List of phytocompounds selected for the evaluation of cytotoxicity

Group of secondary metabolite	Compound name	Group of secondary metabolite	Compound name
Withasteroids	Withametelin	Iridoid glycosides	Deacetyl asperuloside
	Withaphysalin E		Asperulosidic acid
	Withaphysalin D		Feretoside
	12-Deoxy withastramonolide	Anthraquinones	1,2-di methyl-3-hydroxy-9,10-anthracenedione
	Withaperuvin B		1,3-di methyl-3-hydroxy-9,10-anthracenedione
	Physalolactone	Terpenoids	Pedunculoside
Alkaloids	Coptisine	Phenolics	Bergenin
	Eleocarpine		Methyl caffeate
	Tigloidine		Nor bergenin
Coumarins	Cedrelopsin		
	Cleomiscosin A		

4.2 Isolation, purification and characterization of potential phytocompounds

All chemicals, solvents and reagents used were of analytical grade. Solvents used in HPLC analysis were of HPLC grade obtained from Merck Specialities Private Ltd., Mumbai, India. Column chromatography (CC): silica gel (60-120 µm, 100-200 µm and 230-400 µm); Merck Specialities Private Ltd., Mumbai, India Lyophilization was done using Coolsafe 27™, Scanvac. HPLC used for analysis was Shimadzu, Japan, equipped with UV and PDA detectors. ^{1}H NMR spectral analysis for samples dissolved in DMSO was carried out using Bruker *DRX* 300 spectrometer.

4.2.1 Isolation of coptisine from *Fumaria indica*

4.2.1.1 Plant material and general requirements

The seeds of *Fumaria indica* (Family: Fumariaceae) were purchased from local herbal market in Varanasi, Uttar Pradesh and authenticated by Dr. V. K. Joshi, Department of Dravya Guna, Institute of Medical Sciences, Varanasi, India. A voucher specimen (FI/2013/13-2) has been deposited at Department of Pharmacy, BITS-Pilani Hyderabad Campus, Telangana State, India.

4.2.1.2 Isolation and purification of coptisine

Around 1000 g of seeds were powdered and extracted in a Soxhlet Extractor using methanol. The methanolic extract was evaporated under reduced pressure to thick syrup. This was then treated with 7 % aqueous citric acid, stirred for 10 h and filtered. The filtrate was basified by adding ammonium hydroxide (to pH 9) and extracted using chloroform (200 ml) thrice. The chloroform soluble portion was made moisture free by treating with anhydrous sodium sulfate and filtered. The filtrate was evaporated to dryness and subjected to column chromatography over neutral Alumina using solvents of increasing polarity. Elution using 100 % ethyl acetate yielded yellow

coloured solid (20 mg), which on further purification with solvent mixture of chloroform and methanol produced pale yellow powder, identified as coptisine (COP).

4.2.2 Isolation of withametelin from *Datura metel*

4.2.2.1 Plant material

The fresh flowers of *Datura metel* Linn., (Family: Solanaceae) were collected near Jawahar Nagar Village, Hyderabad, Telangana State, India. A voucher specimen (DM/2013/13-1) has been deposited at Department of Pharmacy, BITS-Pilani Hyderabad Campus, Telangana State, India.

4.2.2.2 Isolation and purification of withametelin

Around 1000 g of freshly collected flowers of *D. metel* were macerated and soaked in methanol for one week. The crude methanolic extract was filtered and the filtrate was concentrated under reduced pressure. The residue obtained was diluted with water and extracted with chloroform. The chloroform soluble portion (8 g) was chromatographed over silica gel and eluted using solvents of increasing polarity. The chloroform-ethyl acetate (75:25) eluate on rechromatography using hexane-ethyl acetate (50:50) yielded Withametelin (WM) (35 mg).

4.2.3 Isolation of cedrelopsin from *Hedyotis umbellata*

4.2.3.1 Plant material

The aerial parts of *Hedyotis umbellata* (Family: Rubiaceae) were collected from Virudunagar District, Tamilnadu, India during the months of December-January. The plant material was authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, Chennai, India (PARC/2014/2092). A voucher specimen (HU/2012/12-1) was deposited at Department of Pharmacy, BITS-Pilani Hyderabad Campus, Telangana State, India.

4.2.3.2 Isolation and purification of cedrelopsin

Aerial parts of *H. umbellata* (5.0 kg) were dried and ground to a coarse powder and was subjected for hot extraction using MeOH under heating at 45–50 °C. The methanolic extract was evaporated under reduced pressure to a dry residue (440 g), and was suspended in water. The aqueous suspension was partition separated using 3 × 1.5 L of diethyl ether. The diethyl ether soluble fraction was evaporated under vacuum and lyophilised to yield a dry residue (102 g).

Around 100 g of obtained residue was chromatographed on silica gel using solvents of increasing polarity (hexane-toluene, toluene-EtOAc and EtOAc-MeOH). The fractions eluted with toluene-EtOAc (50:50) (50 g) were re-chromatographed on silica gel using a gradient mixture of toluene-EtOAc. Based on the TLC pattern, the fractions eluted with toluene-EtOAc (70:30) were combined and evaporated to give the residue (12.6 g). The residue was rinsed with hexane and the insoluble residue was treated with EtOAc. The EtOAc soluble portion (2 g) was chromatographed on silica gel with gradient elution of hexane-EtOAc. Elution with hexane-EtOAc (75:25) resulted in the isolation of the cedrelopsin (CDLN) (15 mg).

4.2.4. Characterization of isolated compounds

The isolated compounds were subjected for melting point studies. TLC studies under different solvent systems were carried out to analyse the homogeneity and access the purity. Further, NMR spectral analyses of the compounds were done to characterize and confirm the identity of isolated compounds.

4.3 Biological evaluation

4.3.1 Materials

DMEM, McCoy's 5A and RPMI medium, fetal bovine serum (FBS), trypsin/EDTA, Bovine serum albumin (BSA) and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(MTT) were purchased from Himedia Laboratories (India). Cell cycle reagent, In situ caspase 3/7 detection kit and Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Billercia, USA). Bcl-2, Bax, cdc2, cdc25C, cyt c, cyclin B1 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V/PI Staining Kit was purchased from eBioscience Inc., (San Diego, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA), N-acetyl cysteine (NAC), Bradford reagent and Rhodamine 123 (RH-123) were purchased from Sigma Aldrich (St Louis, MO, USA). Caspase-9, Caspase-8, PARP, β-actin antibody and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Western Blot Chemiluminescence reagent was purchased from Thermo Scientific (Arlington Heights, IL).

4.3.2 Cell lines and culture

Human lung carcinoma cell line A549, human colon adenocarcinoma cells HT-29, breast cancer cells MDA-MB-231 and human embryonic kidney cells HEK-293 were obtained from National Centre for Cell Science (NCCS), Pune, India. A549 and HEK-293 were maintained with DMEM containing 10% FBS and 1% pencillin-streptomycin solution. HT-29 and MDA-MB-231 cells were maintained with McCoy's 5A and RPMI containing 10% FBS (Sigma, St. Louis, MO, USA) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO, USA) at 37 °C in a humidified 5% CO_2 incubator.

4.3.3 Compounds and solubility

All tested compounds, *viz*., withametelin, withaphysalin E, withapysalin D, 12-deoxy withastramonolide, withaperuvin B, physalolactone, coptisine, eleocarpine, tigloidine, cleomiscosin-A, cedrelopsin, feretoside, deacetylasperuloside, asperulosidic acid, 1,2-di methyl-

3-hydroxy-9,10-anthracenedione, 1,3-dimethyl-3-hydroxy-9,10-anthracenedione, pedunculoside, bergenin, nor bergenin and methyl caffeate were lyophilized and dissolved in DMSO solvent for biological assays.

4.3.4 Cell proliferation assay

Cell proliferation was assessed using the MTT staining as described by Mossman (Mosmann T, 1983). The MTT assay was based on the reduction of the tetrazolium salt, MTT, by viable cells. The dehydrogenase using NADH or NADPH as coenzyme converted the yellow form of the MTT salt to insoluble, purple formazan crystals (Liu KZ., *et al*., 1997). Formazan solution was read spectrophotometrically after the crystals were dissolved by organic solvent (DMSO). A549, MDA-MB-231, HT-29 and HEK-293 cells ($5*10^3$ cells/well) were plated in a 96 well plate and treated with appropriate concentrations of test compounds ranging from 100 μM to 0.1 μM and incubated in the presence or absence of 5mM NAC for 48 h at 37 $^{\circ}$C in a 5% CO_2/95% air incubator. Viability of cells was determined by estimating the amount of soluble formazan (in DMSO) formed after the addition of 100 μg MTT and 3.5 h incubation at 37 $^{\circ}$C. Media was removed and the crystals were dissolved in 150 μl DMSO. Absorbance was measured at 450 nm on Spectramax M4 plate reader. Each concentration was tested in three different experiments, run in triplicates. Means and standard errors of mean were calculated and reported as the percentage of growth vs. control.

4.3.5 Cell cycle analysis

The nuclear DNA content of a cell can be quantitatively measured by flow cytometry using propidium iodide, a fluorescent dye that binds stoichiometrically to the DNA. G1 cells would

have one copy of DNA and would therefore show 1X fluorescence intensity. Cells in G2/M phase of the cell cycle would have two copies of DNA and accordingly would show 2X intensity. Since the cells in S phase would be synthesizing DNA they would have fluorescence values between the 1X and 2X populations. Cells were plated at 100,000 per well in a 6-well plate and incubated with the compound for 48 h at 37 $^{\circ}$C in a 5% CO2 incubator. After incubation, cells were fixed in 70% ethanol and stored at 4 $^{\circ}$C till analysis. Cells were stained with Guava Cell Cycle reagent (propidium iodide) according to the manufacturer's instructions. Cell cycle data were obtained using the Guava Personal Cell Analysis System (Millipore, USA).

4.3.6 Annexin V assay

Annexin V-FITC/PI dual labelling identified cells in apoptotic phase. In normal viable cells, phosphatidylserine (PS) was located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS was translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human vascular anticoagulant, annexin V, is a 35–36 kD Ca^{2+}-dependent phospholipid-binding protein that showed high affinity for PS. Annexin V labeled with a fluorophore or biotin could identify apoptotic cells by binding to PS exposed on the outer leaflet (Vermes I, *et al*., 1995). Briefly, 100,000 cells were plated in a 6-well plate and incubated with compound in the presence or absence of 5 mM NAC for 48 h. Cells were trypsinized and Annexin-V-FITC and propidium iodide were added and incubated at 37 $^{\circ}$C for 15 min followed by a wash with PBS and the fluorescent intensity of cells were obtained using the Guava Personal Cell Analysis System (Millipore, USA).

4.3.7 ROS assay

Reactive oxygen species (ROS) in cells were estimated in cells using a fluorescent dye, DCFDA (2',7'-Dichlorodihydrofluorescein diacetate) (Eruslanov E, *et al*., 2010). The cell permeant DCFDA was chemically reduced to fluorescein in presence of ROS upon cleavage of acetate groups by intracellular esterases and oxidation. Briefly, 100,000 cells were plated in a 6-well plate and incubated with compound in the presence or absence of 5 mM NAC for 48 h. Cells were trypsinized and DCFDA was added at 1 μM concentration and incubated at 37 $^{\circ}$C for 15 min followed by a wash with PBS to remove the excess of dye. The fluorescent intensity of cells was obtained using the Guava Personal Cell Analysis System (Millipore, USA).

4.3.8 Caspase-3 assay

Caspase-3 activity in cells was measured using CHEMICON®'s CaspaTagTM In Situ Caspase Detection Kit. The methodology was based on fluorochrome inhibitors of caspases (FLICA) based cell permeable and non- cytotoxic dyes which bound covalently to the active caspase (Ekert PG, et al., 1999). This kit employed a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3 (FAM-DEVD-FMK), which produced a green fluorescence. When added to a population of cells, the FAM-DEVD-FMK probe entered each cell and was covalently bound to a reactive cysteine residue that resided on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled reagent was retained within the cell, while any unbound reagent would diffuse out of the cell and was washed away. The green fluorescent signal was a direct measure of the amount of active caspase-3 or caspase-7 present in the cell at the time the reagent was added. Cells were plated at 100,000 per well in a 6-well plate and incubated with the compound in the presence or absence of 5 mM

NAC for 48 h at 37 °C in a 5% CO_2 incubator. Cells were washed and incubated with FLICA reagent for 30 min. After incubation, cells were washed to remove the excess of FLICA and suspended in PBS. Fluorescence was measured at 485 nm excitation and 520 nm emission on Fluostar Omega (BMG Labtech, USA).

4.3.9 Preparation of cytosolic fraction and mitochondrial fraction

Cells were grown in the presence of drugs or no drug in 6 well plates for 48 h. After incubation the cells were washed with ice cold PBS, lysed in ice cold buffer (10 mM Tris-HCl (pH 7.8), 1% nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride and cocktail protease inhibitor), and homogenized on ice. The homogenate was centrifuged at 1000 g for 10 min at 4 °C. The supernatants were then centrifuged at 12000 g for 30 min at 4 °C and the resulting supernatants were finally collected as cytosolic fraction. The pellet was lysed in lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA (pH 8.0) and cocktail protease inhibitor. After centrifugation at 12000 g for 30 min at 4 °C, the supernatants were collected as mitochondrial fraction.

4.3.10 Western blotting

Cells were washed twice with PBS, trypsinized, and washed twice with ice-cold PBS. Cell pellets were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% Triton X, 0.1% SDS, 25 mM sodium fluoride, 200 μM sodium orthovanadate, 1X protease inhibitor cocktail for 30 min on ice. Cell lysates were clarified by centrifugation for 10 min at 14,000 × g, and the total protein concentration in the resultant supernatants was determined using a Bradford protein assay kit (Biorad, Hercules, CA). Equal amounts (50 μg) of protein was heated in SDS sample buffer with DTT (final concentration, 10

mM) at 98 °C, fractionated by size on 7.5% SDS-polyacrylamide gels, and transferred onto PVDF membranes (Millipore). Membranes were blocked by incubation for 1 h with TBS-T (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20) containing 5% BSA (Bovine serum albumin). Membranes were incubated with antibody at 4 °C overnight in TBS-T containing 5% BSA followed by the corresponding HRP-linked secondary antibody at room temperature for 1 h in TBS-T containing 5% nonfat milk powder. Chemiluminescence substrate was then added to the membranes followed by the exposure to x-ray films. Band intensity was calculated using ImageJ 1.42 (NIH, USA).

4.3.11 Statistical analysis

Calculation of growth inhibition GI_{50} was performed using GraphPad Prism 6.0 (La Jolla, USA) by fitting the data in non-linear regression model with variable slope. Data were expressed as mean ± SEM of at least three independent experiments and statistically analyzed by two way analysis of variance (ANOVA) followed by Bonferroni post test or t-test. The significance level was based on probability of $p<0.05$, $p<0.01$ and $p<0.001$.

CHAPTER 5

Results and Discussion

5.1. Assessment of Cytotoxicity of phytocompounds

5.1.1 Introduction

Global deaths from cancer are projected to continue to rise to over 13.1 million in 2030. Lung cancer is identified as the major causative for this increase in death rate. Lung cancer is the leading cause of tumor mortality associated with a poor survival rate even after complete surgical removal. The search for novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to chemotherapeutic drugs. In addition, the high toxicity usually associated with some cancer chemotherapy drugs and their undesirable side-effects have increased the demand for novel anti-tumour drugs with fewer side-effects and/or with greater therapeutic efficiency. Phytocompounds succeeded in clinical trials and have effectively turned into medicines. Since they offer large structural diversity, they have become sources of new drugs (Demain, AL and Vaishnav P., 2011). Because of structural diversity of phytocompounds and their success rate in clinical trials, several phytocompounds having diversity in their structures from different plant sources were screened to investigate their potentiality as anti cancer drugs. Twenty phytocompounds belonging to different groups of secondary metabolites, *viz*., withasteroids, alkaloids, iridoid glycosides, coumarins, phenolics, terpenoids and anthraquinones (Figure 5.1.1 to 5.1.4) were screened to develop newer anti cancer agents.

5.1.2. MTT assay of compounds:

The effect of selected compounds on the cell viability of human lung cancer cell line A549 and normal cell line (HEK-293) was determined through MTT assay, by exposing the cells for 48 h. The results of the study are presented in Figures 5.1.5 - 5.1.10

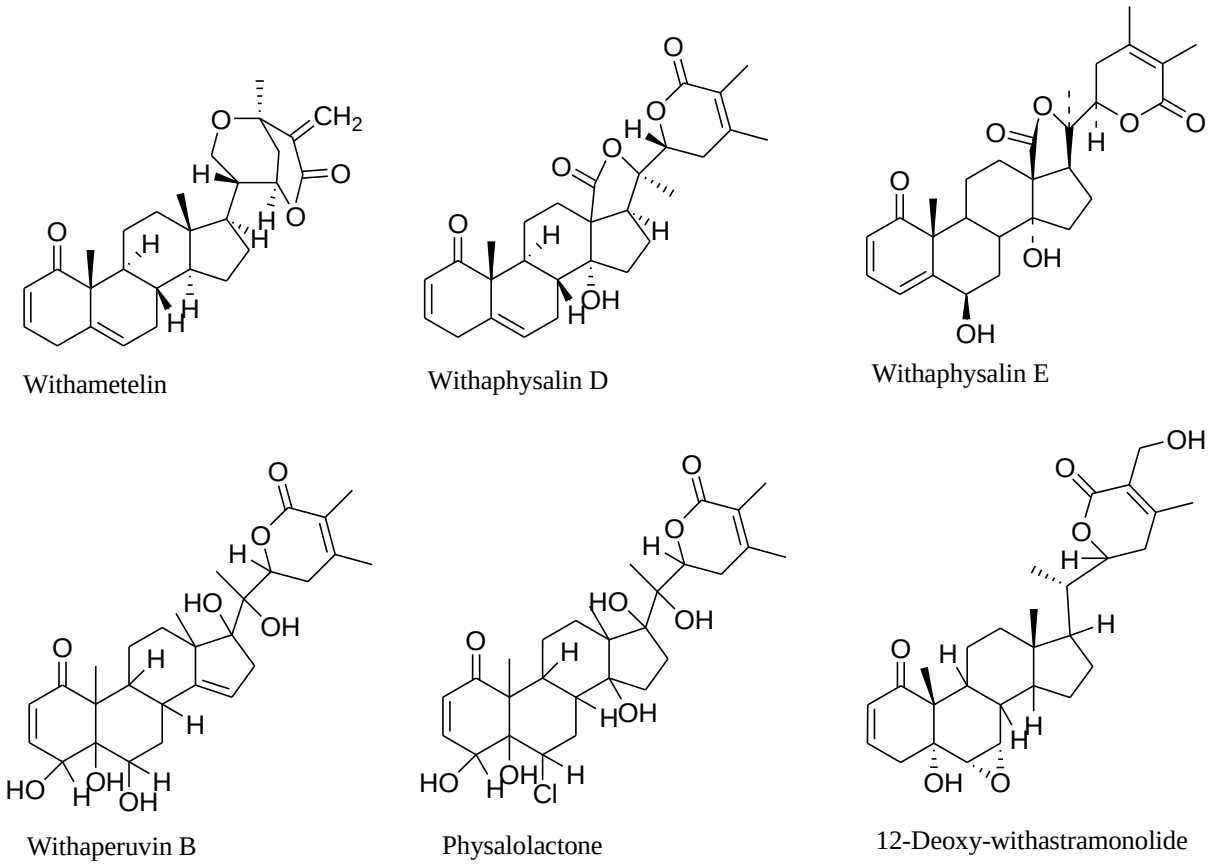
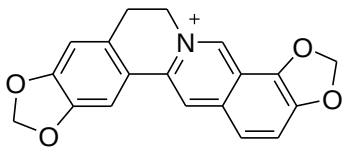
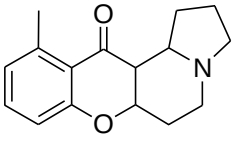


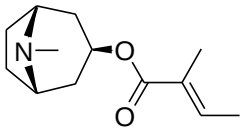
Figure 5.1.1: Structures of selected withasteroids



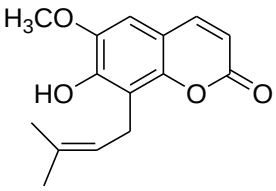
Coptisine



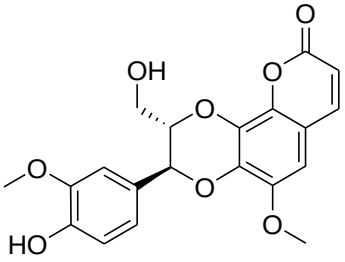
Eleocarpine



Tigloidine

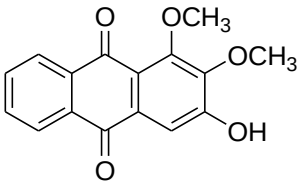


Cedrelopsin

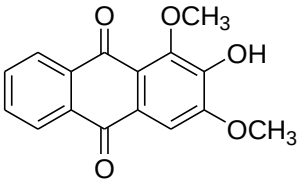


Cleomiscosin A

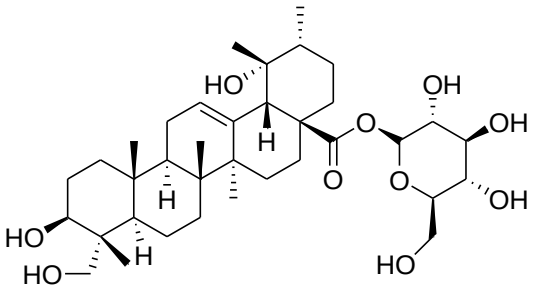
Figure 5.1.2: Structures of selected alkaloids and coumarins



1,2-Dimethoxy-3-hydroxy-9,10-anthacenedione

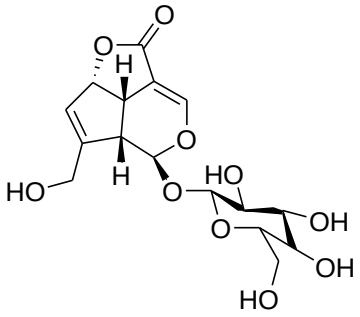


1,3-Dimethoxy-2-hydroxy-9,10-anthacenedione

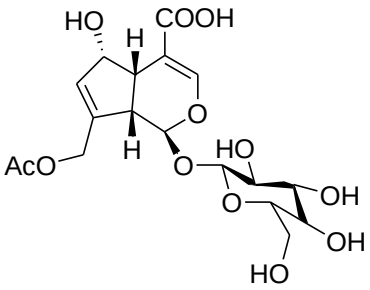


Pedunculoside

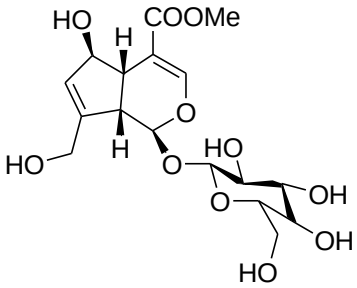
Figure 5.1.3: Structures of selected anthraquinones and pedunculoside



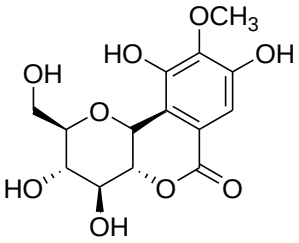
Deacetyl asperuloside



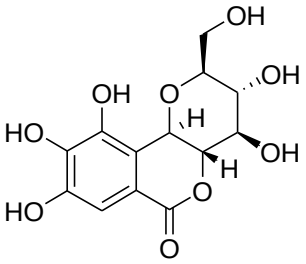
Asperulosidic acid



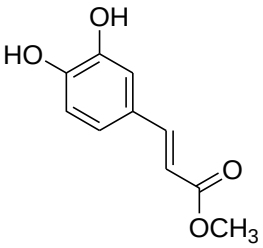
Feretoside



Bergenin



Nor bergenin



Methyl caffeate

Figure 5.1.4 Structures of selected iridoid glycosides and phenolics

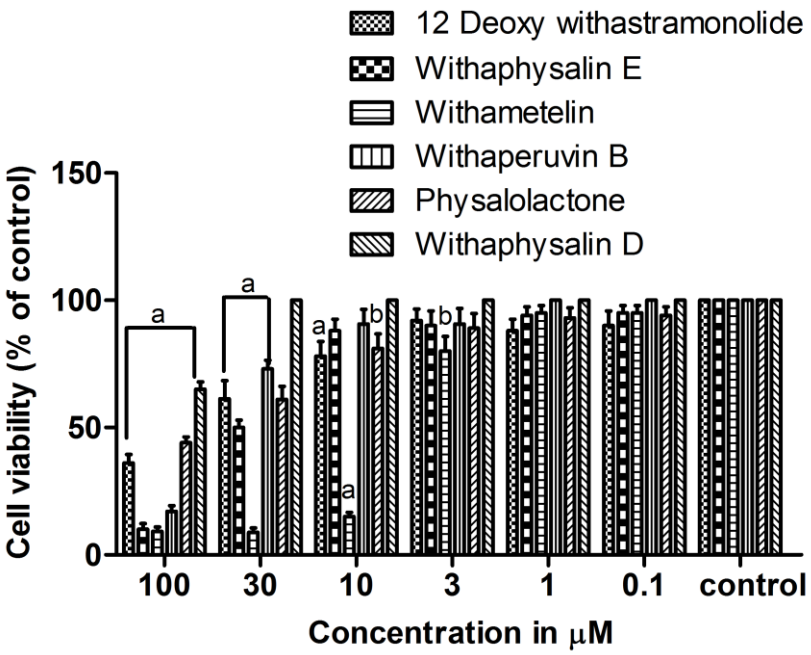


Figure 5.1.5: Effect of withasteroids on cell viability of A549 cells at 24 h. Data were expressed as mean ± SEM of three independent experiments. $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

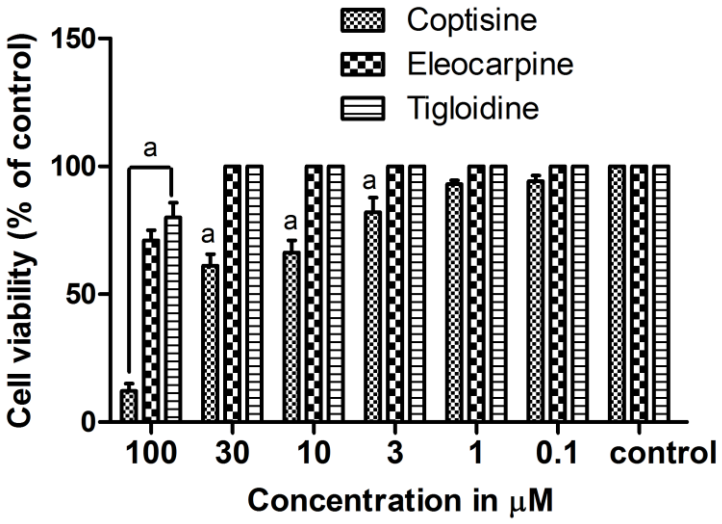


Figure 5.1.6: Effect of alkaloids on cell viability of A549 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{a}p<0.001$ versus control group

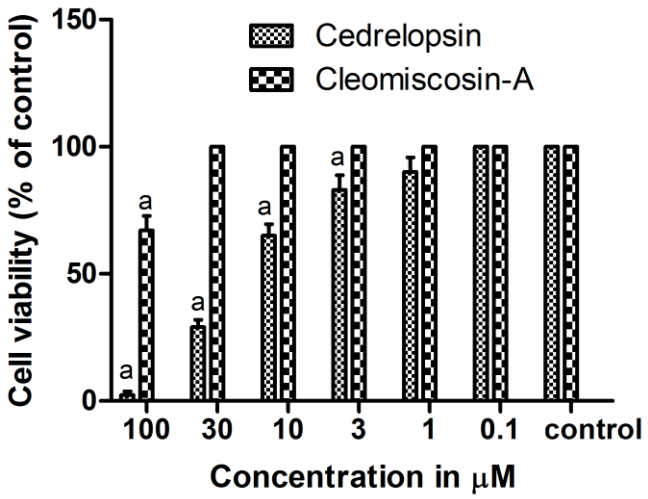


Figure 5.1.7: Effect of coumarins on cell viability of A549 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. [a]p<0.001 versus control group

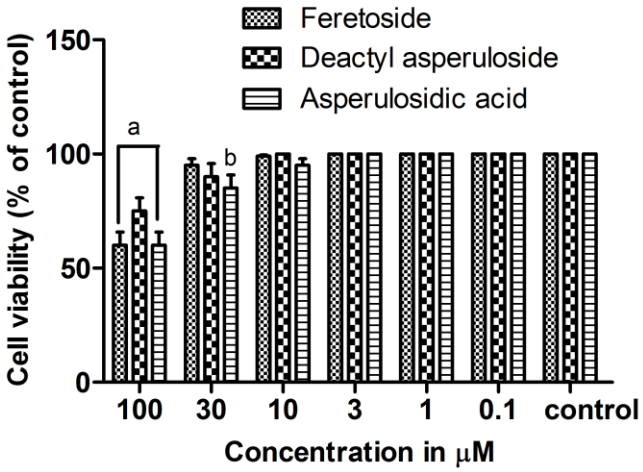


Figure 5.1.8: Effect of iridoid glycosides on cell viability of A549 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. [b]p<0.01 and [a]p<0.001 versus control group

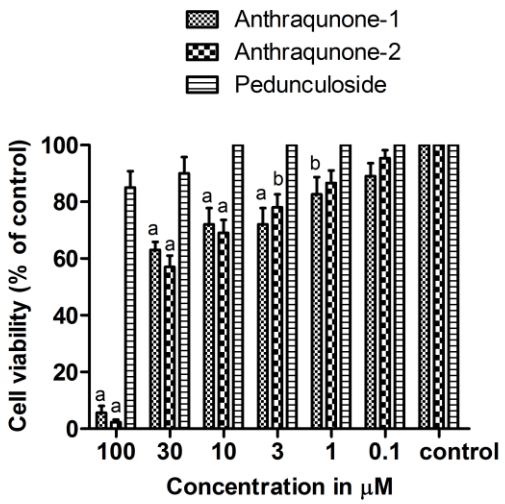


Figure 5.1.9: Effect of anthraquinones and pedunculoside on cell viability of A549 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{b}p<0.01$ and $^{a}p<0.001$ versus control group. Anthraquinone-1 and anthraquinone-2 represents 1,2-dimethoxy-3-hydroxy-9,10-anthracenedione and 1,3-dimethoxy-2-hydroxy-9,10-anthracenedione respectively)

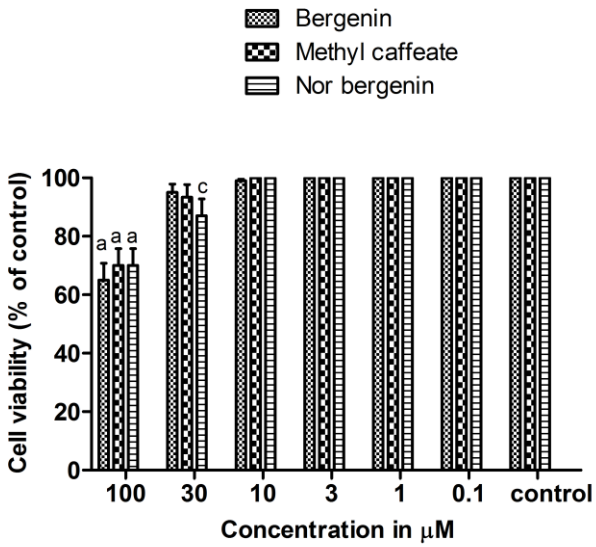


Figure 5.1.10: Effect of phenolics on cell viability of A549 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{c}p<0.1$, $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

Results claimed excellent antiproliferative effect of withametelin (WM) against A549 cells (IC_{50} value of 6.09 µM). WM was the first known C-21 oxygenated withanolide with a novel bicyclic side chain, isolated from the dried leaves of *Datura metel*. Physalolactone, which exhibited significant anti proliferative activity with IC_{50} value of 18.9 µM, was identified as a potent compound next to withametelin. 12-Deoxywithastramonalide, a close relative of WM was found to be only moderately active, differed structurally with 17-β side chain and rings A and B with the presence of hydroxyl at C-5 and an epoxy ring between C-6 and C-7 (Ray *et al*., 1994) was only moderately active. Also, withaphysalins exhibited mild or no cytotoxicity against A549 cell line. It was further observed that the antiproliferative effect differed among withanolides having 17α-side chain, 17β-side chain and withaphysalins. The results from testing of withasteroids on HEK-293 revealed that all withasteroids were found to be moderately safe.

Three alkaloidal compounds, coptisine, eleocarpine and tigloidine were investigated for their anti proliferative activity against A549 cells and HEK-293 cells. Coptisine was demonstrated to be potent in killing A549 cells while eleocarpine and tigloidine were discovered to be inactive. Safety of alkaloids was analyzed by performing cell viability test against HEK-293 cells and selectivity index value for alkaloids indicated the safety of coptisine.

Of the tested coumarins, cedrelopsin exhibited significant anti proliferative activity towards A549 cells. In order to test its selectivity towards A549 cells, cell viability of HEK-293 cells after cedrelopsin treatment was determined. Selectivity index of cedrelopsin was found to be 12.5, which demonstrated its safety. The other coumarin derivative, cleomiscosin A was found to be inactive against A549 cells.

Iridoid glycosides like deacetylasperuloside, asperulosidic acid and feretoside failed to display cytotoxicity against A549 cells. Similar effect was exhibited by all iridoid glycosides against HEK-293 cells.

1,2-dimethyl-3-hydroxy-9,10-anthracenedione and 1,3-dimethyl-3-hydroxy-9,10 anthracenedione, belonging to anthracenedione derivatives exhibited significant anti proliferative activity against A549 cells and the IC_{50} values were found to be 20.8 and 19.13 μM, respectively. When tested against HEK-293 cells moderate safety of those two anthraquinones was determined.

Pedunculoside, a terpenoidal metabolite was found to be inactive against A549 cells. Similar effect was observed when tested against HEK-293 cells.

When phenolics such as bergenin, nor bergenin and methyl caffeate were assessed for anti proliferative activity through MTT assay, none of them affected cell viability of A549 cells.

5.1.3 Conclusion

Based on the screening of cytotoxicity effect through MTT assay, withametelin from withasteroids, coptisine from alkaloids and cedrelopsin from coumarins were identified to be potentially active. They were selected for further investigation to decipher the molecular mechanisms underlying their cytotoxicity against A549 cells.

Table 5.1.1: Consolidated anti proliferative activity (IC_{50} values) of phytocompounds against A549 and HEK-293 cell lines

IC_{50} (μM) (Mean ± SEM)			
Compounds	A549	HEK-293	SI (CC_{50}/IC_{50})
Withasteroids			
Withametelin	6.09 ± 1.2	21.2 ± 3.4	3.53
Withaphysalin E	29.72 ± 4.6	231.0 ± 12.3	7.90
Withaphysalin D	> 100	>300	-
12-Deoxy withastramonolide	47.11 ± 4.7	188.0 ± 8.5	4.00
Withaperuvin B	48.53 ± 4.6	271.0 ± 6.7	5.65
Physalolactone	18.90 ± 2.1	132.1 ± 9.5	6.98
Alkaloids			
Coptisine	18.09 ± 1.6	196.3 ± 7.5	10.88
Eleocarpine	> 100	>300	-
Tigloidine	> 100	>300	-
Coumarins			
Cedrelopsin	14.3 ± 1.7	176.0 ± 8.9	12.50
Cleomiscosin A	> 100	>300	-
Iridoid glycosides			
Deacetylasperuloside	> 100	>300	-
Asperulosidic acid	> 100	>300	-
Feretoside	> 100	>300	-
Anthraquinones			
1,2-dimethyl-3 hydroxy-9,10 anthracenedione	20.8 ± 3.2	161 ± 10.3	8.05
1,3-dimethyl-3 hydroxy-9,10 anthracenedione	19.13 ± 2.5	155 ± 11.2	8.15
Terpenoids			
Pedunculoside	> 100	>300	-
Phenolics			
Bergenin	> 100	>300	-
Methyl caffeate	> 100	>300	-
Nor bergenin	> 100	>300	-
5 Flouro uracil	1.84	-	-

5.2. Characterization of potential cytotoxic molecules

5.2.1 Characterization of COP

COP was obtained as pale yellow powder. It showed single spot under TLC studies carried out using different solvent systems and spraying the plate using 10 % methanolicsulphuric acid and Dragendorff's reagent. The identity of COP was established through spectral analysis. COP displayed $[M]^+$ peak at m/z 320.05 under ESI-MS analysis (Figure 5.2.1), confirming the molecular formula $C_{19}H_{14}NO_4^+$ of coptisine.

The ^1H NMR (300 MHz) spectral analysis of COP dissolved in DMSO-d6 exhibited prominent signals for two methylene dioxy groups at δ 6.54 (s) and 6.18 (s) ppm. The up-field region of the spectrum displayed two triplets integrating for two protons each at δ 4.88 and 3.20 ppm, indicating the presence of mutually coupled methylene groups. The difference in the chemical shift values between the methylene protons corroborated their bonding to different atoms / groups. The down-field region of the spectrum revealed signals for four aromatic protons at 8.04 (d), 7.81 (d), 7.79 (s) and 7.09 (s) ppm alongwith two far-deshielded aromatic proton signals at 9.95 (s) and 8.96 (s) ppm. Further, the two aromatic doublets at 8.04 and 7.81 ppm were found to be ortho-coupled based on their larger coupling constant value (J=8.7 Hz). The interpretation of the NMR spectrum (Figure 5.2.2) clearly revealed the isolated COP as coptisine, which was further ascertained based on the comparison of the observed data (Table 5.2.1) with the reported NMR data. Further, co-TLC studies with authentic sample confirmed the isolated compound COP as coptisine, whose structure is given in Figure 5.2.3.

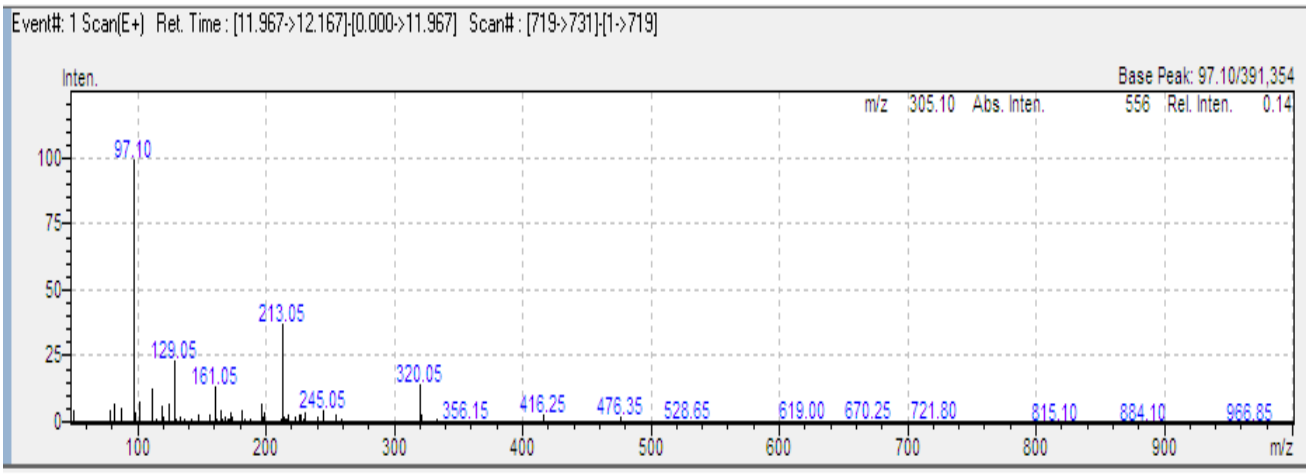


Figure 5.2.1: EI-MS spectrum of COP

COP, broadly belonging to isoquinoline group of alkaloids was first reported by Awe Walther (1951) by processing *Chelidoniummajus* tincture (Walther A *et al*., 1951). Subsequently presence of COP as a major constituent in plants such as *Corydalis ternata* (Lee HY *et al*., 1999), *Fumariaindica* (Pandey V *et al*., 1976), *Chelidoniiherba* (Colombo ML *et al*., 2001), *Coptic japonica* (Lim SY *et al*., 2002), *Corydalis adunca* (Zhao DB*et al*., 2005), *Corydalis yanhusuo* (Chen YU *et al*., 2006), *Stylophorumlasiocarpum* (Kristyna S *et al*., 2015), and *Coptischinensis* (Ye X *et al*., 2015) were identified.

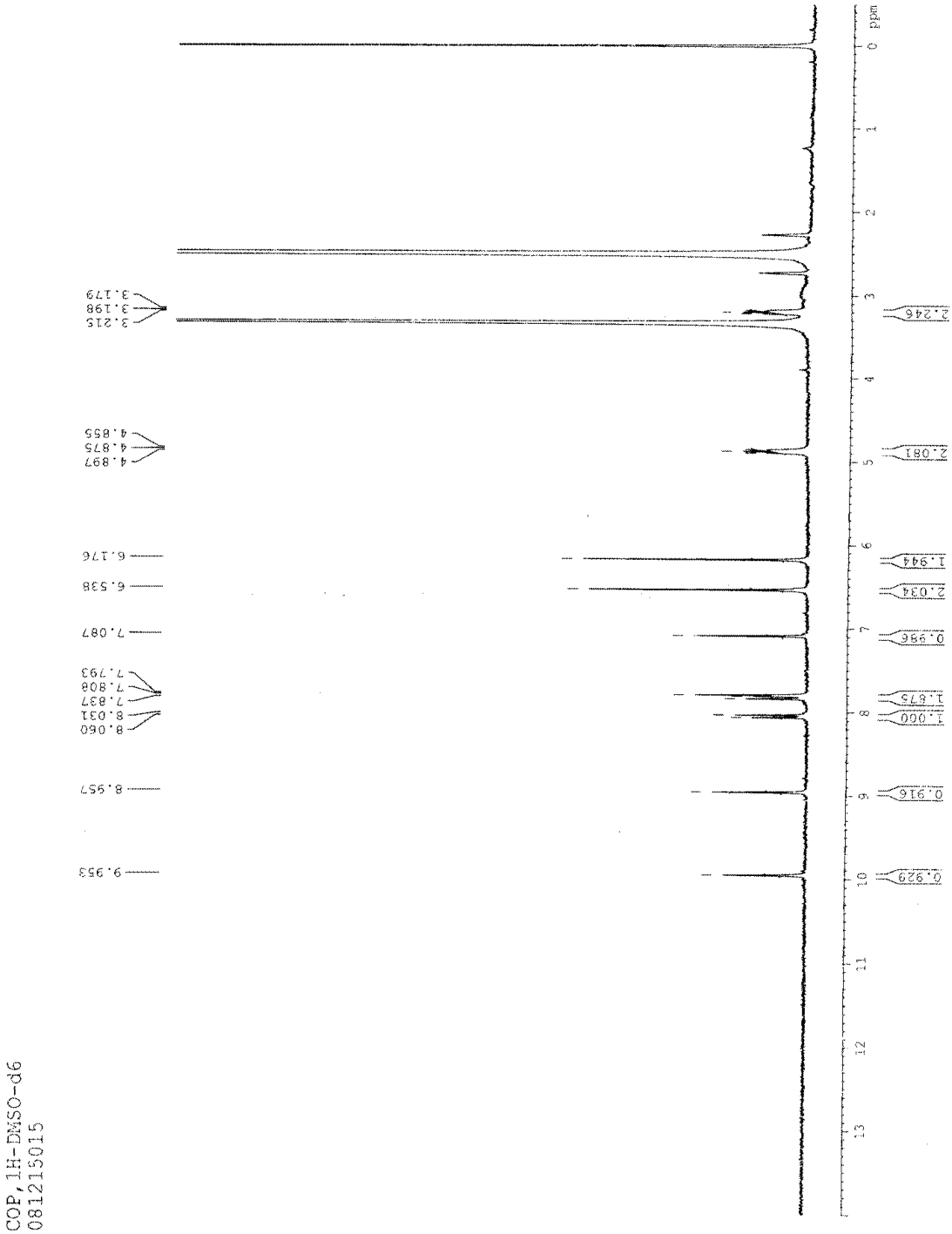


Figure 5.2.2: ^{1}H NMR spectrum of COP

Table 5.2.1.300 MHz ^{1}H NMR spectral data of COP

Chemical Shift (δ ppm)	Integral proton count	Splitting pattern (*J* Hz)	Probable assignment
9.95	1H	s	Ar-C**H**-N^{+}
8.96	1H	s	Ar-C**H**-
8.04	1H	d (8.7)	Ar-C**H**-
7.81	1H	d (8.7)	Ar-C**H**-
7.79	1H	s	Ar-C**H**-
7.09	1H	s	Ar-C**H**-
6.54	2H	s	-O-C**H**$_2$-O-
6.18	2H	s	-O-C**H**$_2$-O-
4.88	2H	t (6.6, 6.0)	-C**H**$_2$-CH_2-
3.20	2H	t (6.3, 5.7)	-CH_2-C**H**$_2$-

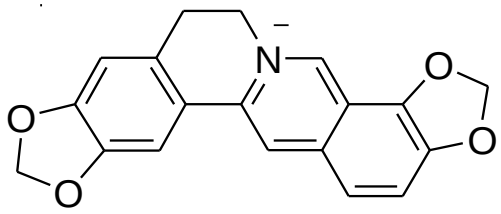


Figure 5.2.3: Structure of COP

5.2.2 Characterization of WM

WM was obtained as colorless needles showing melting point of 209-210 ºC. The purity of the compound was determined by TLC studies (Rf 0.54 in C_6H_6 – EtOAc (3:1) and Rf 0.63 in C_6H_6 – acetone (4:1)TLC solvent system). The TLC plates developed in different solvent system showed single spot when exposed to iodine vapours and charring the plates with 10% methanolicsulphuric acid as well. WM was identified as withametelin based on the spectral

analysis. WM displayed $[M+H]^+$ peak at m/z 437.25 and $[M-H]^-$ peak at m/z 435.30 under positive and negative mode analysis of APCI-MS analysis, respectively (Figure 5.2.4), confirming the molecular mass of 436 and molecular formula $C_{28}H_{36}O_4$ of withametelin.

The 300 MHz ^1H NMR spectrum was measured by dissolving WM in DMSO-d6 (Figure 5.2.5). The spectrum displayed signals for three methyl groups and carbonyl hydrogens signal at δ 4.61 ppm as a broad singlet. The spectrum also showed signals supporting the presence of characteristic features of withanolides like an enone system and unsaturated δ-lactone. Also the exocyclic methylene (6.07 and 6.52 ppm) and an oxymethylene (3.52 and 3.79 ppm) of withametelin were identified in WM through interpreting the NMR spectrum which exhibited corresponding signals in the spectrum. A careful analysis of the proton NMR spectrum and comparison of observed data with those of reported data confirmed WM as withametelin (Sinha*et al*., 1989 and Jahromi*et al*., 1993). Table 5.2.2 presents the comparison of proton NMR data comparison between the observed and reported data. Finally, WM was found to show identical characteristics with the authentic sample of withametelin (mixed melting point and co-TLC) and thus it was ascertained as withametelin, whose structure is presented in Figure 5.2.6.

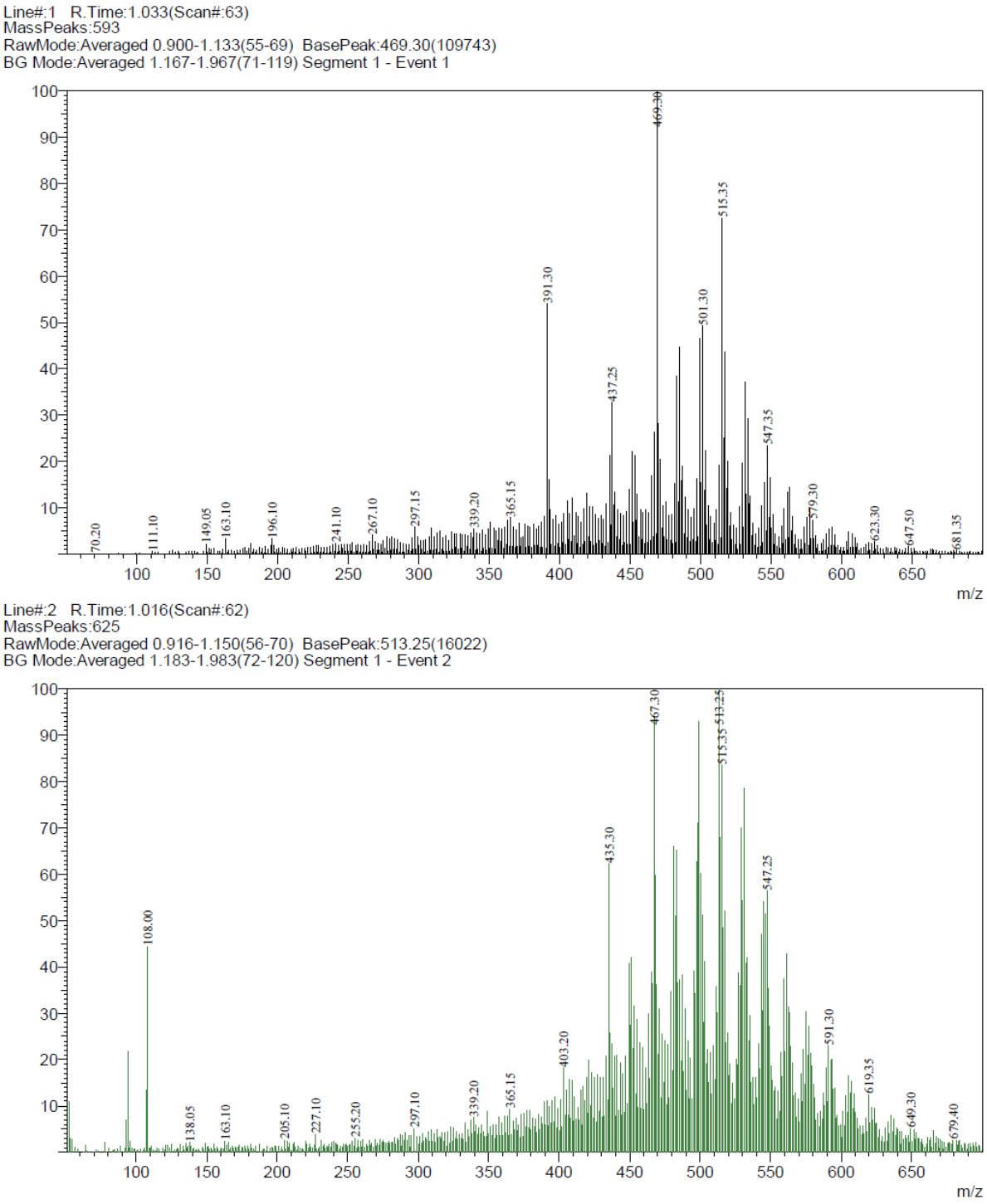


Figure 5.2.4. APCI-MS spectrum of WM

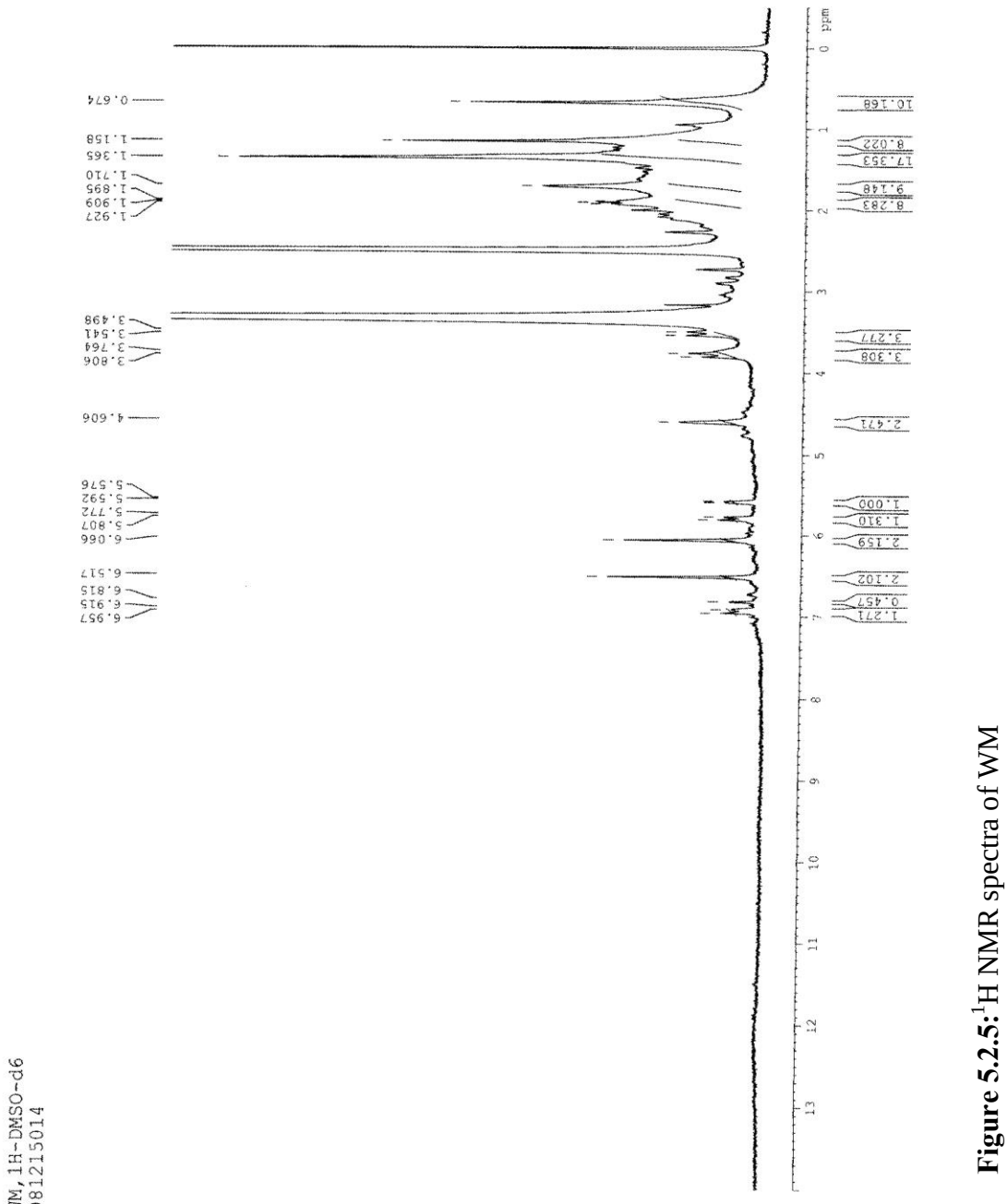


Figure 5.2.5: ^{1}H NMR spectra of WM

Table 5.2.2. Comparative ^{1}H NMR spectral data of observed values of WM and reported values

Proton position	**δ in ppm, splitting pattern (*J* value in Hz)**	
	Observed values (300 MHz, DMSO-d6)	**Reported values (500 MHz, $CDCl_3$**
H-2	5.79 br. d (10.5)	5.88 dd (9.8, 2.5)
H-3	6.93 m	6.77 ddd (9.8, 5.1, 2.5)
H-4	2.6 – 2.9 m	2.84 dd (20, 5.1) 3.30 dt (20, 2.5)
H-6	5.53 br. d (4.8)	5.58 t (5.8)
H-18 and 19	0.67 s / 1.19 s	0.71 s/1.22 s
H-21	3.52 br. d (12.9) 3.79 br. d (12.6)	3.73 dd (12.7, 3) 3.95 d (12.7)
H-22	4.61 br. s	4.65 br. S
H-27	6.07 br. s 6.52 br. s	6.02 br. S 6.76 br. s
H-28	1.37 s	1.44 s

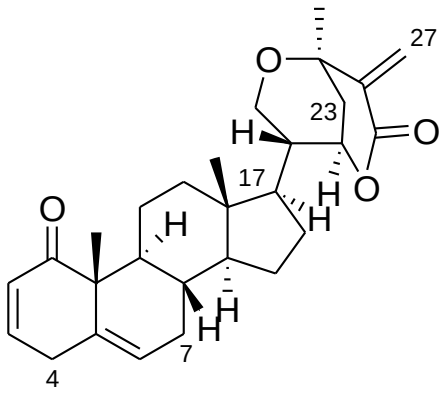


Figure 5.2.6: Structure of WM

5.2.3 Characterization of CDLN

Although cedrelopsin had been reported to be first isolated from *Cedrelopsis grevei* (Um, B.H*et al*., 2003), we preferred its isolation from a locally distributed plant, *Hedyotisumbellata* (Rubiaceae) as reported by Mahibalan et al (2015). CDLN was obtained as yellow amorphous powder, showing melting point of 173-176 ºC. The purity of CDLN was determined using RP-HPLC step gradient method using ACN in H_2O as mobile phase and detection at 280 nm (Figure 5.2.7). The identity of CDLN as a 6,7,8-trisubstituted derivative was established based on the Mass (Figure 5.2.8),^{1}H NMR (Figure 5.2.9), and ^{13}C NMR (Figure 5.2.10) spectral analysis.

The molecular formula $C_{15}H_{16}O_4$deduced based on the spectral analysis was settled based on the $[M-H]^-$ peak at m/z 259.25 apeared in the EI-MS spectrum. The ^{1}H NMR spectrum showed signals corresponding to H-3 and H-4 of coumarin nucleus as mutually coupled doublets (J = 9.5 Hz) at δ 7.57 and 6.25 ppm. Also, the presence of aromatic methoxyl group (3.94 ppm) and a prenyl group [3.57 (d), 5.29 (t), 1.85 (s) and 1.68 (s) ppm] was identified from the spectrum. The interpretation of carbon NMR spectrum confirmed the presence of chromone nucleus, aromatic methoxyl and prenyl group in CDLN. Comprehensive analysis of spectra led to the identification of CDLN as cedrelopsin. Table 5.2.3 presents the ^{1}H NMR and ^{13}C NMR data of the measured spectra of CDLN. Identification of CDLN as cedrelopsin was ascertained based on the comparison of measured spectral data with those of reported data (Eshiett and Taylor J., 1968). The structure of Cedrelopsin is presented in Figure 5.2.11.

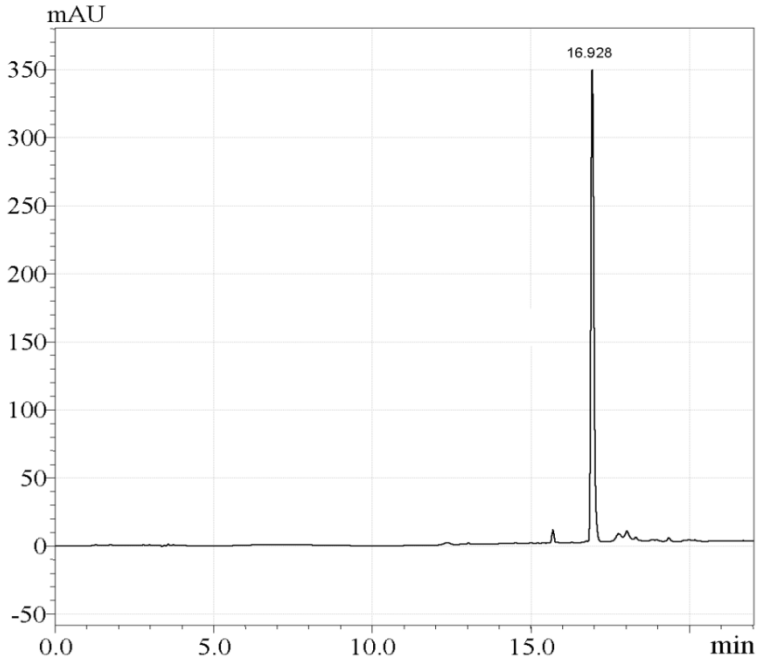


Figure 5.2.7 RP-HPLC chromatogram of cedrelopsin detected at λ_{max}280 nm

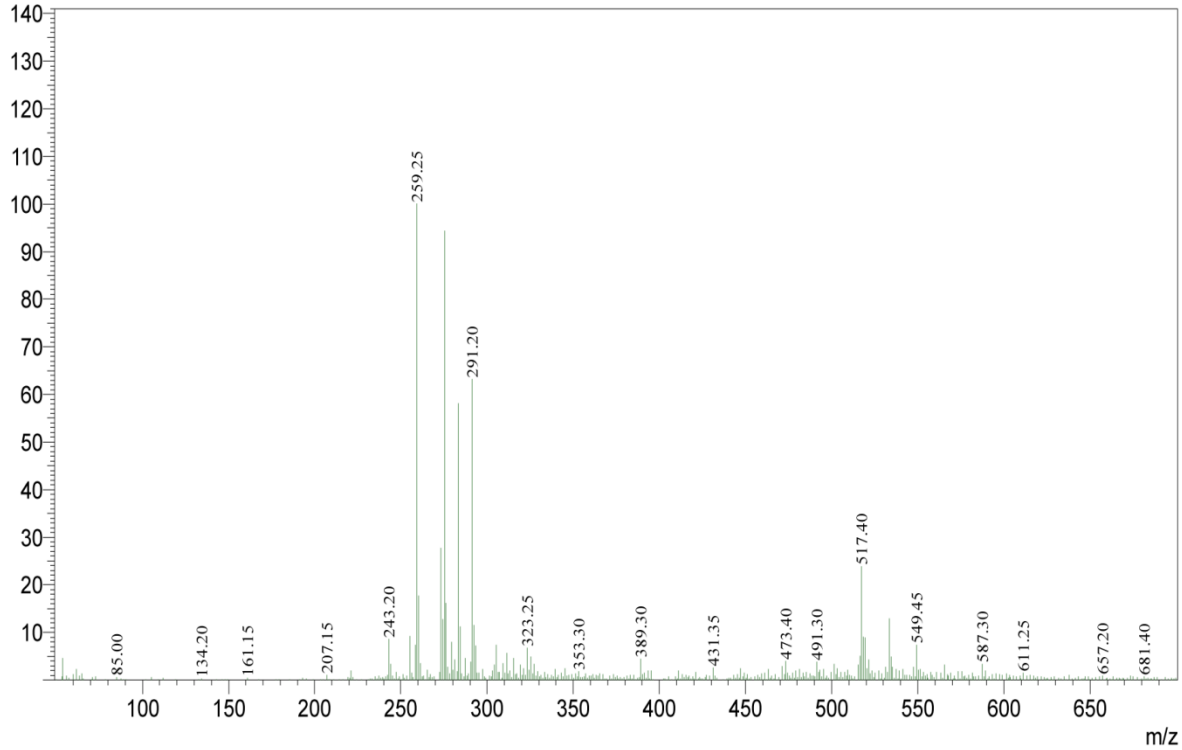


Figure 5.2.8.ESI-Mass spectrum of cedrelopsin

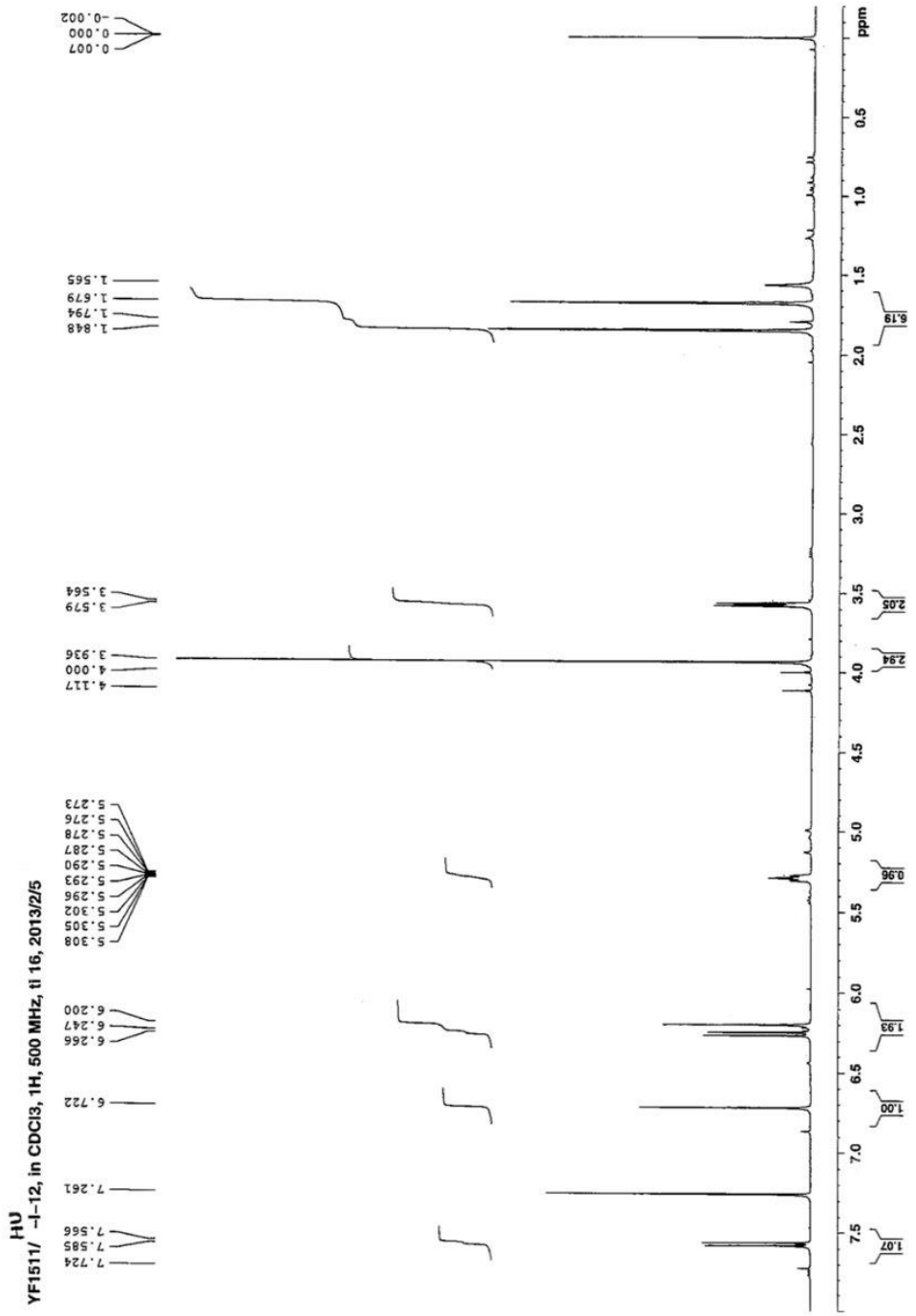


Figure 5.2.9: ^{1}H NMR spectra of cedrelopsin

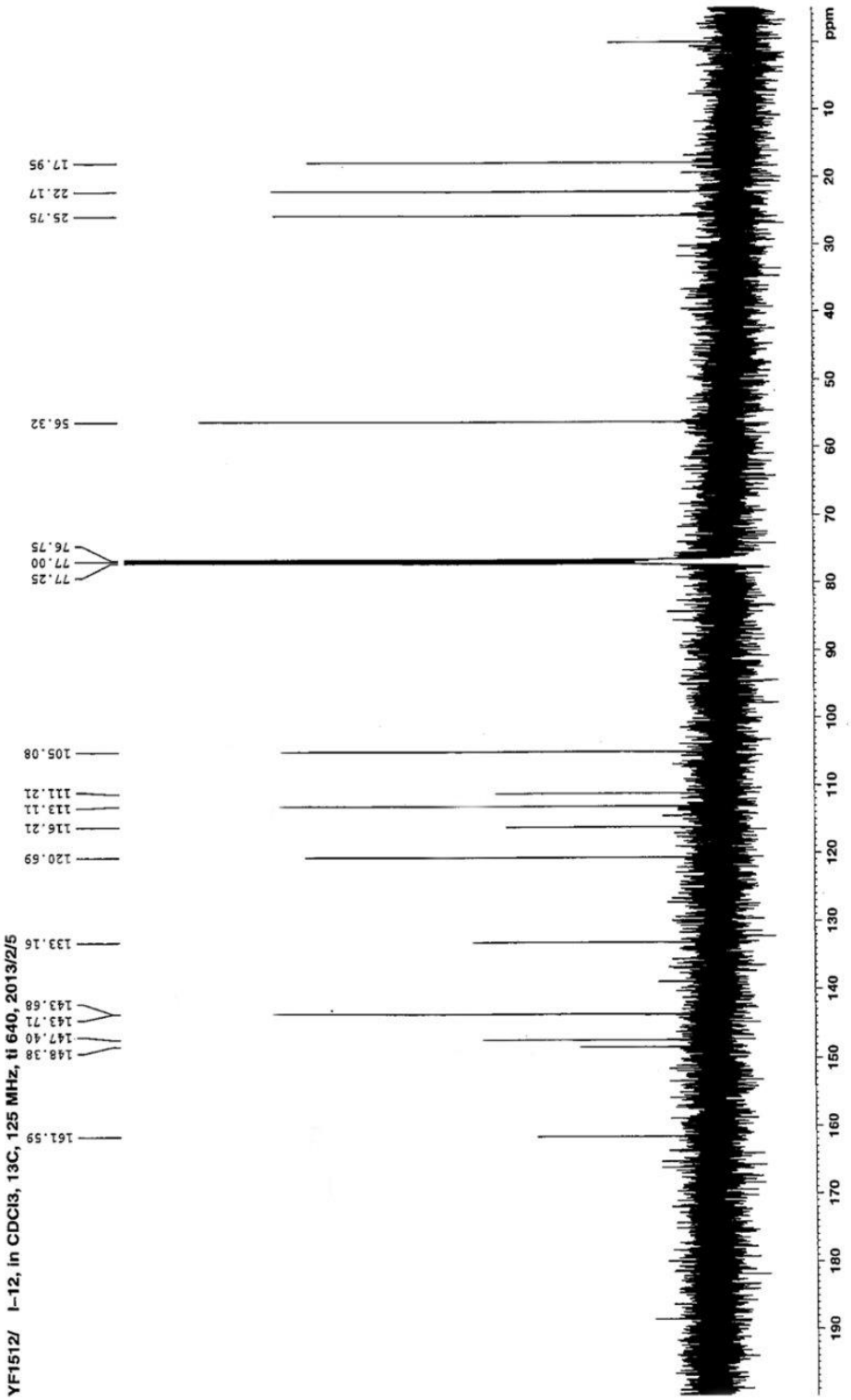


Figure 5.2.10: ^{13}C NMR spectra of cedrelopsin

Table 5.2.3. Proton and Crabon NMR data of cedrelopsin

Position	δ in ppm (^{1}H NMR)	δ in ppm (^{13}C NMR)
2		
3		
4		
5		
6		
7		
8		
9		
10		
1´		
2´		
3´		
4´		
5´		
-OMe		
-OH		

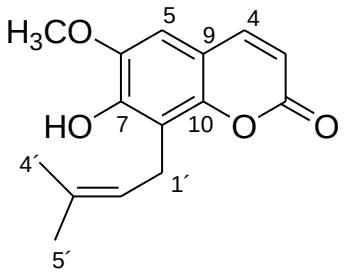


Figure 5.2.11. Structure of CDLN

5.3. Investigation of mechanisms underlying the cytotoxicity of coptisine

5.3. Investigation of mechanisms underlying the cytotoxicity of coptisine

Several plant derived compounds have been recognized to possess anticancer activities both *in vitro* and *in vivo* (Kelloff GJ *et al*., 2000). Quercetin, a bioactive flavonol was explored to inhibit cell proliferation, induce cell cycle arrest and apoptosis in different cancer cell types. In 2009, Chien SY *et al*, investigated the human breast cancer MDA-MB-231 cell death mechanism of quercetin and reported it to be through mitochondrial- and caspase-3-dependent pathways (chien sy *et al*., 2009). Deoxyelephantopin, a sesquiterpene lactone had been spotted to exhibit antiproliferative and apoptosis-inducing properties in SiHa cells. Recently, Farha AK *et al*, had elucidated the underlying molecular mechanisms evidencing that STAT3/p53/p21 signaling, MAPK pathway, PI3k/Akt/mTOR pathway, caspase cascades, and ROS play critical roles in deoxyelephantopin-induced G2/M phase arrest and apoptosis of SiHa cells (Farha AK *et al*., 2014) Further compounds like artemisinin, sanguinarine, cepharanthine have been proved to induce apoptosis through ROS mediated mechanisms (Gao W *et al*., 2013; Han MH *et al*., 2013; Hua P *et al*., 2015). Literature also claims hundreds of natural molecules screened for antiproliferative effects. However, only few among the several known cytotoxic natural compounds have been exploited for their mechanisms of action. Many potential molecules may emerge as chemotherapeutic drugs if relentless research is carried out on such underexploited natural compounds. In view of this, COP which was found to be cytotoxic against A549 cells (Kim KH *et al*., 2010) was selected for exploring the mechanistic pathway.

5.3.1 Inhibition of cell proliferation by COP

The potentiality of COP in the treatment of cancer can be well recognized from its antiproliferative activity on hepatoma cell lines (HepG2, Hep3B, SK-Hep1), leukemia (k562, u937, P3HI and Raji) (Lin C *et al*., 2004), non small cell lung cancer (A549), ovarian cancer (SK-OV-3) and melanoma (HCT-15) (Kim KH *et al*., 2010) and suppression of human breast cancer cell metastasis (Li J *et al*., 2014). However, no mechanistic studies are delineated in the literature. The effect of COP on the proliferation of human lung cancer cell lines A549, MDA-MB-231 and HT-29 was determined through MTT assay by exposing the cells for 48 h. Treatment with various concentrations of COP resulted in a dose dependent inhibition of cell growth in all cell lines (Figure 5.3.1). Maximal inhibition was observed with 100 µM of coptisine, which inhibited the growth of A549 cells at 94.4%. The calculated IC_{50} value was found to be 18.09 µM. Discussions on the killing of cancer cells through reactive oxygen species (ROS) metabolism by increasing ROS levels above the toxic threshold are more particularly discussed in the literature (Fruehauf JP *et al*., 2007; Schumacker PT., 2006; Sabharwal SS *et al*., 2014). NAC, non specific antioxidant, is commonly used to identify and test ROS inducers. Further, to identify if the anti proliferative effect of COP is mediated through ROS, A549 cells were pretreated with NAC. Anti proliferative effect of COP was almost reduced when the cells were pretreated with a ROS scavenger, NAC as compared to the corresponding groups which were not exposed to NAC.

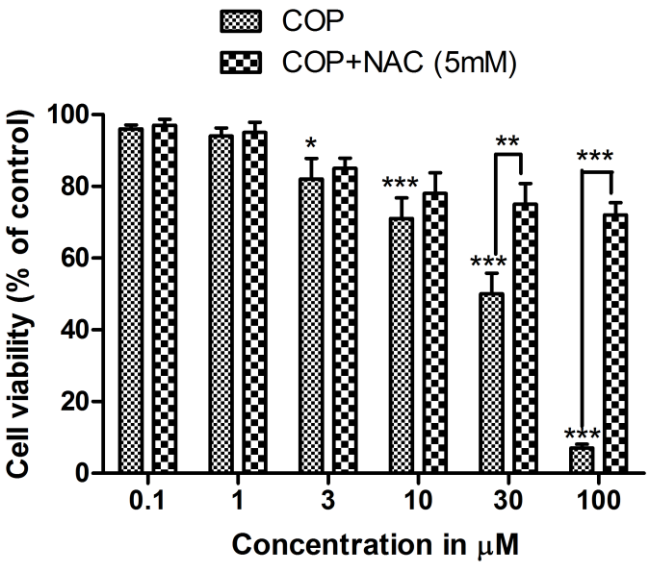


Figure 5.3.1: COP induced cytotoxicity in A549 cells. A549 cells were treated with indicated concentrations of COP for 48 h, with or without NAC addition and the percentage of cell viability was estimated by MTT assay. Data were expressed as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus control group or group with NAC versus without NAC

5.3.2 COP induced G2/M arrest and downregulated G2/M related proteins

The basis of COP cytotoxicity could be either apoptosis or cell cycle arrest since they are the two major mechanisms involved in the induction of cell death. Cell cycle is controlled by numerous mechanisms ensuring correct cell division in normal cells while fundamental alterations in genetic control of cell division results in uncontrolled cell proliferation and eventually leading to the development of cancer (Vermeulen K *et al*., 2003). The G2/M checkpoint prevents the cell from entering mitosis when DNA is damaged, providing an opportunity for repair and terminating proliferation of damaged cells (Stark GR *et al*., 2006). Cyclins and CDKs are necessary for the cells to progress through the different phases of cell cycle. Cyclin B1 forms a complex with cdc2 (Cdk1) that is essential for the cells to enter into

mitosis. During G2phase, dephosphorylation of regulatory residues of cdc2, Thr14 and Tyr 15, by phosphorylating cdc25C to pcdc25C at ser 216, directly activates cyclinB1/cdc2 complex and causes mitosis initiation. When the cells undergo genotoxic stress, phosphorylation of cdc2 weakens the activity of cyclinB1 and cdc2 complex that arrest cells in G2/M phase (Carmazzi Y *et al*., 2012). It was reported that COP does dual blockade of cell progression in vascular smooth muscle at G0/G1 and G2/M phases (Tanabe H *et al*., 2005). To investigate the effect of COP on cell cycle distribution, cells were treated with 50, 25 and 12.5 µM concentrations of COP for 48 h and analyzed cell distribution using flow cytometer. Treatment resulted a concentration dependent arrest of cells in G2/M phase of cell cycle. COP increased the cell population in G2/M phase to 36.8%, 29.9% and 26.5% at concentrations of 50, 25, and 12.5 µM, respectively compared with DMSO control (16.95%) (Figure 5.3.2A). This was accompanied by decrease in cells in G1 phase observed at all concentrations. However, COP did not affect cells in S phase. Data revealed that COP caused cells to arrest only at G2/M in A549 cells in dose dependent manner, suggesting that the effect of COP on cell cycle progression was cell type dependent.

To investigate the molecular mechanism of COP mediated G2/M phase arrest, G2/M regulatory proteins such as cyclin B1, cdc2 and cdc25C in A549 cells were examined. Protein extracts were prepared from cells treated with different concentrations of COP for 48 h and analyzed by western blot assay. As a result, expression of cyclin B1, cdc2 and cdc25C were significantly reduced by COP in a dose dependent fashion in comparison with control (Figure 5.3.2C). Significant reduction in the expression of these proteins confirmed that A549 cells were arrested at G2/M phase by COP.

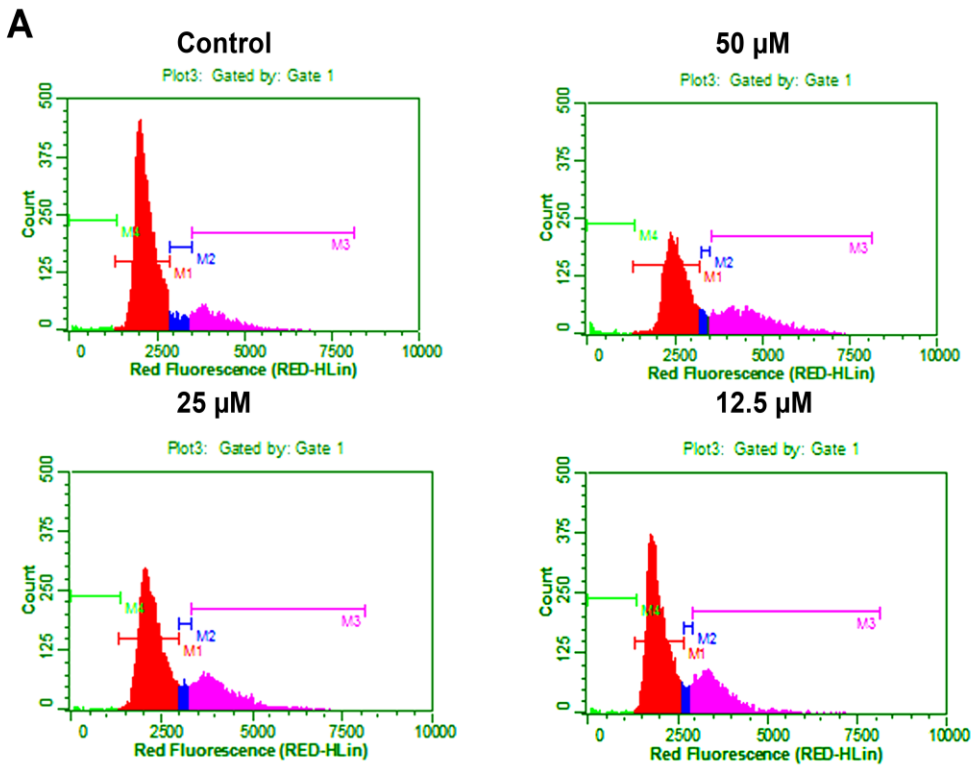


Figure 5.3.2A: COP-induced G2/M arrest. A549 cells were exposed to different concentrations of COP for 48 h stained with PI and analyzed by flow cytometry.

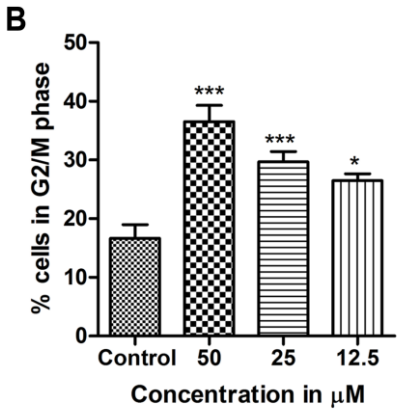


Figure 5.3.2B: Representative histogram of coptisine-induced G2/M arrest. All results were expressed as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus control group.

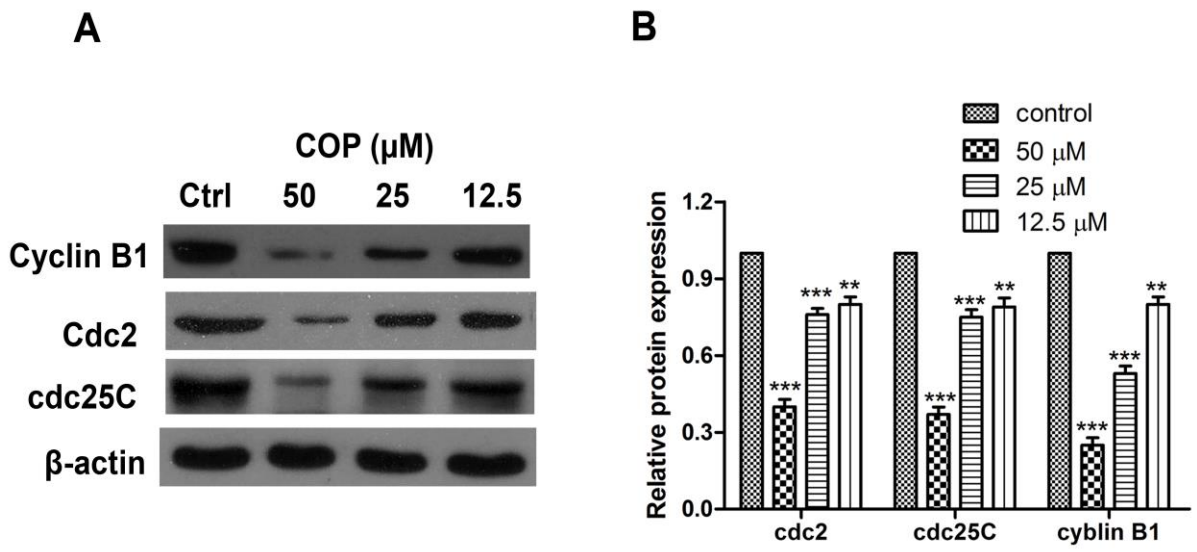


Figure 5.3.2C: COP downregulated G2/M regulatory proteins in A549 cells. Expression of G2/Mregulatory proteins, cyclin B1, cdc2 and cdc25C were detected by western blotting analysis (A) followed by densitometry (B). All results were expressed as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus control group

5.3.3 COP-induced apoptosis

In addition to cell cycle arrest, triggering of apoptosis by COP was found to be another important mechanism in inhibiting cell proliferation. Generally, apoptosis is characterized by morphological and biological changes such as cytoplasmic shrinkage, chromatin condensation, and DNA degradation. Apoptosis serves as a protective mechanism that prevents the process of carcinogenesis resulted from mutations of genetic materials of normal cells (Chowdhary I *et al*., 2006). Berberine, a related compound to COP manifested inhibition of cell proliferation through apoptosis in different cell lines (Hsu W *et al*., 2007; Lin JP *et al*., 2006; Burgeiro A *et al*., 2011).

To further decipher COP-induced cytotoxicity, FITC conjugated annexin V and PI double staining were performed on both untreated and COP treated A549 cells. In the untreated cells, annexin labeled population (early apoptotic) was found to be 8.6% and both PI and annexin labeled population (late apoptotic) was observed to be 4.6%. While the early apoptotic

population of cells was significantly increased to 58.5%, it was found to be 24.2 % of cells in late apoptotic phase under higher concentration of COP i.e. 50 µM. Similarly, at 25 and 12.5 µM concentrations, 26.4% and 10.8% of cells were found to be early apoptotic, respectively (Figure 5.3.3). Flow cytometric analysis delineated COP-induced apoptosis as a dose dependent process.

To examine if COP-induced apoptosis is associated with ROS, COP treated A549 cells were pre-exposed to NAC. Pretreatment with NAC significantly abrogated apoptotic effect of COP. Results confirmed that COP induced apoptosis is mediated through ROS.

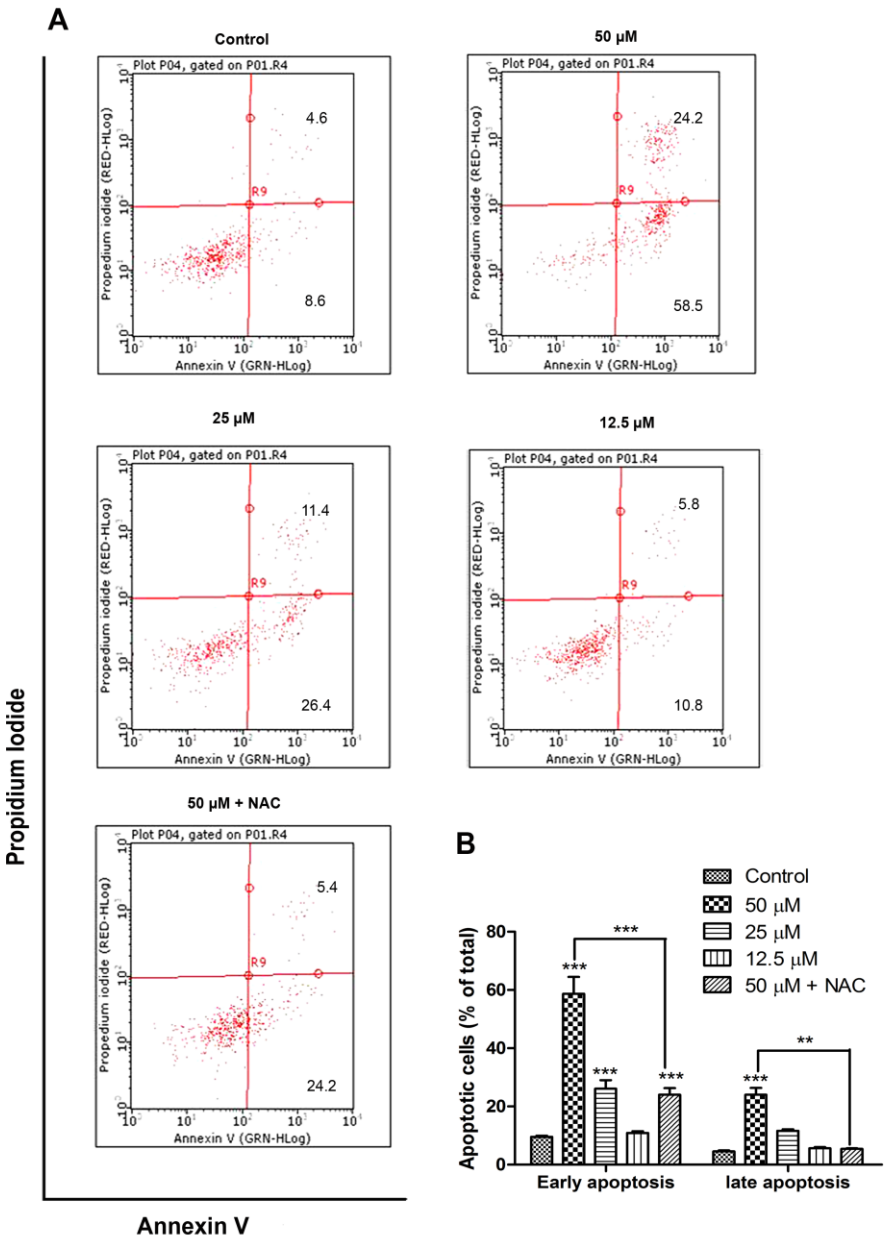


Figure 5.3.3: COP induced apoptosis in A549 cells. Cells treated with various concentrations of COP in the presence or absence of 5 mM NAC for 48 h. Then the cells were double stained with FITC conjugated Annexin V and PI for flow cytometric analysis (A). Quantitative data of A (B). Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group or group with NAC versus without NAC.

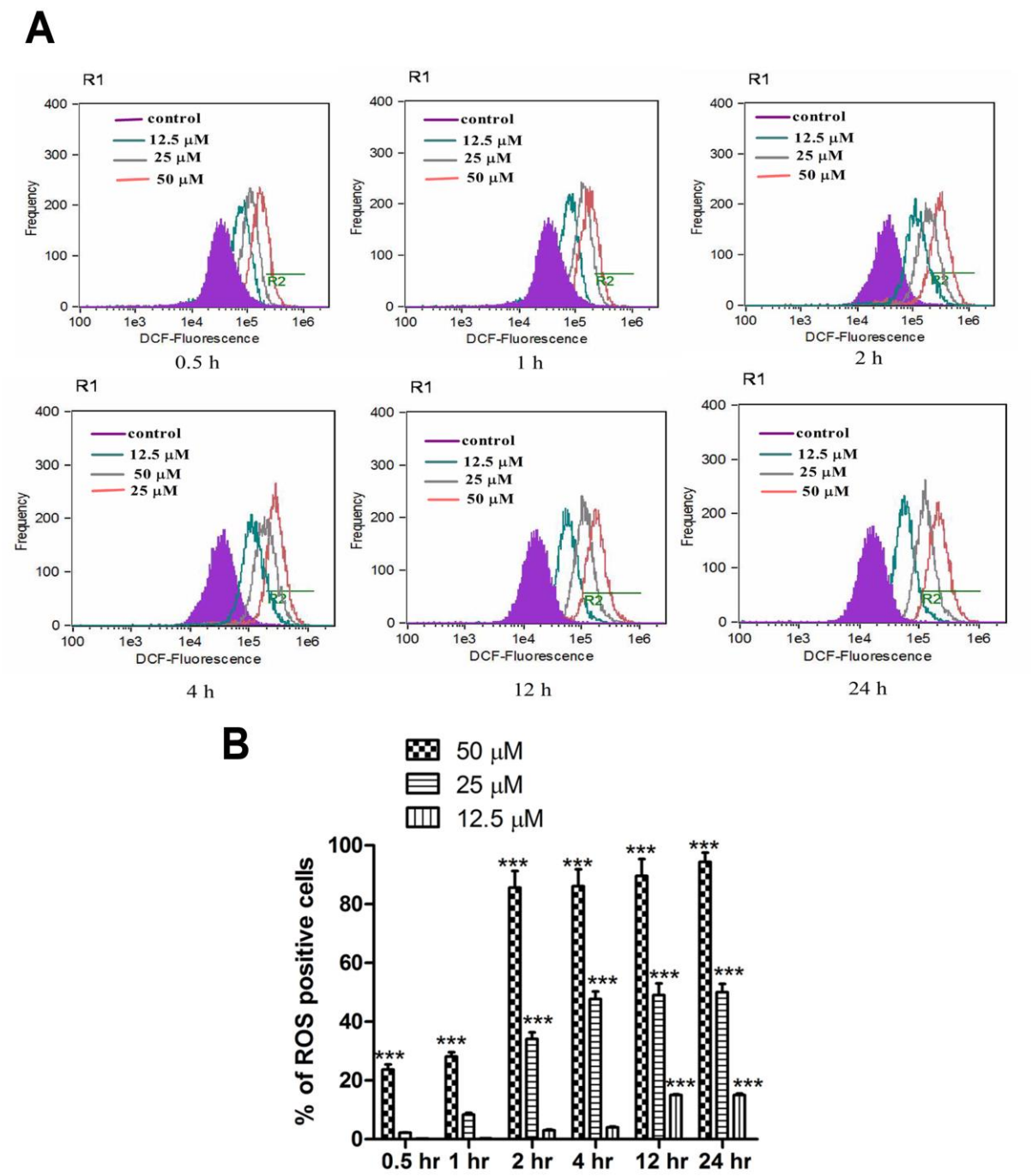


Figure 5.3.4: COP triggered ROS generation in A549 cells. Cells were treated with different concentrations of COP from 0.5 h to 24 h and incubated with DCFDA for 30 min. The intracellular ROS was measured by flow cytometry (A). Histogram depicting the percentage of ROS positive cells of indicated concentrations of COP at different time points (B). Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group

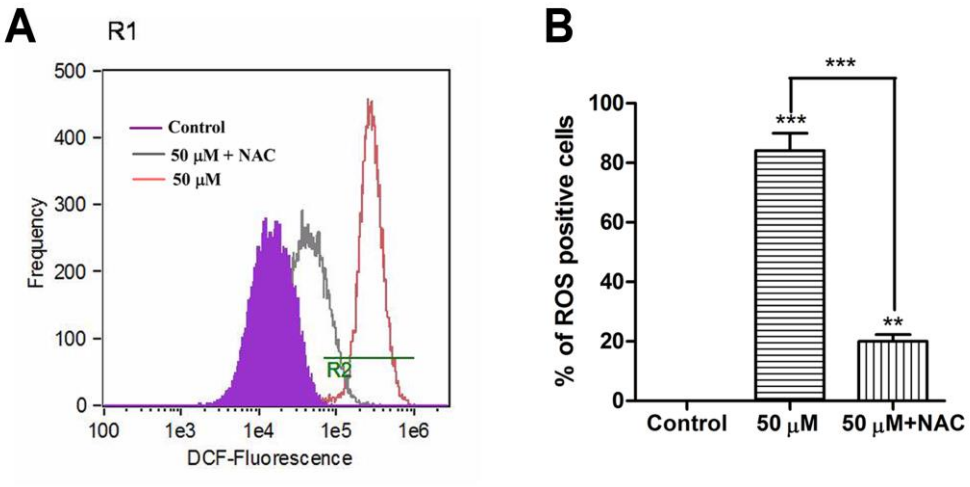


Figure 5.3.5: NAC abrogated COP triggered ROS generation. Cells were pretreated with NAC for 1 h and then treated with COP (50 µM) for 24 h. (A) The histogram depicting NAC abrogated COP-induced ROS generation (B). Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group or group with NAC versus without NAC

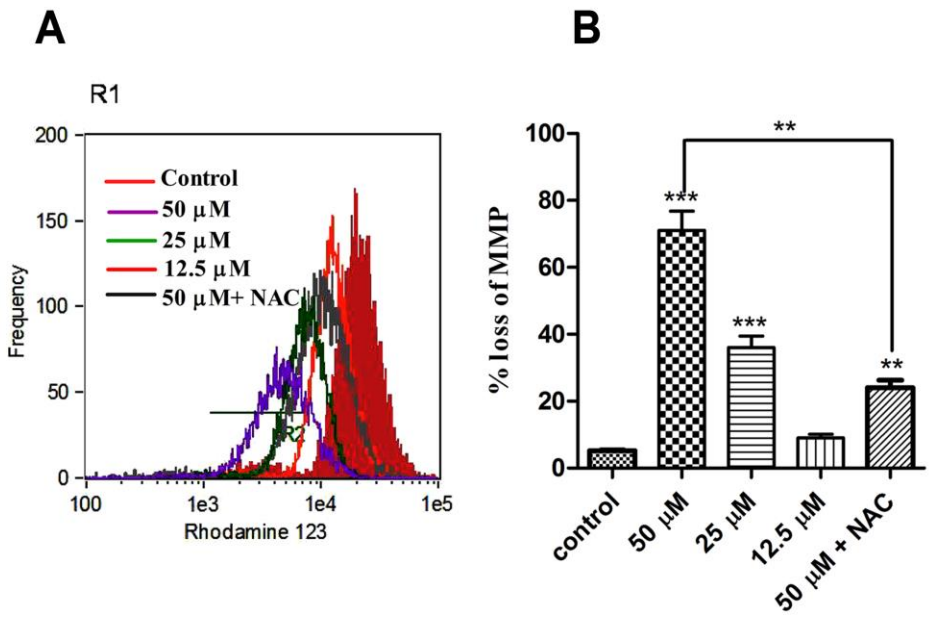


Figure 5.3.6. Effect of COP on mitochondrial function in A549 cells. **A** Cells were treated with different concentrations of COP with or without NAC for 24 h and then incubated with rhodamine 123 for 30 min. MMP was measured by flow cytometry. The histogram depicts COP-caused MMP loss in A549 cells and pretreatment with NAC prevented the COP-induced mitochondrial dysfunction.

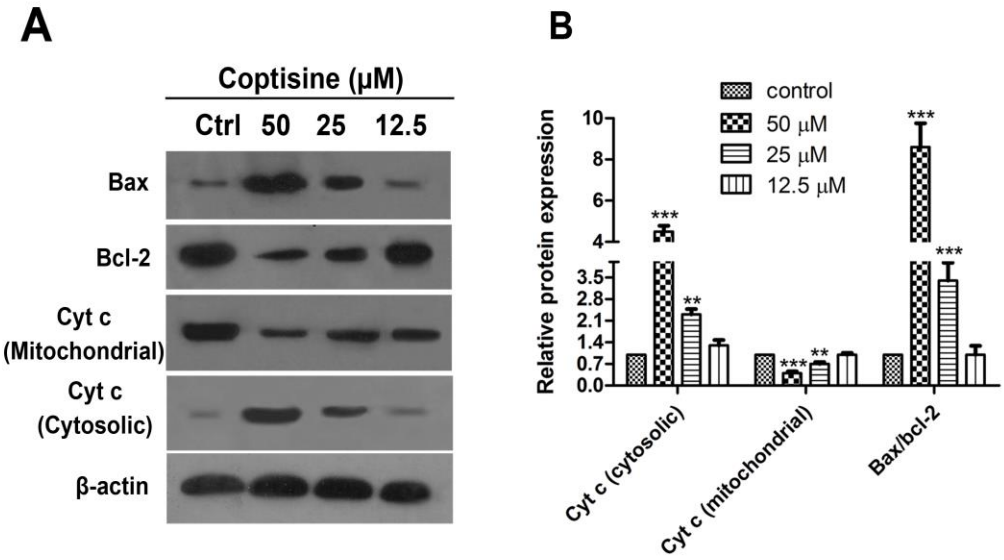


Figure 5.3.7: Effect of COP on mitochondrial related proteins. Expression of Bax, Bcl-2, cyt c in A549 cells were estimated by western blot (A) and densitometric analysis (B). β-actin was used for loading control. Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group or group with NAC versus without NAC.

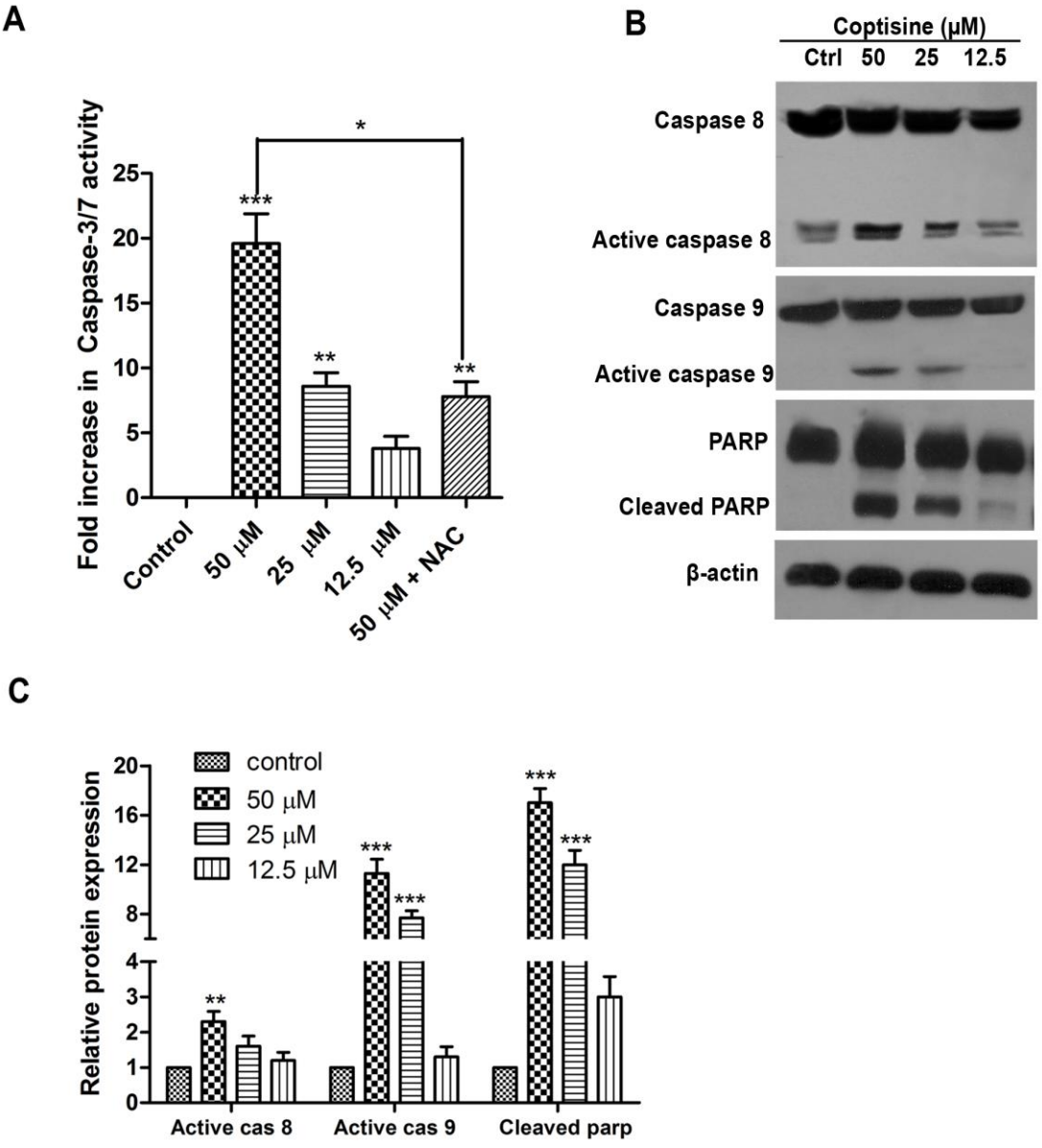


Figure 5.3.8: Effect of COP on activity of caspases. Activity of caspase 3/7 was determined separately by In situ caspase assay kit. A549 cells were treated with different concentrations of COP in the presence or absence of 5 mM NAC for 48 h and fluorescence intensity was measured (A). Expression of caspase 8, caspase 9 and PARP in A549 cells treated with indicated concentration of COP for 48 h was detected by western blot analysis (B) followed by densitometry (C). β-actin was used for loading control. Data were expressed as mean ± SEM of three independent experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ versus control group or group with NAC versus without NAC

5.3.4 Effect of COP on ROS generation

ROS is found to be the mediator of intracellular cascade signaling. The excessive generation of ROS can induce oxidative stress, loss of cell functioning and apoptosis. Hence, to investigate the role of ROS in COP-induced apoptosis, cells treated with COP were stained with DCFDH and ROS was quantified by flow cytometry. The levels of ROS after COP treatment with 50 µM at 0.5, 1, 2, 4, 12 and 24 h were estimated to be 23.7, 28.1, 76, 85.7, 86.2 and 87.6%, respectively. Results demonstrated significantly increased ROS levels in a time and dose dependent manner by COP (Figure 5.3.4). Further NAC significantly inhibited the COP-induced ROS generation (Figure 5.3.5).This finding indicated that ROS generation in A549 cells was triggered by COP.

5.3.5 COP disrupts MMP and induces apoptosis through mitochondrial dependent apoptotic pathway

Mitochondria are vital component of apoptotic machinery. Permeabilization of the mitochondrial outer membrane resulting from depolarization of MMP to release proteins from the intermembrane space is a major event during apoptosis (Waterhouse NJ *et al*., 2002). To determine the effect on mitochondrial function, change of MMP by flow cytometer with rhodamine 123 was performed. Percentage of cells with MMP loss after COP treatment with 50, 25 and 12.5 µM at 24 h was found to be 71.2, 36.5 and 8.7%, respectively (Figure 5.3.6). Outcome of flow cytometry manifested a significant disruption of mitochondrial function.

Further, it has been uncovered that Bcl-2 family plays an important role in regulating mitochondrial pathway (Hong C *et al*., 2002). The balance between the pro apoptotic proteins including Bax and Bad and antiapoptotic proteins including Bcl-2 and Bcl-xl decides the fate of cells. Induction of mitochondrial permeability transition by Bax results in the release of cyt c into

cytosolic portion of cell (VanGurp M *et al*., 2003). To validate if such a mechanism is implicated in COP induced apoptosis, expression of Bax, Bcl-2 and cyt c was scrutinized by western blot analysis. Additionally COP significantly increased expression of cytochrome c in cytosol and reduced its expression in mitochondria along with increase of Bax/bcl-2ratio in a dose dependent manner (Figure 5.3.7). The cells pretreated with NAC significantly revoked the disruption of mitochondrial membrane potential.

Caspases are the family of proteins related to cysteine proteases which function as apoptotic executors to attain programmed cell death through triggering of extrinsic and intrinsic pathways (Cohen GM., 1997). Among them, caspase 9 an activating complex formed by cyt c along with other proteins, plays a vital role in intrinsic apoptosis pathway (Salvesen and Dixit., 1997). Activated caspase 9, in turn, activates caspase3, which is an activated death protease, catalyzing the specific cleavage of many cellular proteins (Adams JM., 2003). The mechanism of COP-induced apoptosis was further understood by examining the levels of caspase 3 through fluorescence assay, and expression of caspase 8, caspase 9 and Poly ADP ribose polymerase (PARP) by western blot assay. COP treatment increased the expression of active caspase 8 and 9 along with the cleaved PARP dose dependently (Figure 5.3.8). Active caspase 3 was increased by about 19 fold in cells treated with COP at 50 µM concentration compared to control and dose dependent induction of caspase 3 was observed. Induction of caspase 3 by COP was inhibited by NAC (Figure 5.3.8). It was proved that COP induced apoptosis is associated with ROS and these results clearly corroborated that ROS dependent mitochondrial apoptotic pathway is the mechanism of COP-mediated apoptosis.

5.3.6 Summary and Conclusion

Non-small cell lung cancer (NSCLC) accounting for about 85% of all lung cancer cases needs immediate attention because of the uncontrollable deaths of NSCLC patients in the world (Jemal A *et al*., 2009). Although, combination of chemotherapy and surgery has been identified as an optimal treatment for patients with early stage disease, no effective single drug therapy is currently available. Hence, discovery of specific and effective chemotherapeutic agents for NSCLC is a desperate need. Previous pharmacological findings on COP have reported it to effectively inhibit proliferation of hepatoma, leukemia cells and ovarian cancer cells. However, the mechanism underneath the COP-induced cytotoxicity was not explored and till date no reports are found in the literature elucidating the anticancer pathway of COP. In order to fulfill this gap, the current study was undertaken targeting A549 cells.

The study clearly demonstrated that COP inhibited proliferation of A549 cells through cell cycle arrest and apoptosis. COP-treated A549 cells were arrested at G2/M phase, accompanied by reduction in the expression of cyclin B1, cdc2 and cdc25C. Treatment of A549 cells with COP resulted in the generation of ROS, upregulation of Bax and downregulation of Bcl-2. The imbalance of Bax and Bcl-2 caused mitochondrial depolarization, which resulted in the cytochrome c release into the cytosol, followed by the activation of caspase 9 and 3 and consequently cleavage of PARP, leading to apoptosis. ROS scavenger, NAC effectively reduced the apoptotic activity, caspase 3 induction, and MMP loss by COP, indicating that COP-induced intrinsic mitochondrial apoptosis was unambiguously mediated by ROS.

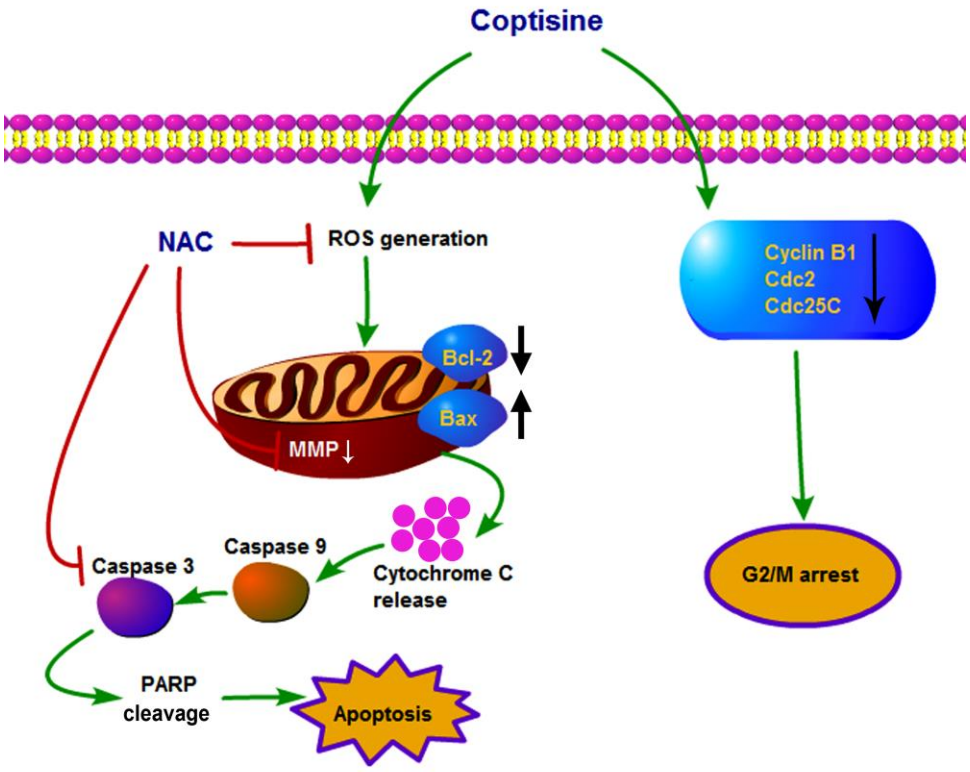


Figure 5.3.9: Schematic diagram of proposed mechanism of COP induced cytotoxicity. The ↓ indicates involvement, ⊥ indicates inhibition or blockade

5.4. Deciphering mechanisms underlying the cytotoxicity of withametelin

5.4. Deciphering mechanisms underlying cytotoxicity of withametelin

Drugs discovered from plants account for a significant proportion of approved anticancer therapeutic agents. Taxol, vinblastine, vincristine and topotecan are the major phyto-anticancer agents that are currently in clinical use all over the world. Also, a number of promising agents such as combrestatin, betulinic acid and silvesterol are in clinical or preclinical development. Exhaustive efforts on exploring cytotoxic leads from natural sources have been undertaken by worldwide researchers. However, only a few among the several identified cytotoxic plant derived molecules have so far been well exploited for their mechanisms of action. Promising chemotherapeutic agents will emerge if extensive research is carried out on such underexploited molecules. On this ground, search for the identification of potent plant derived cytotoxic molecules was initiated which disclosed withasteroids group of molecules, possessing wide spectrum of biological effects including antimicrobial, anti inflammatory, hepatoprotective, immunomodulatory, antidiabetic along with antitumor activities (Ray *et al*., 1994; Reyes-Reyes *et al*., 2012; Gorelick *et al*., 2015).

5.4.1 Molecular mechanistic studies on WM

Over the years, reports describing death of cancer cells by natural molecules *via* ROS metabolism by increasing the ROS levels above the toxic threshold are published. This can be easily attained in cancer cells as they produce higher levels of endogenous ROS compared with their normal cells (Fruehauf JP *et al*., 2007). ROS are known to disrupt mitochondrial function by affecting MMP and cause sequence of mitochondria associated events. ROS production may

contribute to the collapse of MMP, translocation of cyt c, subsequent activation of caspases, and eventually induction of apoptosis. This programmed cell death is characterized by cell shrinkage, blebbing of the plasma membrane and chromosomal DNA fragmentation (Reed J.C., 2000). Two major pathways are found to be involved in the regulation of apoptosis, *viz*., cell death receptor- (extrinsic) and mitochondria dependent apoptotic (intrinsic) pathways (Earnshaw, W.C *et al*., 1999). The extrinsic pathway is mediated through cell surface death receptors, which recruit adaptor molecules Fas-associated death domain (FADD) and caspase 8. Upon stimulation and recruitment, caspase 8 becomes activated and triggers apoptosis. On the other hand, mitochondrial dependent apoptosis is modulated by antiapoptotic and proapoptotic Bcl-2 family members. Loss of MMP causes the translocation of Bax to mitochondria and cyt c to cytosol, which subsequently activates caspase 9, resulting in apoptosis (Jiang X., 2000).

Search of literature on WM had revealed a study showing cell cycle arrest at S phase and induced apoptosis in DLD-1 cells (Bellila A *et al*., 2011). However, no work on A549, MDA-MB-231 and HT-29 cells, and mechanistic studies of WM for its antiproliferative effect against A549 cells are found in the literature and this is the first work to describe on this.

5.4.2 WM induces G2/M arrest in A549 cells

Cell cycle progression is tightly controlled by numerous mechanisms including various checkpoints ensuring correct cell division in normal cells. Any alterations in checkpoints will result in abnormal cell proliferation leading to the development of cancer. Tumor cells frequently acquire defects in checkpoints, resulting in uncontrolled proliferation. However, control of such proliferation of tumor cells can possibly be achieved through pharmacological correction of

defected check points (Kastan MB *et al*., 2004). Hence, the defect corrective effect of WM on cell cycle distribution of A549 cells was examined by cell cycle analysis.

It was observed that WM arrested cells at G2/M phase dose dependently. WM at concentrations of 12, 6 and 3 µM was found to increase cell population in G2/M phase from 20.8 % to 93.7, 35.6 and 29.9%, respectively (Figure 5.4.1). This was accompanied by the decrease in the cells in G1 phase but no effect was found in the S phase. This finding demonstrated WM induced cell cycle arrest at G2/M phase to be one of mechanisms of WM induced cytotoxicity.

5.4.3 Effect of WM on G2/M Regulatory proteins

Several studies have documented the death of cancerous cells by chemotherapeutic agents through cell division arrest at certain check points of cell cycle (Rasul *et al*., 2012a; Rasul *et al*., 2012b; Rasul *et al*., 2012c). Cyclins and CDKs are family of proteins needed for cell progression acting on check points. Cyclin B1 forms complex with cdc2 (Cdk1) which is essential for the cells to enter into mitosis. During G2 phase, dephosphorylation of regulatory residues of cdc2, Thr14 and Tyr 15, by phosphorylating cdc25C to pcdc25C at ser 216, directly activates cyclin b1/cdc2 complex and causes mitosis initiation. When cells undergo genotoxic stress, phosphorylation of cdc2 weakens the activity of cyclin B1 and cdc2 complex that arrest cells in G2/M phase (Carmazzi, Y *et al*., 2012). To further support the inference, G2 regulatory proteins including cyclin B1, cdc2 and cdc25C in A549 cells treated with WM were evaluated. Protein extracts were prepared from the cells treated with different concentrations of WM for 24 h and analyzed by western blot assay. As a result, expression of cyclin B1, cdc2 and cdc25C were significantly reduced by WM in a dose dependent manner compared to control (Figure 5.4.2).

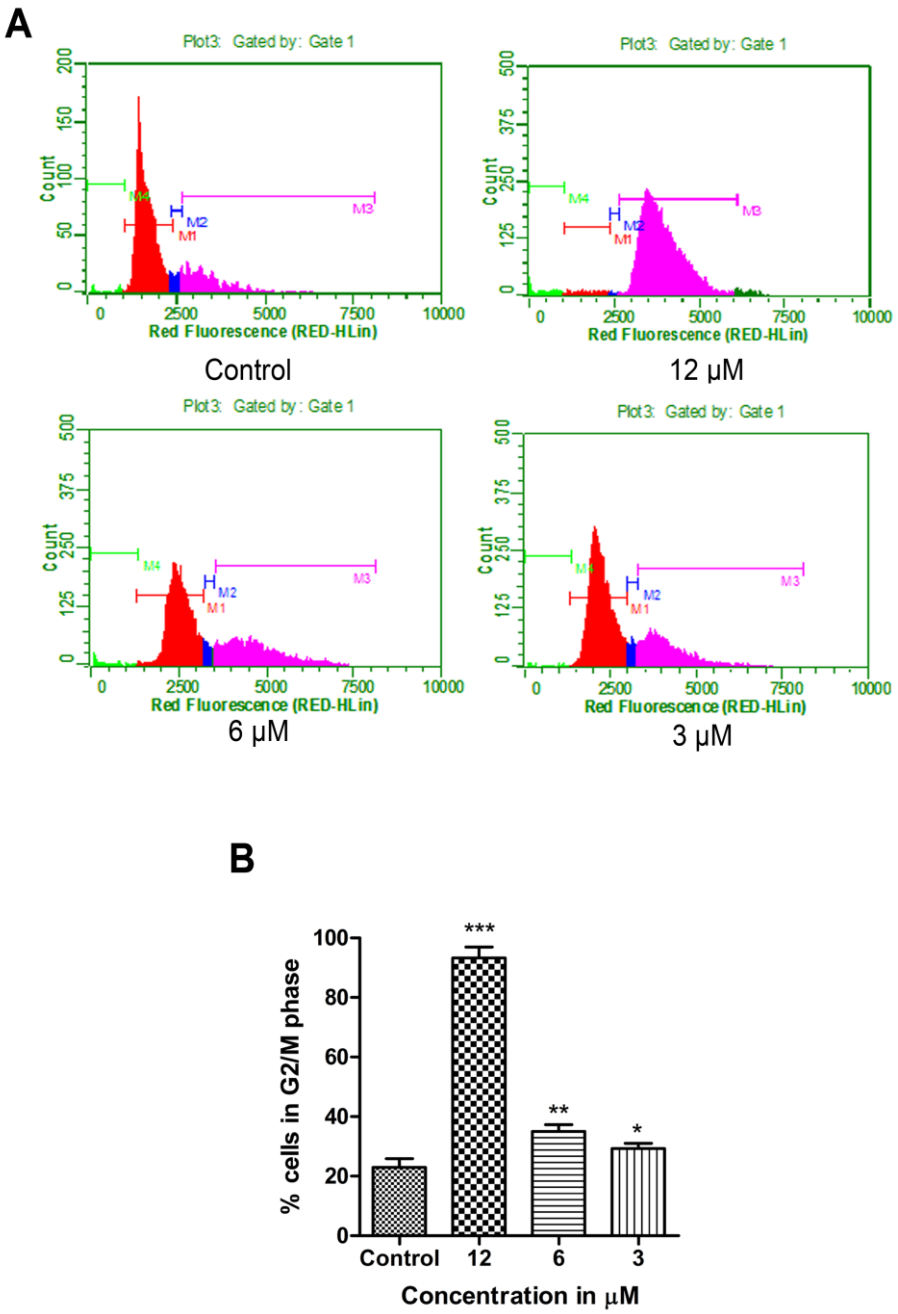


Figure 5.4.1: WM induced G2/M arrest. (A) A549 cells were exposed to different concentrations of WM for 24 h stained with PI and analyzed by flow cytometry. (B) All results were expressed as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus control group.

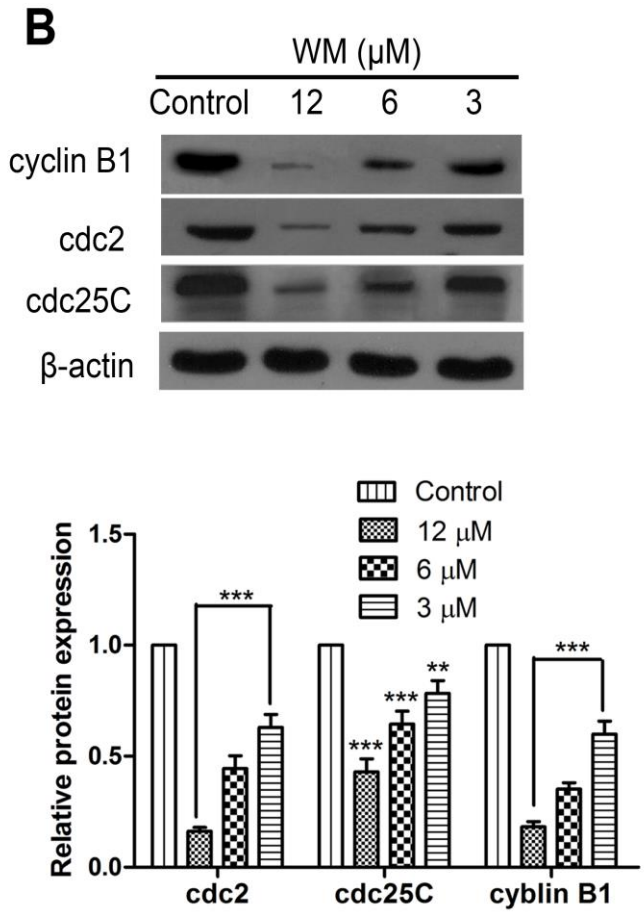


Figure 5.4.2: WM dowregulated G2/M regulatory proteins. Detection of cell cycle regulatory proteins, cyclin B1, cdc2 and cdc25C by western blotting analysis followed by densitometry. All results were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group

5.4.4 WM induces apoptosis in A549 cells

Apoptosis play an important role in the physiological growth control and regulation of tissue homeostasis. Tilting the balance between cell death and proliferation towards cell survival may result in the development of cancer. Hence, modulation of apoptosis in cancer cells is more important in the treatment of cancer. Accumulated literature data has indicated that most

anticancer agents terminate tumor cell progression through the induction of apoptosis (Rasul *et al*., 2012a; Rasul *et al*., 2012b; Rasul *et al*., 2012c).

To further investigate if the inhibition of A549 cells by WM was through the induction of apoptosis, A549 cells were incubated with different concentrations of WM for 24 h and the percentage of apoptotic/necrotic cells were analyzed by staining with Annexin V FITC and PI. WM increased the apoptotic population in treated cells, from 9.8 % to 75, 44.4 and 24% at 12, 6 and 3 µM, respectively. Further at the same tested concentration, PI and Annexin V positive or necrotic population were found to be 24.5, 32 and 11.4% (Figure 5.4.3). The results clearly evidenced that A549 cells underwent apoptosis after exposure to WM.

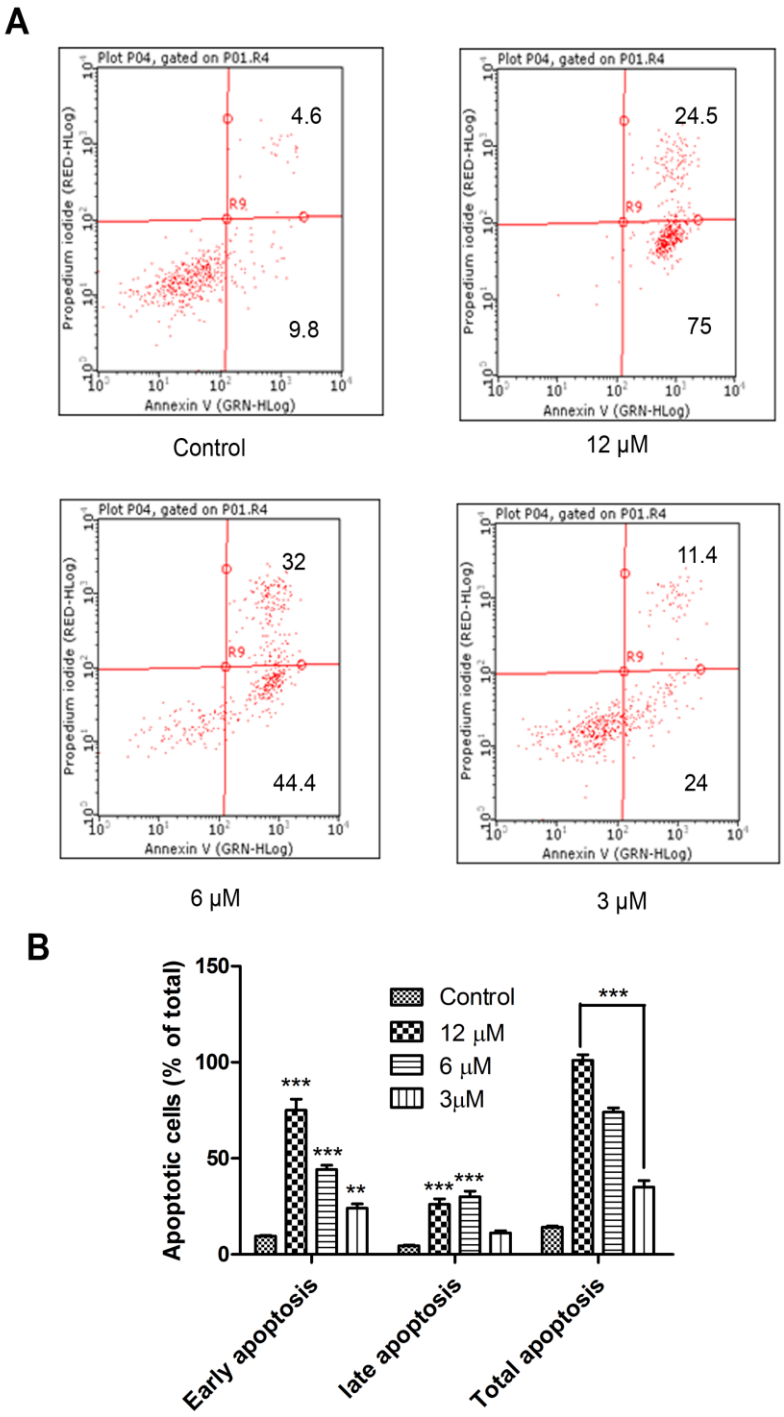


Figure 5.4.3: WM induced apoptosis in A549 cells. (A) A549 cells treated with various concentrations of WM for 24 h. Then cells were double stained with FITC conjugated Annexin V and PI for flow cytometric analysis. (B) Quantitative data of A. Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group.

5.4.5 WM triggers generation of ROS in A549 cells

ROS is found to be the mediator of intracellular cascade signaling. The excessive generation of ROS can induce oxidative stress, loss of cell functioning and apoptosis. Hence, to validate if WM induced apoptosis was a consequence of increased ROS levels, the intracellular ROS was measured. DCFDA, a highly sensitive and accurate dye was used for measuring oxidative stress in irradiated cells. Gradual increase of ROS with increasing concentrations of WM was observed from 1 h to 24 h. ROS levels in A549 cells treated with 12 μM at 1, 2, 4, 12 and 24 h were determined as 24, 30, 33.8, 72.5 and 77%, respectively (Figure 5.4.4). NAC, a nonspecific antioxidant is commonly used to identify and test the ROS inducers. Study with NAC, displayed a significant abrogation of ROS generation by WM (Figure 5.4.5). This finding indicated that ROS generation in A549 cells was triggered by WM. The anticancer agents are likely to cause toxicity to cancer cells by causing increase of oxidative stress because it is involved in the induction of various biological responses like DNA repair, cell cycle arrest, and apoptosis (Pelicano *et al*., 2004).

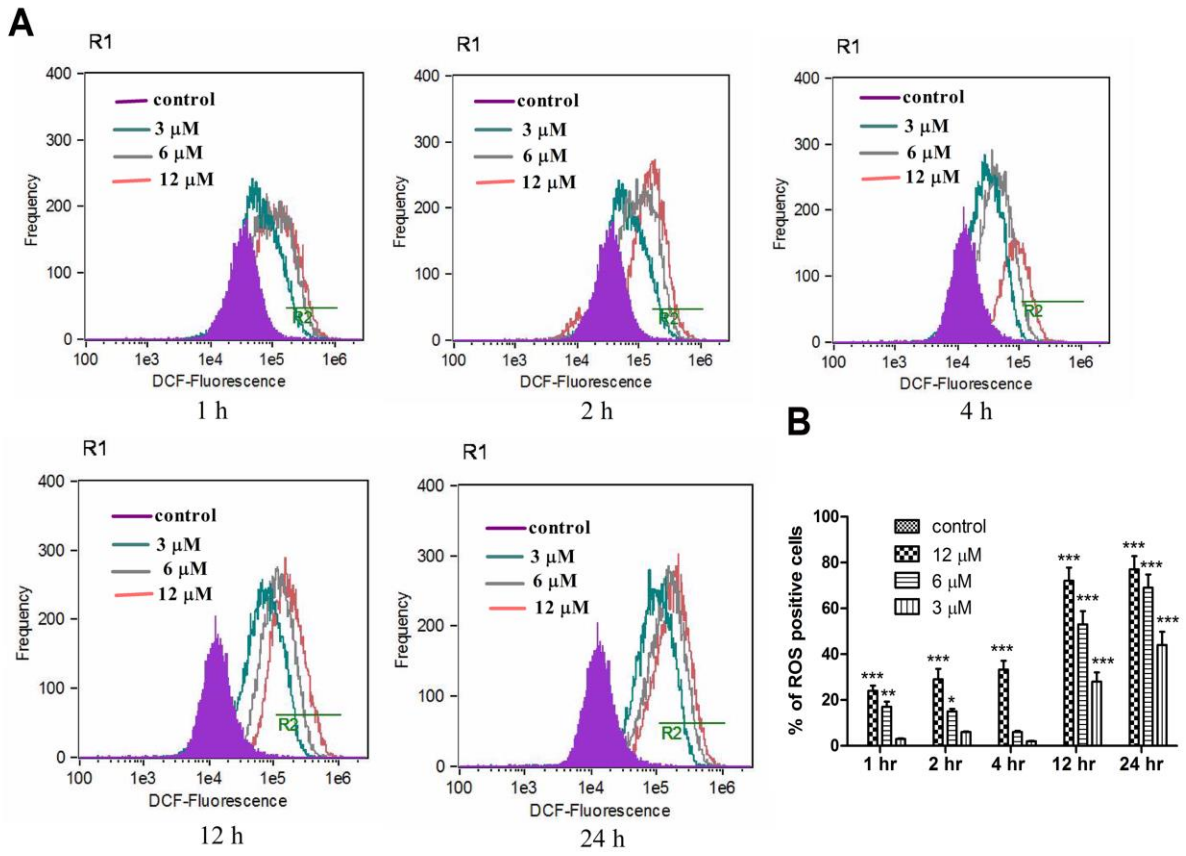


Figure 5.4.4: WM triggered ROS generation in A549 cells. Cells were treated with different concentrations of WM from 30 min to 24 h, incubated with DCFDA (30 min) and the intracellular ROS was measured by flow cytometer (A). The histogram depicts the percentage of ROS positive cells of indicated concentrations of WM at different time points (B). Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group.

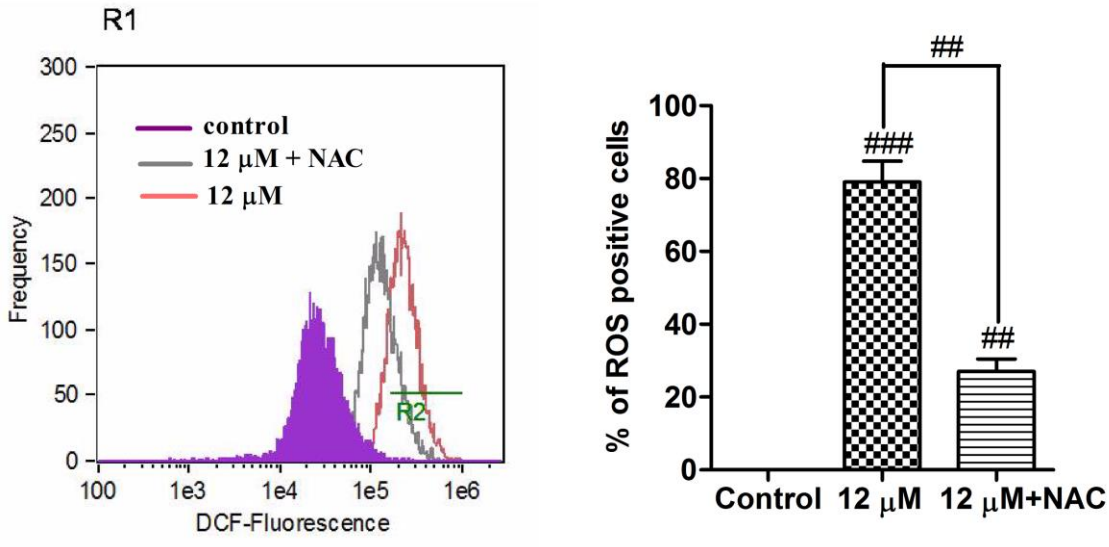


Figure 5.4.5: NAC abrogated WM induced ROS generation. Cells were pretreated with NAC for 2 h and then treated with WM (12 µM) for 24 h. The histogram depicts NAC prevented WM induced ROS generation. Data were expressed as mean ± SEM of three independent experiments. **p<0.01 and ***p<0.001 versus control group.

5.4.6 WM disrupts MMP and induces apoptosis through mitochondria dependent apoptotic pathway

Mitochondria are vital component of apoptotic machinery. Permeabilization of the mitochondrial outer membrane resulting from depolarization of MMP to release proteins from the intermembrane space is a major event during apoptosis (Waterhouse N.J *et al*., 2002). The effect of WM on MMP of treated A549 cells was analyzed by flow cytometry using RH-123. WM caused dose dependent increase in depolarization of mitochondrial membrane. The percentage depletion of MMP after 12, 6 and 3 µM of WM treatment was found to be 70, 47 and 27%, respectively (Figure 5.4.6). Outcome of flow cytometry manifested a significant disruption of mitochondrial function.

Further, it has been uncovered that Bcl-2 family plays an important role in regulating mitochondrial pathway (Hong C *et al*., 2002). The balance between the pro apoptotic proteins including Bax and Bad and antiapoptotic proteins including Bcl-2 and Bcl-xl decides the fate of cells. Induction of mitochondrial permeability transition by Bax results in the release of cyt c into cytosolic portion of cell (Van Gurp *et al*., 2003). To validate if such a mechanism is implicated in WM induced apoptosis, expression of Bax, Bcl-2 and cyt c was scrutinized by western blot analysis. WM significantly increased the expression of cyt c in cytosolic fraction by reducing its expression in mitochondria, suggesting that WM-induced release of cyt c to cytosol from mitochondria was dose dependent. Furthermore, while the expression of Bax was increased, the expression of Bcl-2 was markedly reduced (Figure 5.4.7).

Caspases are the family of proteins related to cysteine proteases which function as apoptotic executors to attain programmed cell death through triggering of extrinsic and intrinsic pathways. Among them, caspase 9 an activating complex formed by cyt c along with other proteins, plays a vital role in intrinsic apoptosis pathway (Salvesen GS *et al*., 1997). Activated caspase 9, in turn, activates caspase 3, which is an activated death protease, catalyzing the specific cleavage of many cellular proteins (Adams JM., 2003).

To examine the involvement of caspases in WM induced apoptosis, activities of caspase 9, caspase 3 and its downstream target PARP were assessed. Results demonstrated that WM treatment increased the activity of caspase 3 by 12 folds at higher concentration compared to control and the induction of caspase 3 was observed to be dose dependent (Figure 5.4.8). Additionally, WM caused the induction of active caspase 9 along with PARP cleavage dose dependently (Figure 5.4.8). A549 cells were subjected to different concentrations of WM to determine its involvement in the extrinsic apoptotic pathway as well. Interestingly, the activity of

caspase 8 was not affected by WM treatment. Results confirmed the action of WM through intrinsic or mitochondria mediated apoptotic pathway.

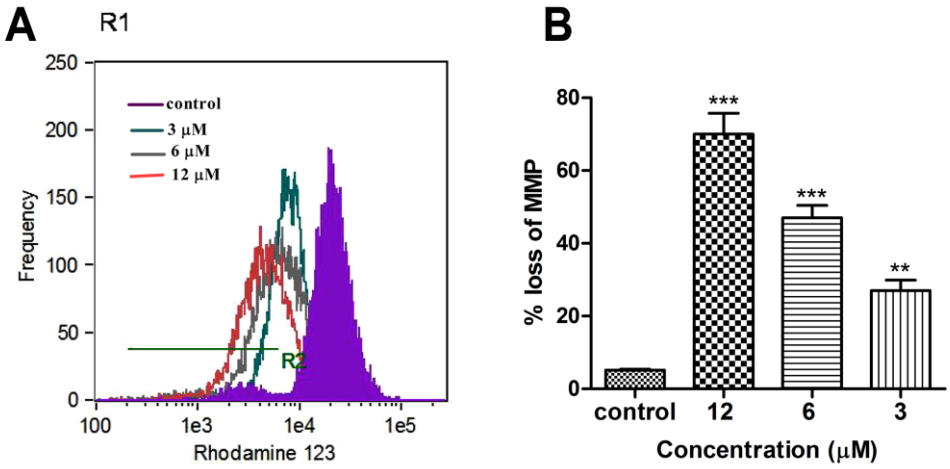


Figure 5.4.6: Effect of WM on mitochondrial function in A549 cells. (A) Cells were treated with different concentrations of WM for 24 h and then incubated with RH-123 for 30 min. MMP was measured by flow cytometry. (B) The histogram depicts WM-caused MMP loss in A549 cells.

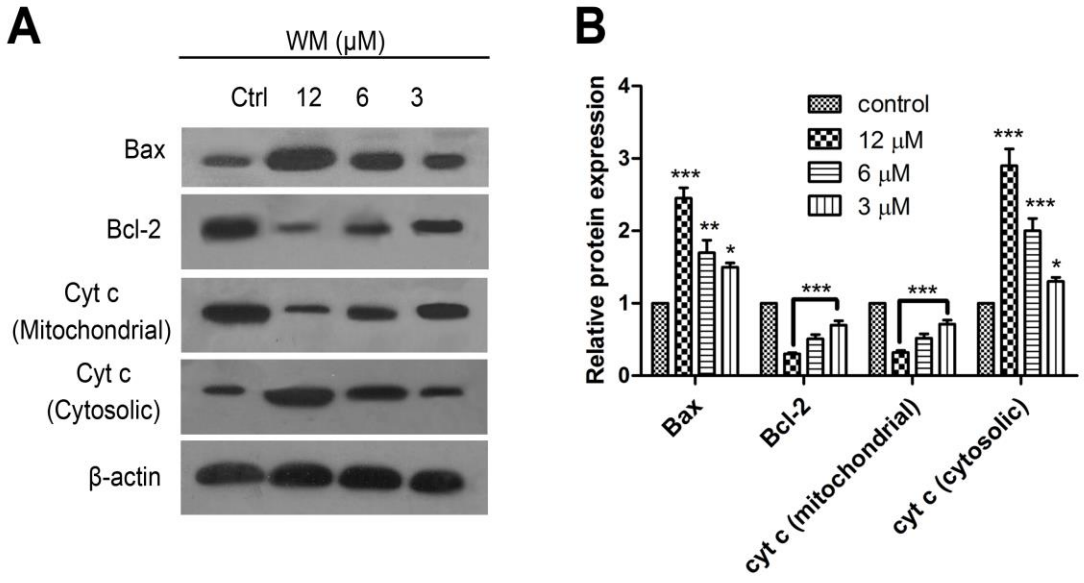


Figure 5.4.7: Effect of WM on mitochondrial related proteins. Expression of Bax, Bcl-2, cytochrome c (cyt c) estimation by western blot analysis (A) and densitometric analysis results (B). β-actin was used as loading control. Data were expressed as mean ± SEM of three independent experiments.*p<0.05, **p<0.01 and ***p<0.001 versus control group.

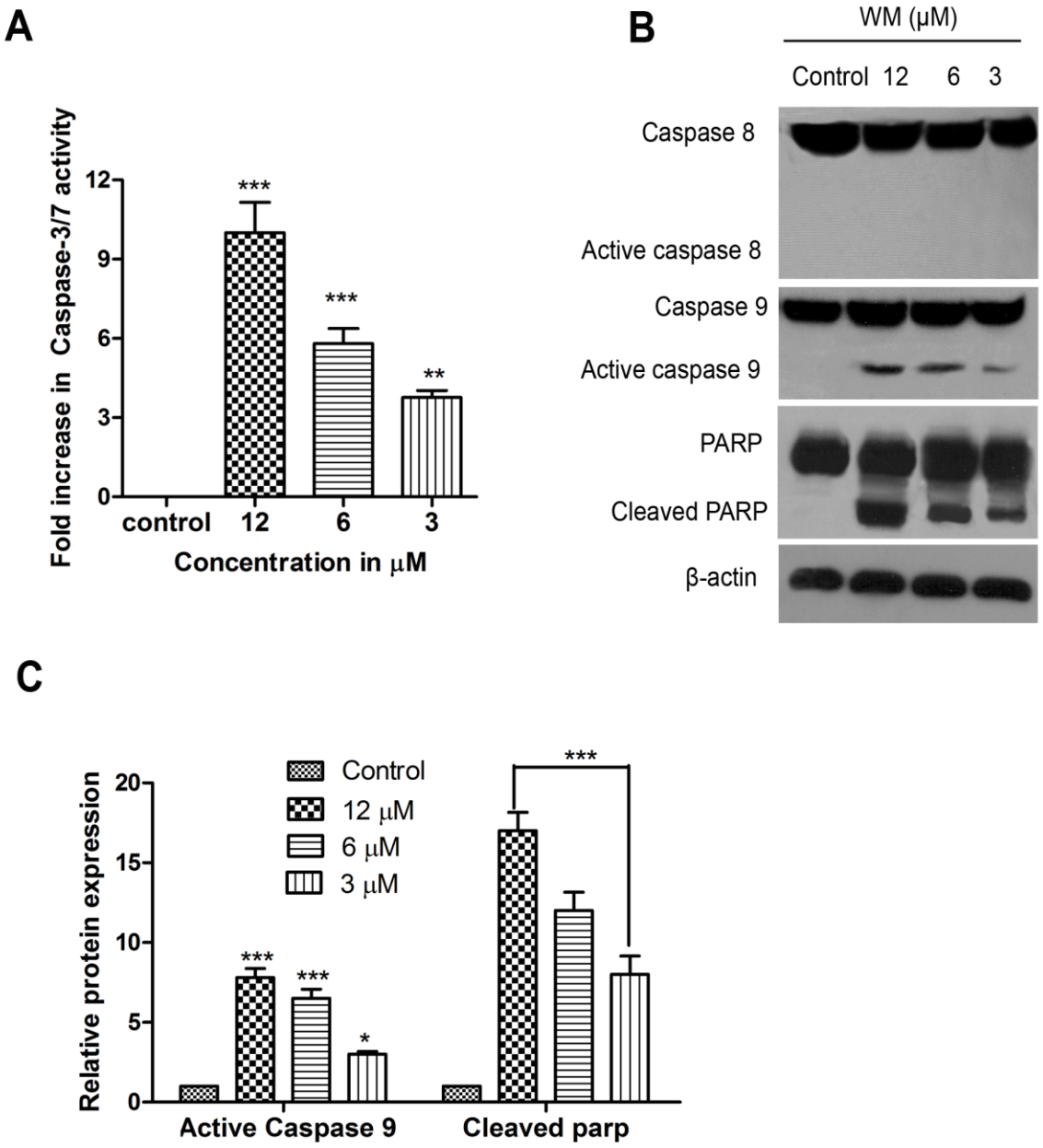


Figure 5.4.8: Effect of WM on the activity of caspases. (A) Activity of caspase 3/7 was determined separately by In situ caspase assay kit. Cells were treated with different concentrations of WM for 24 h and fluorescence intensity was measured. (B) Expression of caspase 8, caspase 9 and PARP in A549 cells treated with indicated concentration of WM for 24 h and detection by western blot analysis. (C) Followed by densitometry. β-actin was used for loading control. Data were expressed as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus control group

5.4.7 Effect of NAC on WM induced anti proliferative effect

Further, to identify if the anti proliferative effect of WM is mediated through ROS, A549 cells were pretreated with NAC and subsequently antiproliferative effect was assessed by MTT assay. MTT results revealed that anti proliferative effect of WM was not affected when the cells were pretreated with a ROS scavenger, NAC (Figure 5.4.9).

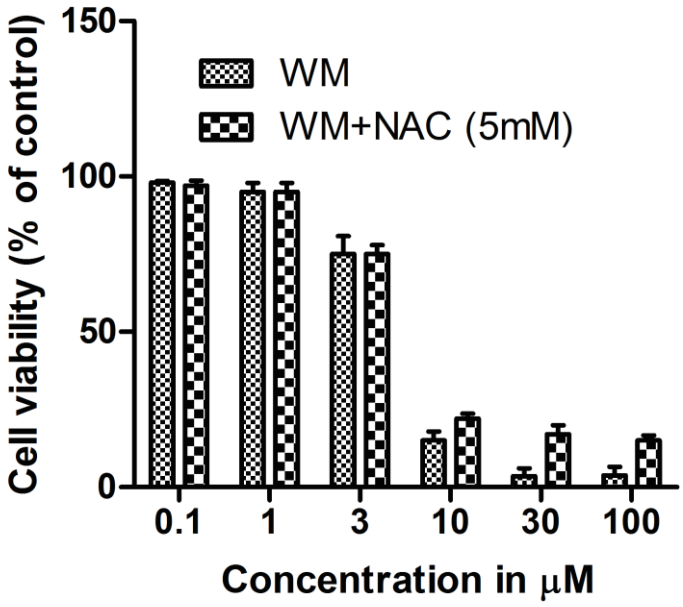


Figure 5.4.9: Effect of NAC on WM induced anti proliferative effect. The cells were treated with various concentrations of WM with or without NAC for 24 h and percentage of cell viability was estimated by MTT assay. Data were expressed as mean ± SEM of three independent experiments.

5.4.8 Effect of NAC on WM induced apoptosis

To confirm whether NAC abrogated WM-induced cytotoxicity**,** A549 cells were pretreated with NAC, followed by A549 cells were stained with FITC conjugated annexin V and PI double staining. Pretreatment with NAC failed to prevent apoptotic effect of COP. Results confirmed that WM induced apoptosis is not mediated through ROS (Figure 5.4.10).

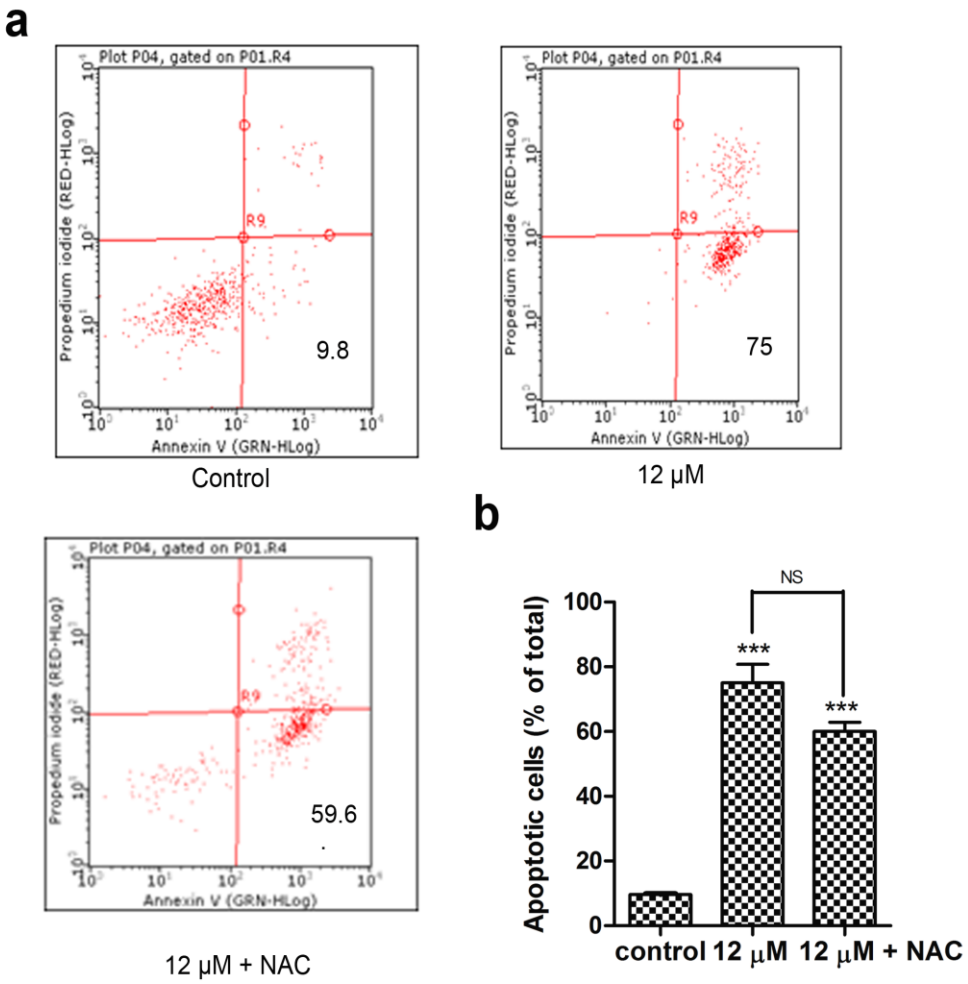


Figure 5.4.10: Effect of NAC on WM induced apoptosis. (A) A549 cells treated with various concentrations of WM with or without NAC for 24 h. Then cells were double stained with FITC conjugated Annexin V and PI for flow cytometric analysis. (B) Quantitative data of A. Data were expressed as mean ± SEM of three independent experiments. ***p<0.001 versus control group. NS represents non significant.

5.4.9 Summary and Conclusion

Considerable interest has been gained by withasteroids because of their structural uniqueness and wide spectrum of biological activities. However limited systematic studies for proving their cytotoxic potential have so far been reported. Hence, an attempt was made to test the cytotoxicity of seven withasteroids *viz.*, withametelin (WM). In conclusion, the present study demonstrated the cytotoxic effect and mechanism of action of WM. The substitutions at ring A and B and at

Carbon 17 could be accountable for the difference in the inhibitory effect of various withasteroids. Further the very significant cytotoxic potential of WM against A549 cells, was revealed to be mediated by G2/M arrest and induction of apoptosis. G2/M phase arrest was ascertained from the downregulation of cyclin B1, cdc2 and cdc25C expression. WM induced cell death in A549 cells through mitochondria dependent apoptotic pathway, causing the generation of ROS, upregulation of Bax and parallel dowregulation of Bcl-2. Eventually, dissipation of MMP, translocation of cyt c and sequential activation of caspase-9 and -3 and its downstream substrate PARP occurred, leading to apoptosis. Thus, the current study explored the mechanism behind the cytotoxicity of WM, which has the potential to be developed as a new therapeutic class for lung cancer. Also, the cytotoxicity results of withasteroids project them as an interesting group of natural molecules that is worthy of continuing anticancer research.

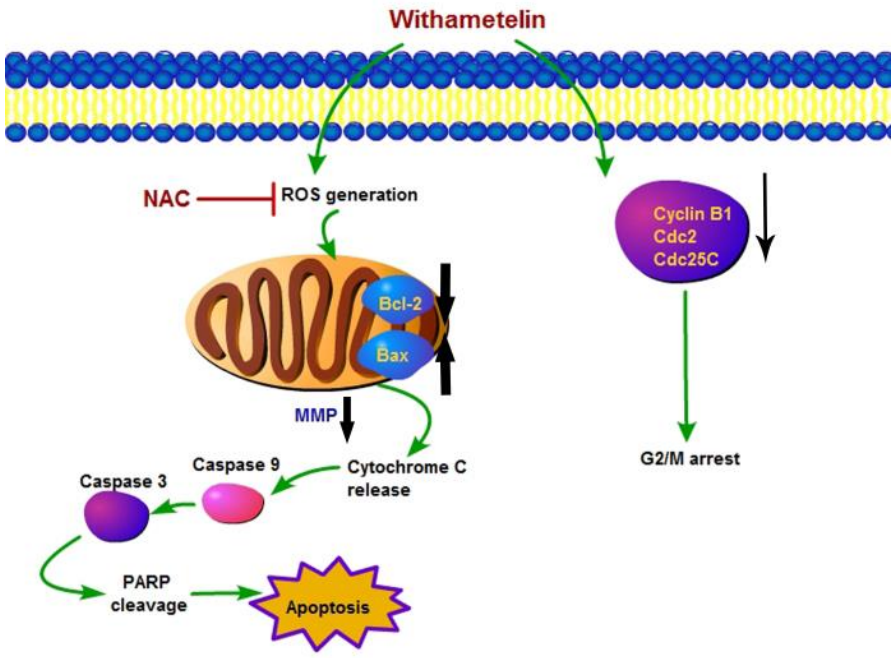


Figure 5.4.11: Illustration of proposed mechanism of WM-induced cytotoxicity

5.5 Mechanism underlying the cytotoxicity of cedrelopsin

5.5 Mechanism underlying the cytotoxicity of cedrelopsin

Prognosis for lung cancer persists depressing with 5-year survival period still lingering around 15%, unlike significant developments made in prognosis of certain cancers (prostrate, breast etc). Non-small cell lung cancer is leading by 85% among all lung cancers despite decades of relentless progress in chemotherapeutic and surgical interventions. Thus, our primary thrust was to develop effective anti cancer agents and apprehend the mechanism by which they cause cytotoxicity. Some species from the genus *Oldenlandia*, belonging to the family Rubiaceae, have exhibited remarkable anti-cancer effects. *Oldenlandia diffusa* is clinically used for treatment of cancer at a dose of 30-60 g/day and it has been incorporated in about 15% Chinese anti cancer herbal formulations on account of its safety (Shao J *et al*. 2011). Another species, *Oldenlandia corymbosa* (syn. *Hedyotis corymbosa*) was reported to display significant anticancer activity against human leukemia cells K562 and human breast carcinoma dependent hormone cells MCF-7 (Sivaprakasam S *et al*. 2014).

O. umbellata (syn. *Hedyotis umbellata* L.), commonly known as Indian madder or Chay root, is widely grown in India, Ceylon, Burma, Pakistan and west Tropical Africa. The Indian Siddha System of Medicine documented that it can treat tuberculosis, haemoptysis, bronchitis and asthma (Yoganarasimhan., 2000). Pharmacological reports disclosed that the plant possesses different activities including anti-tussive (Hema *et al*., 2007), hepatoprotective and anti-oxidant (Malaya *et al*., 2007), antibacterial (Rekha *et al*., 2006), anti-inflammatory and anti-pyretic (Padhy and Endale., 2014) and anti-tumor activities (Sethuramani *et al*., 2014). Mahibalan S *et al* described the isolation and characterization of Cedrelopsin (CDLN) from *O.*

umbellata and tested its anti proliferative activity against different human cancer cell lines including breast, colorectal and non-small cell lung cancer. Interestingly, it was found to be active only against A549 cells with an IC_{50} of 14.2 µM. However, further mechanistic studies were not performed to study the mechanism of action of its anti proliferative effect. Therefore, molecular mechanism underlying its activity has been explored in the present work.

CDLN, a dimethylallyl coumarin derivative has so far been isolated from *Melicope borbonica* (Simonsen HT *et al*., 2004), *Cedrelopsis grevei* (Um BH *et al*., 2003), *Rubia wallichiana* (Wu T *et al*., 2003), *Clausena excavata* (Huang S *et al*., 1997), *Harrisonia perforata* (Tanaka T *et al*., 1995), *Citrus grandis* (Wu T *et al*., 1988), and, *Angelicada hurica* (Kozawa M *et al*., 1981). Further regioselective synthesis of cedrelopsin (Patre *et al*., 2011) had been reported.

5.5.1 Effect of cedrelopsin on apoptosis of A549 cells

CDLN caused concentration dependent inhibition of cell proliferation of A549 cells (IC_{50} 14.2 µM) under MTT assay (Fig. 5.5.1). Apoptosis plays an important role in the physiological growth control and regulation of tissue homeostasis. Tilting the balance between cell death and proliferation towards cell survival may result in the development of cancer. Thus, modulation of apoptosis in cancer cells could be used as a strategy to treat cancer (Fulda S *et al*., 2004). Accumulated literature data has indicated that many of the anticancer agents induce apoptosis to inhibit tumor growth (Rasul *et al*., 2012a; Rasul *et al*., 2012b; Rasul *et al*., 2012c). The mechanism behind the antiproliferative effect of CDLN was assumed as apoptosis, which was then evaluated by incubating A549 cells with different concentrations of CDLN for 48 h and the percentage of apoptotic/necrotic cells were analyzed by staining with Annexin V FITC and PI. As shown in 5.5.2, early apoptotic population or Annexin V positive cells as well as late

apoptotic population or PI and annexin V positive cells of treated A549 cells were not affected by CDLN.

Caspases are the family of proteins related to cysteine proteases which function as apoptotic executors to attain programmed cell death through triggering of extrinsic and intrinsic pathways (Cohen GM., 1997). To further scrutinize if the anti proliferative effect of cedrelopsin is not mediated by the induction of apoptosis, executor caspase, caspase 3 levels were estimated using Insitu caspase 3/7 assay kit. Results revealed failure of CDLN to induce active caspase 3 in A549 cells at all concentrations (Figure 5.5.3). This unambiguously corroborated that the antiproliferative effect of CDLN against A549 cells was not due to induction of apoptosis.

5.5.2 Cedrelopsin induced G2/M arrest

Several check points at different stages tightly regulate cell cycle progression by constantly checking defects to ensure proper cell division in normal cells. Tumor cells frequently obtain defects in checkpoints, which subsequently lead to abnormal cell proliferation, eventually resulting in the development of cancer. G2/M check point stops the cells entering mitosis in response to DNA damage, providing an opportunity for repair and terminating proliferation of defected cells (Grana X and Reddy EP., 1995). Coumarin derivatives have been shown to inhibit neoplastic cell proliferation by arresting cells at G2/M phase (Haghighi F *et al*., 2014; Nasr T *et al*., 2014; Singh RK *et al*., 2011). To investigate if the mechanism of anti proliferative effect of CDLN was related to cell cycle control, its effect on cell cycle distribution in A549 cells was examined by cell cycle analysis. It was observed that cedrelopsin arrested cells at G2/M phase dose dependently. CDLN at concentrations of 100, 30 and 10 µM was found to increase cell

population in G2/M phase from 20.8 % to 92.9, 39.2 and 30.7%, respectively (Figure 5.5.4). This finding demonstrated CDLN-induced cell cycle arrest at G2/M phase.

G2/M transition provides effective checkpoint, which is regulated by activation of specific cyclin and cyclin dependent kinase complexes. Cyclin B1 forms complex with cdc2 (Cdk1) which is essential for the cells to enter into mitosis. During G2 phase, dephosphorylation of regulatory residues of cdc2, Thr14 and Tyr 15, by phosphorylating cdc25C to pcdc25C at ser216, directly activates cyclin B1/cdc2 complex and causes mitosis initiation. When cells undergo genotoxic stress, phosphorylation of cdc2 weakens the activity of cyclin B1 and cdc2 complex that arrest cells in G2/M phase (Carmazzi Y *et al* 2012). To further support the inference, G2 regulatory proteins including cyclin B1, cdc2 and cdc25C were evaluated in A549 cells treated with CDLN. Protein extracts were prepared from the cells treated with different concentrations of CDLN for 48 h and analyzed by western blot assay. As a result, expression of cyclin B1, cdc2 and cdc25C were significantly reduced by cedrelopsin in a dose dependent manner compared to control (Figure 5.5.4). This finding ascertained that cedrelopsin inhibited the proliferation of A549 cells by arresting them at G2/M phase.

5.5.3 Summary and conclusion

The study on CDLN demonstrated the inhibition of A549 cell proliferation mediated by G2/M arrest. CDLN induced G2/M arrest, as evidenced by downregulation of G2/M regulatory proteins such as Cyclin B1, cdc2 and cdc25C. However, CDLN did not induce the apoptosis in A549 cells. Thus, current study explored the mechanism of antiproliferative activity of CDLN, which has the potential to be developed as a drug for treating NSCLC.

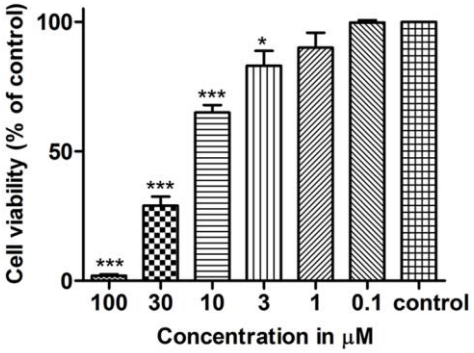


Figure 5.5.1 Effect of cedrelopsin on viability of A549 cells. The cells were treated with indicated concentrations of cedrelopsin for 48 h and the percentage of cell viability was estimated by MTT assay. Data were expressed as mean ± SEM of three independent experiments. . *p<0.05, **p<0.01 and ***p<0.001 versus control group.

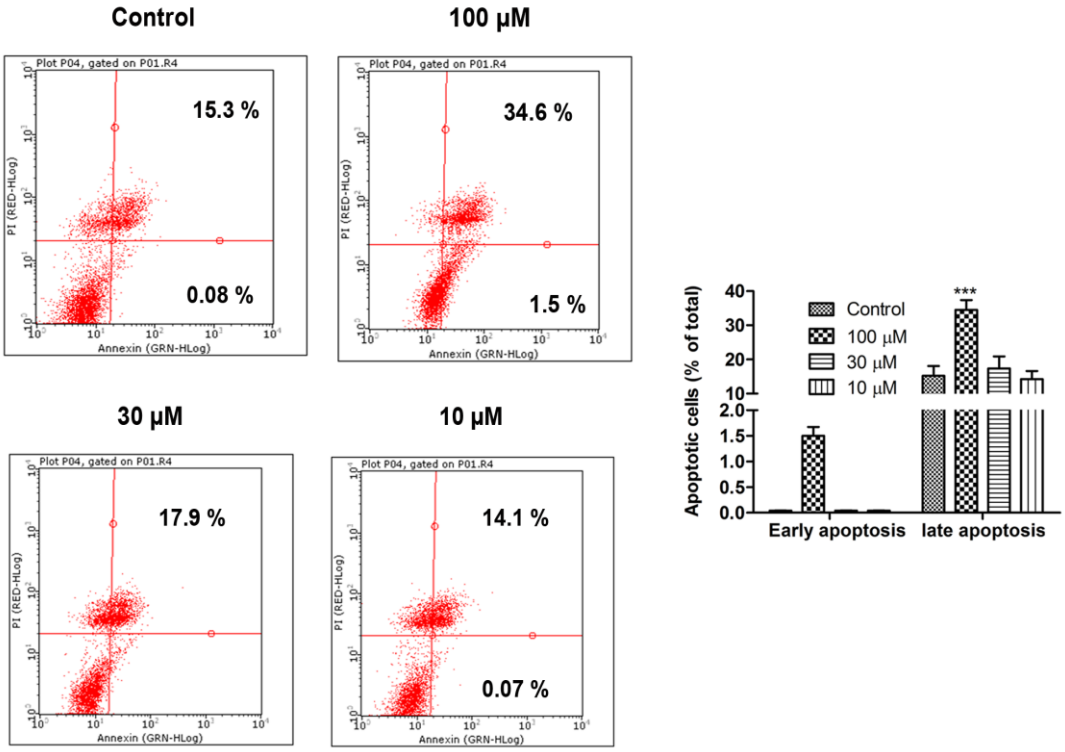


Figure 5.5.2 Effect of cedrelopsin on apoptosis of A549 cells. A549 cells treated with various concentrations of Cedrelopsin for 48 h. Then cells were double stained with FITC conjugated Annexin V and PI for flow cytometric analysis. Data were expressed as mean ± SEM of three independent experiments. . *p<0.05, **p<0.01 and ***p<0.001 versus control group.

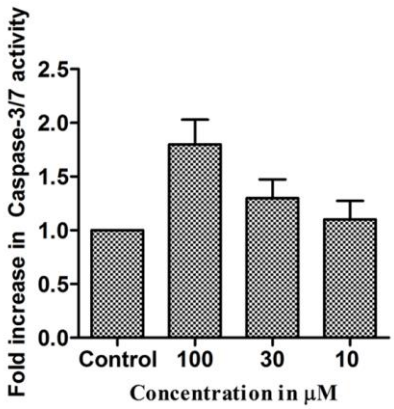


Figure 5.5.3 Effect of cedrelopsin on induction of caspase 3/7 in A549 cells. A549 cells treated with various concentrations of cedrelopsin for 48 h. Then cells were incubated with FLICA reagent and fluorescence was analyzed by plate reader. Data were expressed as mean ± SEM of three independent experiments.

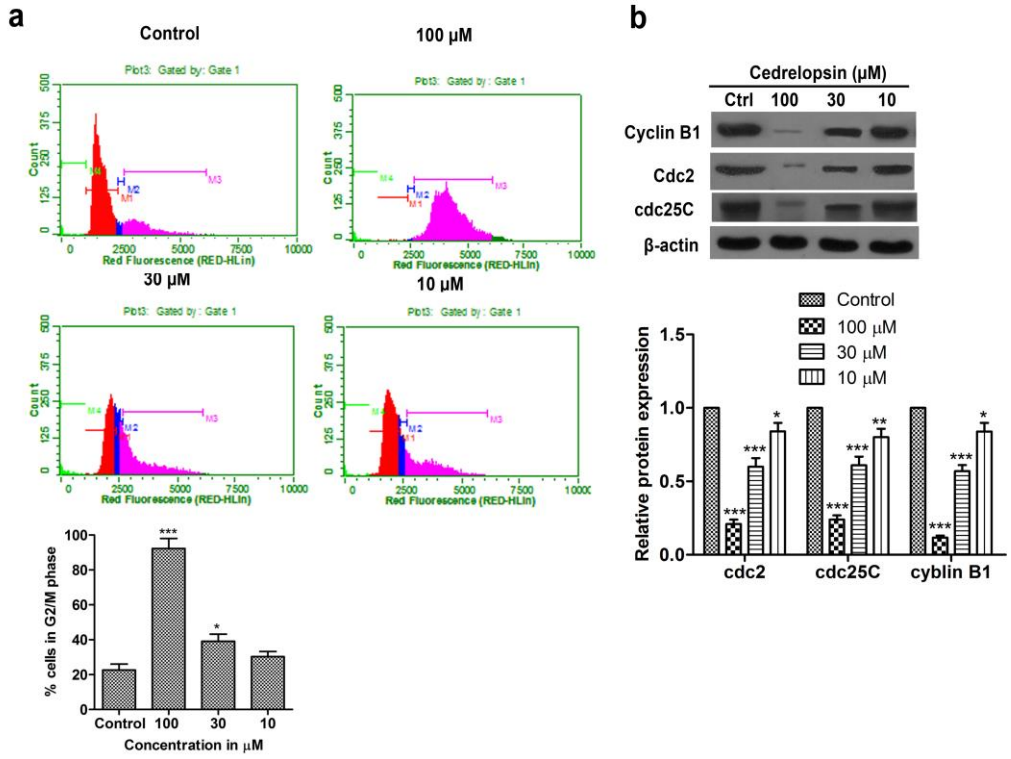


Figure 5.5.4 Cedrelopsin induced G2/M arrest. **a** A549 cells were exposed to different concentrations of cedrelopsin for 48 h stained with PI and analyzed by flow cytometry. **b** Detection of cell cycle regulatory proteins, cyclin B1, cdc2 and cdc25C by western blotting analysis followed by densitometry**.** All results were expressed as mean ± SEM of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus control group.

5.6. Assessment of anti proliferative activity of phytocompounds against other cancer cell lines

5.6 Assessment of anti proliferative activity of phytocompounds against other cancer cell lines

5.6.1. Antiproliferative effect against MDA-MB-231 and HT-29 cell lines

In order to test the cytotoxic effect of selected molecules on cancer cells other than lung cancer (A549), the study was extended to test the efficacy against colorectal (HT-29) and breast cancers (MDA-MB-231). Colorectal (6,94,000 deaths) and breast (5,21,000 deaths) cancers are also the major causes of cancer deaths (American Cancer Society., 2016).

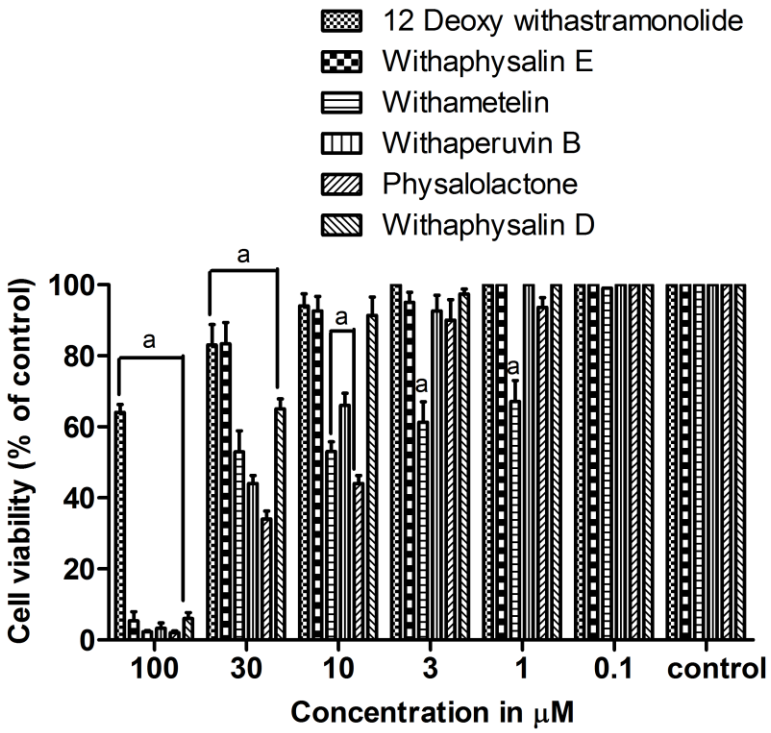


Figure 5.6.1: Effect of withasteroids on cell viability of MDA-MB-231 cells at 24 h. Data were expressed as mean ± SEM of three independent experiments. [a]$p<0.001$ versus control group

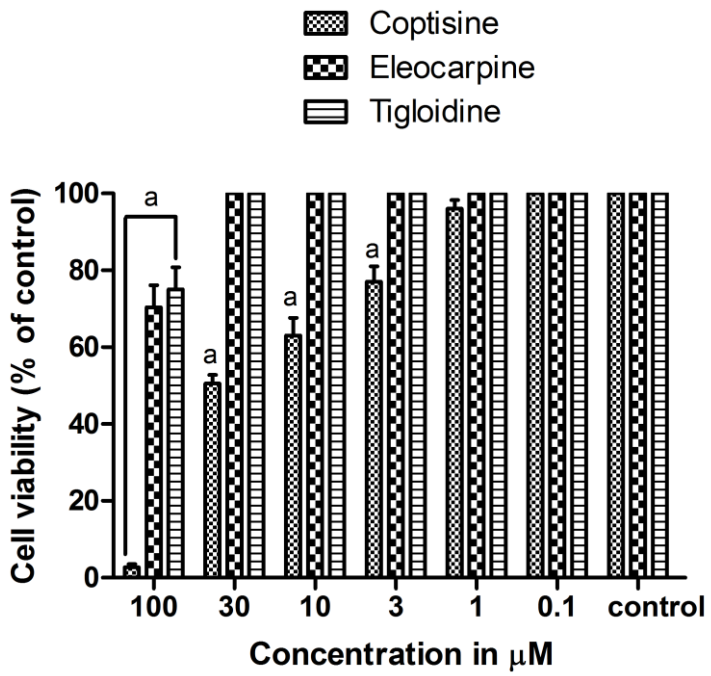


Figure 5.6.2: Effect of alkaloids on cell viability of MDA-MB-231 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. [a]p<0.001 versus control group

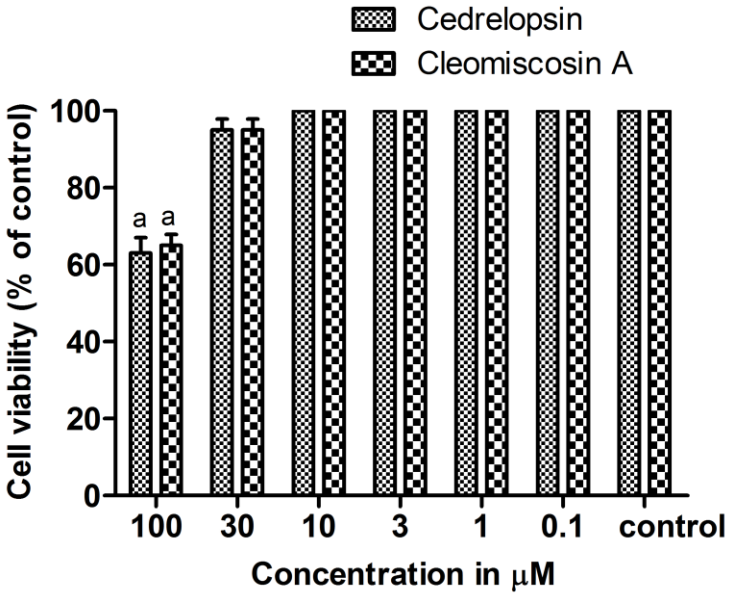


Figure 5.6.3: Effect of coumarins on cell viability of MDA-MB-231 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. [a]p<0.001 versus control group

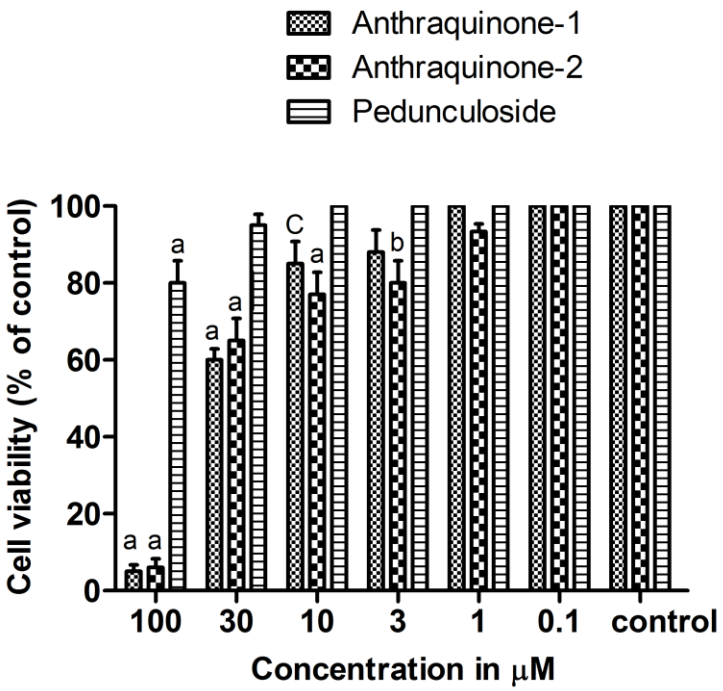


Figure 5.6.4: Effect of anthraquinones and pedunculoside on cell viability of MDA-MB-231 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{c}p<0.1$, $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

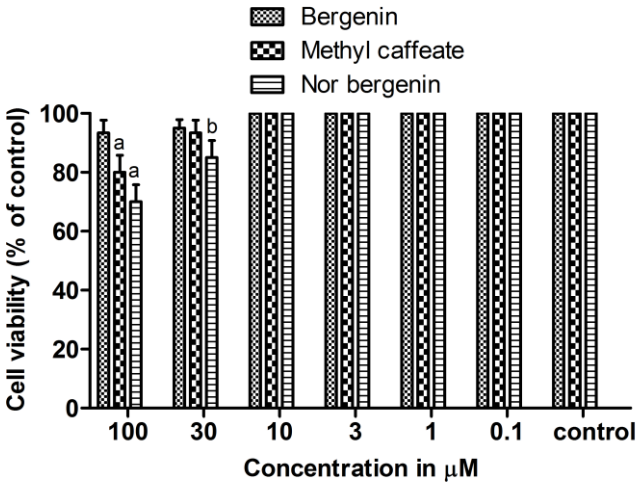


Figure 5.6.5: Effect of phenolics on cell viability of MDA-MB-231 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

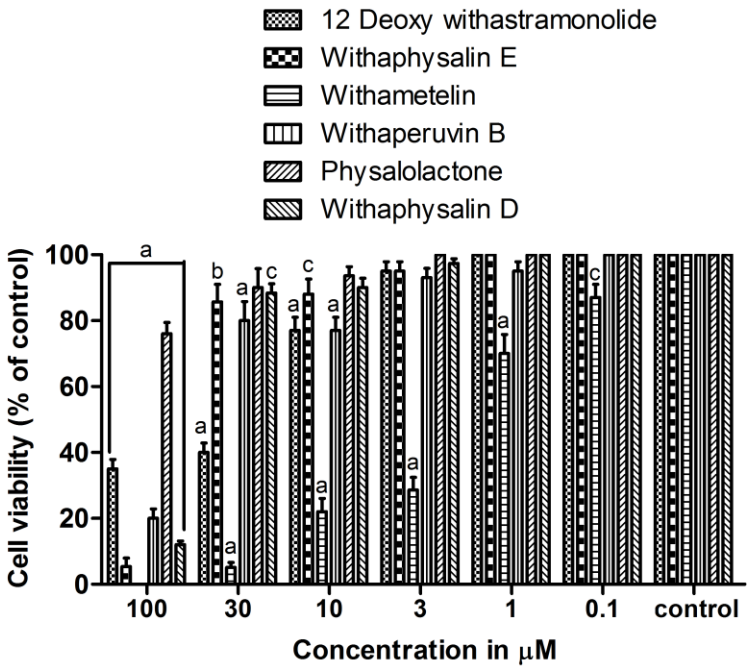


Figure 5.6.6: Effect of withasteroids on cell viability of HT-29 cells at 24 h. Data were expressed as mean ± SEM of three independent experiments. $^{c}p<0.1$, $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

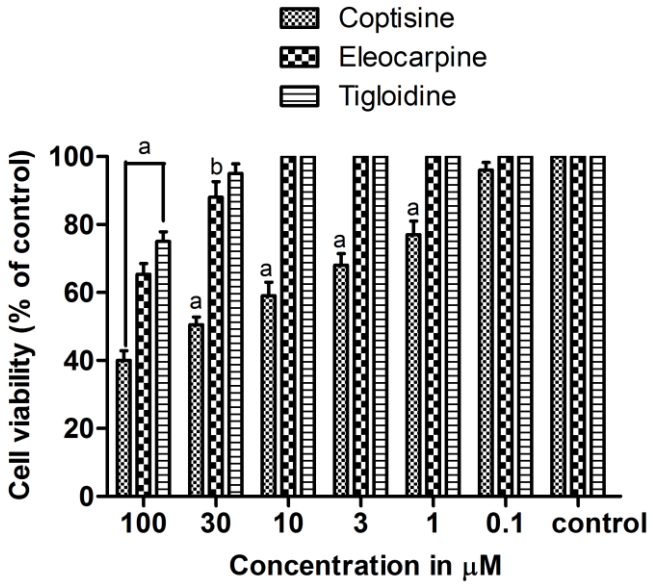


Figure 5.6.7: Effect of alkaloids on cell viability of HT-29 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

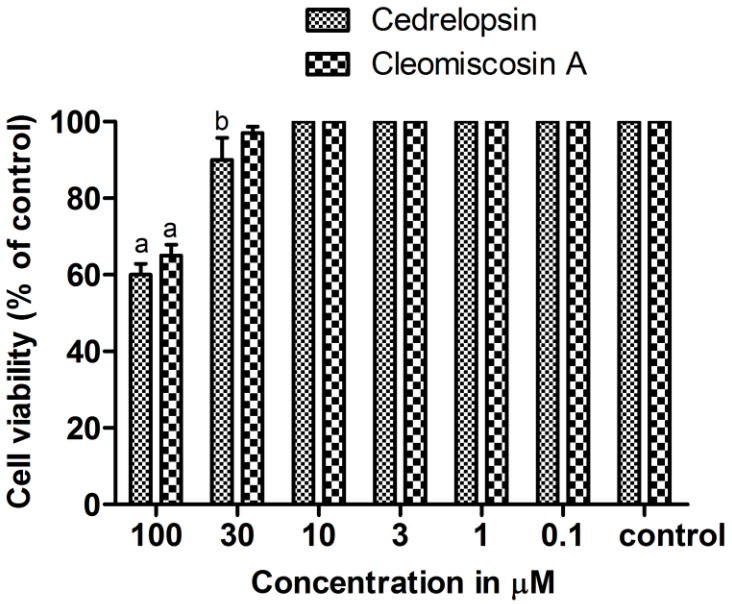


Figure 5.6.8: Effect of coumarins on cell viability of HT-29 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{b}p<0.01$ and $^{a}p<0.001$ versus control group

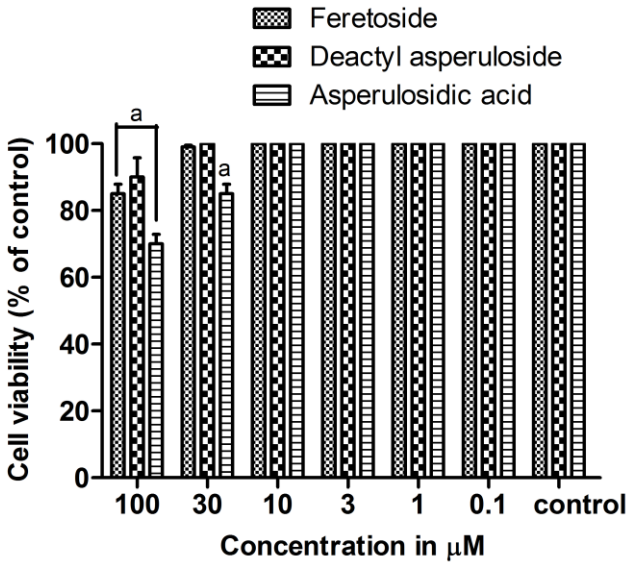


Figure 5.6.9: Effect of iridoid glycosides on cell viability of HT-29 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. $^{a}p<0.001$ versus control group

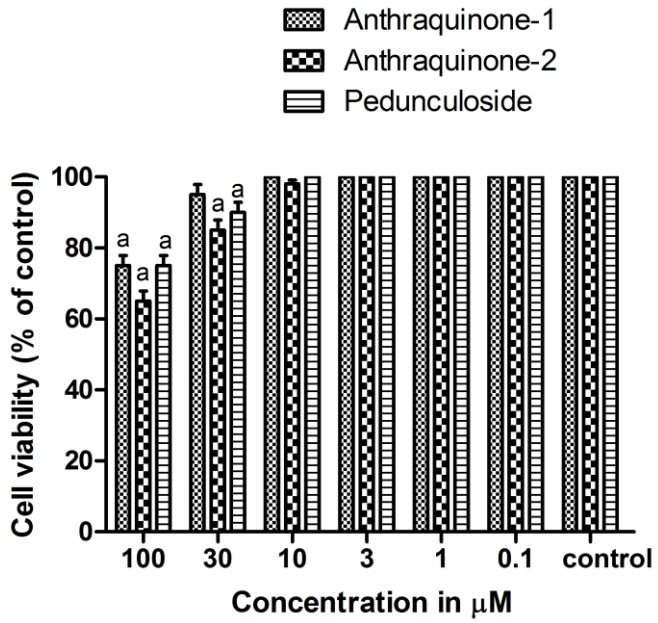


Figure 5.6.10: Effect of anthraquinones and pedunculoside on cell viability of HT-29 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. [a]p<0.001 versus control group

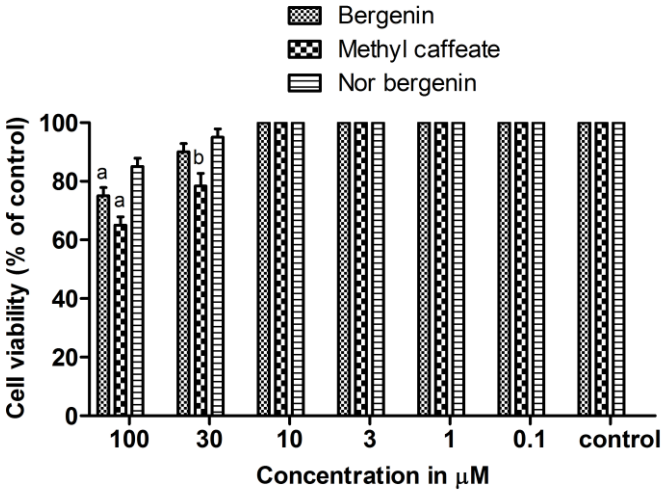


Figure 5.6.11: Effect of phenolics on cell viability of HT-29 cells at 48 h. Data were expressed as mean ± SEM of three independent experiments. [b]p<0.01 and [a]p<0.001 versus control group

Table 5.6.1: Consolidated anti proliferative activity (IC_{50} values) of phytocompounds against various human cancer cell lines

IC_{50} (µM)		
Compounds	**MDA-MB-231**	**HT-29**
Withasteroids		
Withametelin	7.6 ± 1.3	8.28 ± 1.1
Withaphysalin E	39.1 ± 4.2	45.7 ± 3.2
Withaphysalin D	35.0 ± 3.2	40.74 ± 3.9
12-Deoxy withastramonolide	> 100	29.8 ± 3.2
Withaperuvin B	18.8 ± 1.6	52.7 ± 4.8
Physalolactone	10.8	> 100
Alkaloids		
Coptisine	20.2 ± 2.6	26.6 ± 2.1
Eleocarpine	> 100	> 100
Tigloidine	> 100	> 100
Coumarins		
Cedrelopsin	> 100	> 100
Cleomiscosin A	> 100	> 100
Iridoid glycosides		
Deacetylasperuloside	> 100	> 100
Asperulosidic acid	> 100	16.2 ± 2.7
Feretoside	> 100	> 100
Anthraquinones		
1,2-dimethyl-3 hydroxy-9,10anthracenedione	31.0 ± 3.5	> 100
1,3-dimethyl-3 hydroxy-9,10anthracenedione	32.0 ± 2.9	> 100
Terpenoids		
Pedunculoside	> 100	> 100
Phenolics		
Bergenin	> 100	> 100
Methyl caffeate	> 100	> 100
Nor bergenin	> 100	> 100
5 Flouro uracil	7.87	9.17

The anti proliferative effect of withasteroids was tested against MDA-MB-231 and HT-29 cell lines by MTT assay and the IC_{50} values are displayed in Table 5.6.1. Results demonstrated potent antiproliferative effect of WM against both MDA-MB-231 and HT-29 cells. Physalolactone was found to be potent compound next to withametelin against MDA-MB-231 cells while the viability of HT-29 cells was unaffected. 12-Deoxywithastramonalide, a close relative of WM differed structurally with 17-β side chain and rings A and B with the presence of hydroxyl at C-5 and an epoxy ring between C-6 and C-7 (Ray AB *et al*., 1994) was moderately active against HT-29 cells but not against MDA-MB-231 cells. Withaphysalins displayed mild cytotoxicity against both cell lines. Anti proliferative activity of withaperuvin B was found to be more selective towards MDA-MB-231, compared to that of HT-29 cells.

Among the alkaloidal compounds, coptisine exhibited significant anti proliferative activity against both MDA-MB-231 and HT-29 cell lines. Eleocarpine and tigloidine were found to be inactive.

Cedrelopsin (CDLN) and cleomiscosin A exhibited no anti proliferative activity against both MDA-MB-231 and HT-29 cells. Iridoid glycosides like deacetylasperuloside, asperulosidic acid and feretoside failed to display cytotoxicity against MDA-MB-231 cells. Except aspersulodic acid (IC_{50} 16.2 µM), other two iridoid glycosides were demonstrated to be inactive against HT-29 cells.

1,2-dimethyl-3-hydroxy-9,10-anthracenedione and 1,3-dimethyl-3-hydroxy-9,10 anthracenedione, belonging to anthraquinones, indicated moderate anti proliferative activity against MDA-MB-231 cells where as anti proliferative activity against HT-29 cells was unaffected by treatment with anthraquinones.

The MTT results revealed pedunculoside to be inactive against both the tested cell lines.

None of the tested phenolic derivatives, bergenin, nor bergenin and methyl caffeate exhibited anti proliferative activity against MDA-MB-231 and HT-29 cells.

5.6.2 Conclusion

Withametelin (WM) exhibited greater anti proliferative activity against MDA-MB-231 cells. However, cedrelopsin (CDLN) which was found to be active against A549 cells was discovered to be inactive against MDA-MB-231 cells. WM potentially inhibited proliferation of HT-29 cells compared to other compounds. Surprisingly, aspersulodic acid which was demonstrated to be inactive against A549 and MDA-MB-231 cells, was found to be active against HT-29 cells.

CHAPTER 6

RECAPTULATION AND FUTURE PERSPECTIVES

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RECAPTULATION AND FUTURE PERSPECTIVES

Given the incidences and mortality related to lung cancer among global population, development of novel treatment strategies is of utmost importance. Though in the last few decades, prevailing chemotherapy in combination with surgery has emanated in relieving symptoms and ameliorating quality of patient lives suffering from lung cancer, death remains to be the ending for most of lung cancer patients. In addition, the high toxicity usually associated with some cancer chemotherapy drugs and their undesirable side-effects have increased the demand for novel anti-cancer drugs with fewer side-effects and/or with greater therapeutic efficiency. Investigation on underexploited natural compounds may yield potential molecules to develop as chemotherapeutic drugs. In view of this, we focused on exploring cytotoxic lead molecules along with characterization of mechanism of activity of potential molecules.

In summary,

- 20 phytocompounds were selected from different groups of plant secondary metabolites including withasteroids, coumarins, alkaloids, coumarins, iridoid glycosides, terpenoids, anthraquinones and phenolics based on literature survey.
- Selected phytocompounds were screened for anti proliferative activity against NSCLC cancer (A549) cells using MTT assay and further tested against HEK-293 cells to determine their safety. Coptisine (COP), withametein (WM) and cedrelopsin (CDLN) emerged to be potent in

inhibiting proliferation of A549 cells. While COP and CDLN were determined to be safe, WM exhibited moderate safety.

- Three cytotoxic molecules COP, WM and CDLN were selected based on their potency and subsequently they were investigated to understand molecular mechanisms behind their anti proliferative activities.

- COP, WM and CDLN were isolated from *Fumaria indica, Datura metel* and *Hedyotis umbellata,* respectively and the identity of compounds were established through spectral analysis.

- Cell cycle analysis indicated that coptisine inhibited cell proliferation by causing G2/M cell cycle arrest. It was further corroborated by downregulation of G2/M regulatory markers including cyclin B1, cdc2 and cdc25C.

- COP upregulated ROS generation both dose and time dependently. It caused the downregulaton of anti apoptotic protein Bcl-2 and upregulation of apoptotic protein Bax.

- COP treatment resulted in the depolarization of mitochondrial membrane along with translocation of cytochrome c into cytosol, followed by activation of caspase-9 and -3 and consequently cleavage of PARP, leading to apoptosis.

- To determine if coptisine-induced apoptosis was mediated by ROS, A549 cells were pretreated with NAC. Results indicated that NAC effectively abrogated the apoptotic activity, caspase-3 induction and MMP loss by COP, indicating that COP-induced intrinsic mitochondrial apoptosis was unambiguously mediated by ROS.

- Results of cell cycle analysis revealed that WM inhibited cell proliferation through arresting the A549 cells in G2/M phase. WM-induced G2/M arrest was supported by dowregulation of G2/M regulatory proteins like cyclin B1, cdc2 and cdc25C.

- WM induced apoptosis by increasing cells in early apoptotic phase evidenced by Annexin V staining.

- WM triggered ROS generation and disturbed the balance of Bax and Bcl-2 by increasing the expression of Bax and decreasing the expression of bcl-2

- WM caused translocation of cytochrome c into cytosol from mitochondria. It induced the expression of caspase 9 but not caspase 8. Followed by activation of caspase-3 and cleavage of PARP was evidenced with treatment, suggesting that WM induced apoptosis was mediated by intrinsic apoptotic pathway

- To determine if WM-induced apoptosis was mediated by ROS, A549 cells were pretreated with NAC. Results indicated that NAC failed to abrogate the anti proliferative activity and apoptosis caused by WM, indicating that WM-induced intrinsic mitochondrial apoptosis was not mediated by ROS.

- The mechanism underlying the antiproliferative effect of CDLN was assumed as apoptosis, which was evaluated by staining with Annexin V FITC and PI. Results claimed that early apoptotic population and late apoptotic population were not affected by CDLN.

- The mechanism of anti proliferative effect of CDLN related to cell cycle control was examined by cell cycle analysis. The finding from cell cycle analysis demonstrated cedrelopsin induced cell cycle arrest at G2/M phase. Further it was corroborated by

downregulaton of cyclin B1, cdc2 and cdc25c, suggesting that anti proliferative effect of cedrelopsin is due to G2/M arrest.

- To determine broader cytotoxic activity of phytocompounds, all compounds were tested against MDA-MB-231 and HT-29 cells. MTT results revealed that coptisine and withametelin displayed lower anti proliferative activity against MDA-MB-231 and HT-29 cells as compared to that of A549 cells. However, viability of both MDA-MB-231 and HT-29 cells were not affected by cedrelopsin, corroborating cancer specific activity.

Future Perspectives of This Study Include

- The demonstrated activity of cytotoxic compounds on cell cycle analysis and apoptosis, could be extended to various other cancers along with identification of upstream biomarkers that could be possibly affected by cytotoxic compounds.

- Effect of potent cytotoxic compounds on angiogenesis and metastasis could be evaluated.

- Anti tumor potential of potent compounds could be assessed in tumor xenograft model.

- Pharmacokinetics of those cytotoxic compounds in both healthy and disease rat/mouse models could be evaluated.

- These compounds can be used as lead molecules for synthesising more active analogues in future.

REFERENCES

REFERENCES

Achanta, G., Huang, P., 2004. Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents. Cancer Res. 64, 6233-6239.

Achanta, G., Sasaki, R., Feng, L., Carew, J.S., Lu, W., Pelicano, H., Keating, M.J., Huang, P., 2005. Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol gamma. EMBO J. 24, 3482-3492.

Adams, J.M., 2003. Ways of dying: multiple pathways to apoptosis. Genes Dev. 17, 2481-2495.

Alexandre, J., Nicco, C., Chereau, C., Laurent, A., Weill, B., Goldwasser, F., Batteux, F., 2006. Improvement of the therapeutic index of anticancer drugs by the superoxide dismutase mimic mangafodipir. J. Natl. Cancer Inst. 98, 236-244.

Armstrong, R.C., Aja, T., Xiang, J., Gaur, S., Krebs, J.F., Hoang, K., Bai, X., Korsmeyer, S.J., Karanewsky, D.S., Fritz, L.C., Tomaselli, K.J., 1996. Fas-induced activation of the cell death-related protease CPP32 Is inhibited by Bcl-2 and by ICE family protease inhibitors. J. Biol. Chem. 271, 16850-16855.

Attardi, L.D., Donehower, L.A., 2005. Probing p53 biological functions through the use of genetically engineered mouse models. Mutat. Res-Fund. Mol. M. 576, 4-21.

Awe Walther., 1951. Isolation of the alkaloid coptisine from the root of the Chelidonium plant. Arzneimittelforschung 1, 287-8.

Bailly, F., Toillon, R., Tomavo, O., Jouy, N., Hondermarck, H., Cotelle, P., 2013. Antiproliferative and apoptotic effects of the oxidative dimerization product of methyl caffeate on human breast cancer cells. Bioorg. Med. Chem. Lett. 23, 574-578.

Barber, D.A., Harris, S.R., 1994. Oxygen free radicals and antioxidants: a review. Am. Pharm. NS34, 26-35.

Barlogie, B., Drewinko, B., 1978. Cell cycle stage-dependent induction of G 2 phase arrest by different antitumor agents. Eur. J. Cancer. (1965) 14, 741-745.

Barlogie, B., Drewinko, B., Johnston, D.A., Freireich, E.J., 1976. The effect of adriamycin on the cell cycle traverse of a human lymphoid cell line. Cancer Res. 36, 1975-1979.

Behrend, L., Henderson, G., Zwacka, R.M., 2003. Reactive oxygen species in oncogenic transformation. Biochem. Soc. Trans. 31, 1441-1444.

Bellila, A., Tremblay, C., Pichette, A., Marzouk, B., Mshvildadze, V., Lavoie, S., Legault, J., 2011. Cytotoxic activity of withanolides isolated from Tunisian Datura metel L. Phytochemistry 72, 2031-2036.

Beutler, B., van Huffel, C., 1994. Unraveling function in the TNF ligand and receptor families. Science 264, 667-668.

Bey, E.A., Bentle, M.S., Reinicke, K.E., Dong, Y., Yang, C.R., Girard, L., Minna, J.D., Bornmann, W.G., Gao, J., Boothman, D.A., 2007. An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by beta-lapachone. Proc. Natl. Acad. Sci. U. S. A. 104, 11832-11837.

Blasina, A., Paegle, E.S., McGowan, C.H., 1997. The role of inhibitory phosphorylation of CDC2 following DNA replication block and radiation-induced damage in human cells. Mol. Biol. Cell 8, 1013-1023.

Bohr, V.A., Dianov, G.L., 1999. Oxidative DNA damage processing in nuclear and mitochondrial DNA. Biochimie 81, 155-160.

Booher, R.N., Holman, P.S., Fattaey, A., 1997. Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. J. Biol. Chem. 272, 22300-22306.

Boonstra, J., Post, J.A., 2004. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. Gene 337, 1-13.

Borg, A., Zhang, Q.X., Alm, P., Olsson, H., Sellberg, G., 1992. The retinoblastoma gene in breast cancer: allele loss is not correlated with loss of gene protein expression. Cancer Res. 52, 2991-2994.

Budhiraja, R. D., Krishnan, P., and S. Sudhir., 2000. Review of biological activity of withanolides." J. Sci. Ind. Res 59, 904-911.

Burgeiro, A., Gajate, C., Dakir el, H., Villa-Pulgarin, J.A., Oliveira, P.J., Mollinedo, F., 2011. Involvement of mitochondrial and B-RAF/ERK signaling pathways in berberine-induced apoptosis in human melanoma cells. Anticancer Drugs 22, 507-518.

Butler, M. S., Robertson, A. A., Cooper, M. A., 2014. Natural product and natural product derived drugs in clinical trials. Natural product reports. 31, 1612-1661.

Çalis, I., Kirmizibekmez, H., & Sticher, O., 2001. Iridoid Glycosides from Globularia t richosantha. J. Nat. Prod. 64, 60-64.

Carmazzi, Y., Iorio, M., Armani, C., Cianchetti, S., Raggi, F., Neri, T., Cordazzo, C., Petrini, S., Vanacore, R., Bogazzi, F., 2012. The mechanisms of nadroparin-mediated inhibition of proliferation of two human lung cancer cell lines. Cell Prolif. 45, 545-556.

Carr, A.M., 1997. Control of cell cycle arrest by the Mec1 sc/Rad3 sp DNA structure checkpoint pathway. Curr. Opin. Genet. Dev. 7, 93-98.

Cavalli, L.R., Liang, B.C., 1998. Mutagenesis, tumorigenicity, and apoptosis: are the mitochondria involved? Mutat. Res-Fund. Mol. M. 398, 19-26.

Chattopadhyay, S. K., Kumar, S., Kaur, R., Tandon, S., & Rane, S., 2009. Identification and quantification of two antihepatotoxic coumarinolignoids cleomiscosin A and cleomiscosin B in the seeds of Cleome viscosa using liquid chromatography–tandem mass spectrometry. Biomed. Chromatogr. *23*, 340-356.

Chen, YU., Yan, J., 2006. Isolation of alkaloids from *corydalis yanhusuo* W.T. Wang and its RP-HPLC quantitative determination. Shizhen Guoyi Guoyao 17, C3-C4

Chien, S.Y., Wu, Y.C., Chung, J.G., Yang, J.S., Lu, H.F., Tsou, M.F., Wood, W.G., Kuo, S.J., Chen, D.R., 2009. Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. Hum. Exp. Toxicol. 28, 493-503.

Chou, Y., Bischoff, J.R., Beach, D., Goldman, R.D., 1990. Intermediate filament reorganization during mitosis is mediated by p34 cdc2 phosphorylation of vimentin. Cell 62, 1063-1071.

Chowdhury, I., Tharakan, B., Bhat, G.K., 2006. Current concepts in apoptosis: the physiological suicide program revisited. Cell. Mol. Biol. Lett. 11, 506-525.

Cobb, J.P., Hotchkiss, R.S., Karl, I.E., Buchman, T.G., 1996. Mechanisms of cell injury and death. Br. J. Anaesth. 77, 3-10.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. Biochem. J. 326, 1-16.

Colombo, M.L., Bugatti, C., Mossa, A., Pescalli, N., Piazzoni, L., Pezzoni, G., Menta, E., Spinelli, S., Johnson, F., Gupta, R.C., 2001. Cytotoxicity evaluation of natural coptisine and synthesis of coptisine from berberine. Il Farmaco 56, 403-409.

Cragg, G. M.; Newman, D. J., 2005. Plants as a source of anticancer agents. Journal of Ethnopharmacology 100, 72-79.

Cragg, G.M., Newman, D.J., 2013. Natural products: a continuing source of novel drug leads. Biochimica et Biophysica Acta (BBA)-General Subjects 1830, 3670-3695.

Datta, R., Banach, D., Kojima, H., Talanian, R.V., Alnemri, E.S., Wong, W.W., Kufe, D.W., 1996. Activation of the CPP32 protease in apoptosis induced by 1-beta-D-arabinofuranosyl cytosine and other DNA-damaging agents. Blood 88, 1936-1943.

Demain, A.L., Vaishnav, P., 2011. Natural products for cancer chemotherapy. Microbial biotechnology 4, 687-699.

Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A., Weinberg, R.A., 1993. Physical interaction of the retinoblastoma protein with human D cyclins. Cell 73, 499-511.

Draetta, G., Beach, D., 1988. Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. Cell 54, 17-26.

Dragovich, T., Gordon, M., Mendelson, D., Wong, L., Modiano, M., Chow, H.H., Samulitis, B., O'Day, S., Grenier, K., Hersh, E., Dorr, R., 2007. Phase I trial of imexon in patients with advanced malignancy. J. Clin. Oncol. 25, 1779-1784.

Earnshaw, W.C., Martins, L.M., Kaufmann, S.H., 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68, 383-424.

Editor., 2007. "Hard to swallow". Nature 448, 105–6. doi:10.1038/448106a.

Eischen, C.M., Kottke, T.J., Martins, L.M., Basi, G.S., Tung, J.S., Earnshaw, W.C., Leibson, P.J., Kaufmann, S.H., 1997. Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. Blood 90, 935-943.

Ekert, P., Silke, J., Vaux, D., 1999. Caspase inhibitors. Cell. Death. Differ. 6, 1081-1086.

Elledge, S.J., 1996. Cell cycle checkpoints: preventing an identity crisis. Science 274, 1664-1672.

Emili, A., 1998. MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol. Cell 2, 183-189.

Enari, M., Talanian, R.V., Wrong, W.W., Nagata, S., 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 380, 723-726.

Eruslanov E., Kusmartsev S., 2010. Identification of ROS using oxidized DCFDA and flow-

Faleiro, L., Kobayashi, R., Fearnhead, H., Lazebnik, Y., 1997. Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. EMBO J. 16, 2271-2281.

Eshiett, IT., Taylor, DA., 1968. The isolation and structure elucidation of some derivatives of dimethylallyl-coumarin, chromone, quinoline, and phenol from Fagara species, and from Cedrelopsis grevei. Journal of the Chemical Society C:Organic 481-4.

Farha, A.K., Dhanya, S.R., Mangalam, S.N., Geetha, B.S., Latha, P.G., Remani, P., 2014. Deoxyelephantopin impairs growth of cervical carcinoma SiHa cells and induces apoptosis by targeting multiple molecular signaling pathways. Cell Biol. Toxicol. 30, 331-343.

Flores-Rozas, H., Kelman, Z., Dean, F.B., Pan, Z.Q., Harper, J.W., Elledge, S.J., O'Donnell, M., Hurwitz, J., 1994. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc. Natl. Acad. Sci. U. S. A. 91, 8655-8659.

Fruehauf, J.P., Meyskens, F.L.,Jr, 2007. Reactive oxygen species: a breath of life or death? Clin. Cancer Res. 13, 789-794.

Fulda, S., DEBATIN, K., 2004. Apoptosis signaling in tumor therapy. Ann. N. Y. Acad. Sci. 1028, 150-156.

Fulda, S., Debatin, K., 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25, 4798-4811.

Fulda, S., Susin, S.A., Kroemer, G., Debatin, K.M., 1998. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. Cancer Res. 58, 4453-4460.

Gansauge, S., Gansauge, F., Ramadani, M., Stobbe, H., Rau, B., Harada, N., Beger, H.G., 1997. Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. Cancer Res. 57, 1634-1637.

Gao, W., Xiao, F., Wang, X., Chen, T., 2013. Artemisinin induces A549 cell apoptosis dominantly via a reactive oxygen species-mediated amplification activation loop among caspase-9,-8 and-3. Apoptosis 18, 1201-1213.

Garg, K., Goswami, K., Khurana, G., 2013. A pharmacognostical review on Elaeocarpus sphaericus. Int. J. Pharmacy Pharma. Science 5, 3-8.

Gartel, A.L., Serfas, M.S., Tyner, A.L., 1996. P21--Negative Regulator of the Cell Cycle. Proc. Soc. Exp. Biol. Med. 213, 138-149.

Garvik, B., Carson, M., Hartwell, L., 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell. Biol. 15, 6128-6138.

Glotter, E., Kirson, I., Lavie, D., & Abraham, A., 1978. The withanolides--a group of natural steroids [Withania somnifera, Solanaceae]. Bioorg. Chem.

Goel, R. K., Maiti, R. N., Manickam, M., & Ray, A. B., 1997. Antiulcer activity of naturally occurring pyrano-coumarin and isocoumarins and their effect on prostanoid synthesis using human colonic mucosa. Indian J. Exp. biol. *35*, 1080-1083.

Gong, L., Fang, L., Wang, S., Sun, J., Qin, H., Li, X., Wang, S., Du, G., 2012. Coptisine exert cardioprotective effect through anti-oxidative and inhibition of RhoA/Rho kinase pathway on isoproterenol-induced myocardial infarction in rats. Atherosclerosis 222, 50-58.

Gorelick J., Rosenberg R., Smotrich A., HanuÅ Lr., Bernstein N., 2015. Hypoglycemic activity of withanolides and elicitated withania somnifera. Phytochemistry.116, 283-289

Grana, X., Reddy, E.P., 1995. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11, 211-219.

Gupta, M., Mazumder, U., Thamilselvan, V., Manikandan, L., Senthilkumar, G., Suresh, R., Kakotti, B., 2007. Potential hepatoprotective effect and antioxidant role of methanol extract of Oldenlandia umbellata in carbon tetrachloride induced hepatotoxicity in Wistar rats. Iran. J. Pharmacol. Ther. 6, 5-9.

Haghighi, F., Matin, M.M., Bahrami, A.R., Iranshahi, M., Rassouli, F.B., Haghighitalab, A., 2014. The cytotoxic activities of 7-isopentenyloxycoumarin on 5637 cells via induction of apoptosis and cell cycle arrest in G2/M stage. DARU J. Pharm. Sci. 22, 1.

Hall, M., Peters, G., 1996. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Adv. Cancer Res. 68, 67-108.

Halloran, P.J., Fenton, R.G., 1998. Irreversible G2-M arrest and cytoskeletal reorganization induced by cytotoxic nucleoside analogues. Cancer Res. 58, 3855-3865.

Han, M.H., Kim, G., Yoo, Y.H., Choi, Y.H., 2013. Sanguinarine induces apoptosis in human colorectal cancer HCT-116 cells through ROS-mediated Egr-1 activation and mitochondrial dysfunction. Toxicol. Lett. 220, 157-166.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., Elledge, S.J., 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805-816.

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., 1995. Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell 6, 387-400.

Hartwell, L., 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell 71, 543-546.

Hema, V; Venkidesh, R; Maheswari, E., 2007. Antitussive activity of Oldenlandia umbellata.. Indian J. of Pharm. Sci. 68, 236-238.

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., Draetta, G., 1993. Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. 12, 53-63.

Hong, C., Firestone, G.L., Bjeldanes, L.F., 2002. Bcl-2 family-mediated apoptotic effects of 3, 3′-diindolylmethane (DIM) in human breast cancer cells. Biochem. Pharmacol. 63, 1085-1097.

Hsu, W., Hsieh, Y., Kuo, H., Teng, C., Huang, H., Wang, C., Yang, S., Liou, Y., Kuo, W., 2007. Berberine induces apoptosis in SW620 human colonic carcinoma cells through generation of reactive oxygen species and activation of JNK/p38 MAPK and FasL. Arch. Toxicol. 81, 719-728.

http://www.cancer.org/cancer/lungcancer-non-smallcell/detailedguide/non-small-cell-lung-cancer-key-statistics

Hu, Y., Ding, L., Spencer, D.M., Nunez, G., 1998. WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. J. Biol. Chem. 273, 33489-33494.

Hua, P., Sun, M., Zhang, G., Zhang, Y., Tian, X., Li, X., Cui, R., Zhang, X., 2015. Cepharanthine induces apoptosis through reactive oxygen species and mitochondrial dysfunction in human non-small-cell lung cancer cells. Biochem. Biophys. Res. Commun. 460, 136-142.

Huang, S., Wu, P., Wu, T., 1997. Two coumarins from the root bark of *Clausena excavata*. Phytochemistry 44, 179-181.

Ibrado, A.M., Huang, Y., Fang, G., Liu, L., Bhalla, K., 1996. Overexpression of Bcl-2 or Bcl-xL inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. Cancer Res. 56, 4743-4748.

Itoh, N., Nagata, S., 1993. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. J. Biol. Chem. 268, 10932-10937.

Izumi, T., Maller, J.L., 1993. Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. Mol. Biol. Cell 4, 1337-1350.

Jahromi, M. F., Gupta, M., Manickam, M., Ray, A. B., & Chansouria, J. P. N., 1999. Hypolipidemic activity of pedunculoside, a constituent of Ilex doniana. Pharm. Biol. *37*, 37-41.

Janicke, R.U., Ng, P., Sprengart, M.L., Porter, A.G., 1998. Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J. Biol. Chem. 273, 15540-15545.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Thun, M.J., 2009. Cancer statistics, 2009. CA: Cancer J. Clini.59, 225-249.

Jiang, X., Wang, X., 2000. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. J. Biol. Chem. 275, 31199-31203.

Juarez, J.C., Manuia, M., Burnett, M.E., Betancourt, O., Boivin, B., Shaw, D.E., Tonks, N.K., Mazar, A.P., Donate, F., 2008. Superoxide dismutase 1 (SOD1) is essential for H2O2-mediated oxidation and inactivation of phosphatases in growth factor signaling. Proc. Natl. Acad. Sci. U. S. A. 105, 7147-7152.

Jung, H.A., Yoon, N.Y., Bae, H.J., Min, B., Choi, J.S., 2008. Inhibitory activities of the alkaloids from Coptidis Rhizoma against aldose reductase. Arch. Pharm. Res. 31, 1405-1412.

Kastan, M.B., Bartek, J., 2004. Cell-cycle checkpoints and cancer. Nature 432, 316-323.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., Craig, R.W., 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51, 6304-6311.

Kato, J., Matsuoka, M., Polyak, K., Massague, J., Sherr, C.J., 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27 Kip1) of cyclin-dependent kinase 4 activation. Cell 79, 487-496.

Kaufmann, W.K., Levedakou, E.N., Grady, H.L., Paules, R.S., Stein, G.H., 1995. Attenuation of G2 checkpoint function precedes human cell immortalization. Cancer Res. 55, 7-11.

Kawanishi, S., Hiraku, Y., Pinlaor, S., Ma, N., 2006. Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. Biol. Chem. 387, 365-372.

Keane, R.W., Srinivasan, A., Foster, L.M., Testa, M., Örd, T., Nonner, D., Wang, H., Reed, J.C., Bredesen, D.E., Kayalar, C., 1997. Activation of CPP32 during apoptosis of neurons and astrocytes. J. Neurosci. Res. 48, 168-180.

Kelloff, G.J., Crowell, J.A., Steele, V.E., Lubet, R.A., Malone, W.A., Boone, C.W., Kopelovich, L., Hawk, E.T., Lieberman, R., Lawrence, J.A., Ali, I., Viner, J.L., Sigman, C.C., 2000. Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. J. Nutr. 130, 467S-471S.

Kerr, J.F., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239-257.

Kim, K.H., Lee, I.K., Piao, C.J., Choi, S.U., Lee, J.H., Kim, Y.S., Lee, K.R., 2010. Benzylisoquinoline alkaloids from the tubers of Corydalis ternata and their cytotoxicity. Bioorg. Med. Chem. Lett. 20, 4487-4490.

Kimler, B.F., Schneiderman, M.H., Leeper, D.B., 1978. Induction of concentration-dependent blockade in the G2 phase of the cell cycle by cancer chemotherapeutic agents. Cancer Res. 38, 809-814.

Kirshner, J.R., He, S., Balasubramanyam, V., Kepros, J., Yang, C.Y., Zhang, M., Du, Z., Barsoum, J., Bertin, J., 2008. Elesclomol induces cancer cell apoptosis through oxidative stress. Mol. Cancer. Ther. 7, 2319-2327.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R., Newmeyer, D.D., 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275, 1132-1136.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, B.R., Roberts, J.M., 1992. Formation and activation of a cyclin E-Cdk2 complex during the G1 phase of the human cell cycle. Science 257, 1689-1694.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J.M., Massague, J., 1993. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. Science 260, 536-539.

Kozawa, M., Baba, K., Okuda, K., Fukumoto, T., Hata, K., 1981. Studies on chemical components of “Bai Zhi”(Supplement 1). On coumarins from “Japanese Bai Zhi”. Shoyakugaku Zasshi 35, 90-95.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P., Craig, R.W., 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc. Natl. Acad. Sci. U. S. A. 90, 3516-3520.

Krek, W., Nigg, E.A., 1991. Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. EMBO J. 10, 3331-3341.

Krishan, A., Frei, E., 1976. Effect of adriamycin on the cell cycle traverse and kinetics of cultured human lymphoblasts. Cancer Res. 36, 143-150.

Kuida, K., Haydar, T.F., Kuan, C., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., Flavell, R.A., 1998. Reduced apoptosis and cytochrome c–mediated caspase activation in mice lacking caspase 9. Cell 94, 325-337.

Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W., Hoffmann, I., 1998. The cdc25B phosphatase is essential for the G2/M phase transition in human cells. J. Cell. Sci. 111 (Pt 16), 2445-2453.

Langan, T.A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J., Sclafani, R.A., 1989. Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. Mol. Cell. Biol. 9, 3860-3868.

Leach, S.D., Scatena, C.D., Keefer, C.J., Goodman, H.A., Song, S.Y., Yang, L., Pietenpol, J.A., 1998. Negative regulation of Wee1 expression and Cdc2 phosphorylation during p53-mediated growth arrest and apoptosis. Cancer Res. 58, 3231-3236.

Lee, H., Kim, C., 1999. Isolation and quantitative determination of berberine and coptisine from tubers of Corydalis ternata. Korean J. Pharmacognosy 30, 332-334.

Lee, I.R., Yang, M.Y., 1994. Phenolic compounds from *Duchesnea chrysantha* and their cytotoxic activities in human cancer cell. Arch. Pharm. Res. 17, 476-479.

Lee, M., Nurse, P., 1988. Cell cycle control genes in fission yeast and mammalian cells. Trends Genet. 4, 287-290.

Lee, M.S., Ogg, S., Xu, M., Parker, L.L., Donoghue, D.J., Maller, J.L., Piwnica-Worms, H., 1992. Cdc25+ Encodes a Protein Phosphatase that Dephosphorylates P34cdc2. Mol. Biol. Cell 3, 73-84.

Li, H., Han, T., Liu, R., Zhang, C., Chen, H., Zhang, W., 2008. Alkaloids from *Corydalis saxicola* and Their Anti-Hepatitis B Virus Activity. Chem. biodiversity 5, 777-783.

Li, H., Zhu, H., Xu, C., Yuan, J., 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94, 491-501.

Li, J., Qiu, D.M., Chen, S.H., Cao, S.P., Xia, X.L., 2014. Suppression of human breast cancer cell metastasis by coptisine in vitro. Asian Pac. J. Cancer. Prev. 15, 5747-5751.

Li, J., Qiu, D.M., Chen, S.H., Cao, S.P., Xia, X.L., 2014. Suppression of human breast cancer cell metastasis by coptisine in vitro. Asian Pac. J. Cancer. Prev. 15, 5747-5751.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479-489.

Lim, S., Kaldis, P., 2013. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development 140, 3079-3093.

Lim, S., Kim, D., Shin, T., Lim, J., Eom, D., 2002. Isolation and Determination of Alkaloids in Coptis Rhizome by Forming Complex. J. Pharm. Society Korea 46, 226-230.

Lin JP., Yang JS., Lee JH., Chung JG., 2006. Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line. World. J. Gastroenterol. 12, 21-28. doi:10.3748/wjg.v12.i1.21

Lin, C., Ng, L.T., Hsu, F., Shieh, D., Chiang, L., 2004. Cytotoxic effects of *Coptis chinensis* and *Epimedium sagittatum* extracts and their major constituents (berberine, coptisine and icariin) on hepatoma and leukaemia cell growth. Clin. Exp. Pharmacol. P. 31, 65-69.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86, 147-157.

Liu, X., Zou, H., Slaughter, C., Wang, X., 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89, 175-184.

Lock, R.B., Keeling, P.K., 1993. Responses of HELA and Chinese-hamster ovary p34 (cdc2)/cyclin-b kinase in relation to cell-cycle perturbations induced by etoposide. Int. J. Oncol. 3, 33-42.

Lock, R.B., Ross, W.E., 1990. Inhibition of p34cdc2 kinase activity by etoposide or irradiation as a mechanism of G2 arrest in Chinese hamster ovary cells. Cancer Res. 50, 3761-3766.

Lopez-Girona, A., Furnari, B., Mondesert, O., Russell, P., 1999. Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature 397, 172-175.

Lu, J., Chew, E.H., Holmgren, A., 2007. Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. Proc. Natl. Acad. Sci. U. S. A. 104, 12288-12293.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., Wang, X., 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94, 481-490.

Lynch, Thomas J., et al. 2012. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non–small-cell lung cancer: Results from a randomized, double-blind, multicenter phase II study. J. Clin. Oncol. 30.17, 2046-2054.

Maeda, H., Hori, S., Ohizumi, H., Segawa, T., Kakehi, Y., Ogawa, O., Kakizuka, A., 2004. Effective treatment of advanced solid tumors by the combination of arsenic trioxide and L-buthionine-sulfoximine. Cell Death Differ. 11, 737-746.

Magda, D., Miller, R.A., 2006. Motexafin gadolinium: a novel redox active drug for cancer therapy. Seminars Cancer Biol. 16, 466-476.

Martins, L.M., Mesner, P.W., Kottke, T.J., Basi, G.S., Sinha, S., Tung, J.S., Svingen, P.A., Madden, B.J., Takahashi, A., McCormick, D.J., Earnshaw, W.C., Kaufmann, S.H., 1997. Comparison of caspase activation and subcellular localization in HL-60 and K562 cells undergoing etoposide-induced apoptosis. Blood 90, 4283-4296.

Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J., Hanks, S.K., Roussel, M.F., Sherr, C.J., 1992. Identification and properties of an atypical catalytic subunit (p34 PSK-J3/Cdk4) for mammalian D type G1 cyclins. Cell 71, 323-334.

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J., Kato, J.Y., 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. 14, 2066-2076.

Matsushime, H., Roussel, M.F., Ashmun, R.A., Sherr, C.J., 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65, 701-713.

Matsushime, H., Roussel, M.F., Sherr, C.J., 1991. Novel mammalian cyclins (CYL genes) expressed during G1. Cold Spring Harb. Symp. Quant. Biol. 56, 69-74.

Michalides, R., Hageman, P., van Tinteren, H., Houben, L., Wientjens, E., Klompmaker, R., Peterse, J., 1996. A clinicopathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer. Br. J. Cancer 73, 728-734.

Migliore, L., Migheli, F., Spisni, R., Coppede, F., 2011. Genetics, cytogenetics, and epigenetics of colorectal cancer. J. Biomed. Biotechnol. 2011, 792362.

Morgan, D.O., De Bondt, H.L., 1994. Protein kinase regulation: insights from crystal structure analysis. Curr. Opin. Cell Biol. 6, 239-246.

Mosmann T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65, 55-63

Nam, J. W., Kim, S. Y., Yoon, T., Lee, Y. J., Kil, Y. S., Lee, Y. S., & Seo, E. K., 2013. Heat shock factor 1 inducers from the bark of Eucommia ulmoides as cytoprotective agents. Chem. & biodiversity. 10, 1322-132

Nasr, T., Bondock, S., Youns, M., 2014. Anticancer activity of new coumarin substituted hydrazide–hydrazone derivatives. Eur. J. Med. Chem. 76, 539-548.

Nazir, N., Koul, S., Qurishi, M. A., Taneja, S. C., Ahmad, S. F., Bani, S., & Qazi, G. N., 2007. Immunomodulatory effect of bergenin and norbergenin against adjuvant-induced arthritis—A flow cytometric study. J. Ethnopharmacol. 112, 401-405

Newman, D. J.; Cragg, G. M.; Snader, K. M., 2003. Natural products as source of new drugs over the period 1981-2002. J. Nat. Prod. 66, 1022-1037.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis.

Nigg, E., 1992. Assembly and cell cycle dynamics of the nuclear lamina. 3, 245-253.

Nishio, K., Fujiwara, Y., Miyahara, Y., Takeda, Y., Ohira, T., Kubota, N., Ohta, S., Funayama, Y., Ogasawara, H., Saijo, N., 1993. CIS diamminedichloroplatinum (II) inhibits p34cdc2 protein kinase in human lung cancer cells. Int. J. Cancer. 55, 616-622.

Norbury, C., Blow, J., Nurse, P., 1991. Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates. EMBO J. 10, 3321-3329.

Okamoto, S., Sakai, M., Uchida, J., Saito, H., 1996. 5-Fluorouracil induces apoptotic cell death with G2 phase arrest in human breast cancer grafted in nude mice. Anticancer Res. 16, 2699-2704.

Padhy, I., Endale, A., 2014. Evaluation of anti-inflammatory and anti-pyretic activity of Oldenlandia umbellata Linn. roots. Int. J. Pharm. Healthc. Res. 2, 12-14.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., Draetta, G., 1992. Cyclin A is required at two points in the human cell cycle. EMBO J. 11, 961-971.

Pan, G., O'Rourke, K., Dixit, V.M., 1998. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. J. Biol. Chem. 273, 5841-5845.

Pandey, V., Ray, A., Dasgupta, B., 1976. Quaternary alkaloids of Fumaria indica. Phytochemistry 15, 545-546.

Pardee, A.B., 1989. G1 events and regulation of cell proliferation. Science 246, 603-608.

Parker, L.L., Atherton-Fessler, S., Piwnica-Worms, H., 1992. P107wee1 is a Dual-Specificity Kinase that Phosphorylates P34cdc2 on Tyrosine 15. Proc. Natl. Acad. Sci. U. S. A. 89, 2917-2921.

Parker, L.L., Piwnica-Worms, H., 1992. Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science. 257, 1955-1957.

Patre, R.E., Parameswaran, P.S., Tilve, S.G., 2011. Synthesis of the naturally occurring prenylated coumarins balsamiferone and cedrelopsin by domino reactions.

Paules, R.S., Levedakou, E.N., Wilson, S.J., Innes, C.L., Rhodes, N., Tlsty, T.D., Galloway, D.A., Donehower, L.A., Tainsky, M.A., Kaufmann, W.K., 1995. Defective G2 checkpoint function in cells from individuals with familial cancer syndromes. Cancer Res. 55, 1763-1773.

Pelicano, H., Carney, D., Huang, P., 2004. ROS stress in cancer cells and therapeutic implications. Drug Resistance Updates 7, 97-110.

Pelicano, H., Feng, L., Zhou, Y., Carew, J.S., Hileman, E.O., Plunkett, W., Keating, M.J., Huang, P., 2003. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. J. Biol. Chem. 278, 37832-37839.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., Piwnica-Worms, H., 1997. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science 277, 1501-1505.

Perry, G., Raina, A.K., Nunomura, A., Wataya, T., Sayre, L.M., Smith, M.A., 2000. How important is oxidative damage? Lessons from Alzheimer's disease. Free Radical Bio. Med. 28, 831-834.

Pines, J., Hunter, T., 1989. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34 cdc2. Cell 58, 833-846.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A., Ashkenazi, A., 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 271, 12687-12690.

Plati, J., Bucur, O., Khosravi-Far, R., 2011. Apoptotic cell signaling in cancer progression and therapy. Integr. Biol. 3, 279-296.

Rasul, A., Ding, C., Li, X., Khan, M., Yi, F., Ali, M., Ma, T., 2012a. Dracorhodin perchlorate inhibits PI3K/Akt and NF-κB activation, up-regulates the expression of p53, and enhances apoptosis. Apoptosis 17, 1104-1119

Rasul, A., Khan, M., Yu, B., Zhang, K., Iqbal, F., Ma, T., Yang, H., 2012b. Magnolol, a natural compound, induces apoptosis of SGC-7901 human gastric adenocarcinoma cells via the mitochondrial and PI3K/Akt signaling pathways. Int. J. Oncol. 40, 1153-1161

Rasul, A., Yu, B., Zhong, L., Khan, M., Yang, H., Ma, T., 2012c. Cytotoxic effect of evodiamine in SGC-7901 human gastric adenocarcinoma cells via simultaneous induction of apoptosis and autophagy. Oncol. Rep. 27, 1481-1487.

Rates., Stela, MK., 2001. "Plants as source of drugs." Toxicon 39.5, 603-613.

Ray AB., Gupta M., 1994. Withasteroids, a growing group of naturally occurring steroidal lactones; Fortschritte der chemie organischer naturstoffe/progress in the chemistry of organic natural products; Springer. pp 1-106.

Ray, AB., Sahai, M., Das, BC., 1978. Physalolactone, new withanolides from physalis peruviana. J. Indian. chem. Soc. 55, 1175.

Reed, J.C., 2000. Mechanisms of apoptosis. Am. J. Pathol. 157, 1415-1430.

Rekha, S., Srinivasan, V., Vasanth, S., Gopal, R.H., 2006. The in vitro antibacterial activity of Hedyotis umbellata. Indian J. Pharma. Sci. 68, 236.

Reyes-Reyes EM., Jin Z., Vaisberg AJ., Hammond GB., Bates PJ., 2012. Physangulidine a, a withanolide from physalis angulata, perturbs the cell cycle and induces cell death by apoptosis in prostate cancer cells. J. Nat. Prod.76, 2-7.

Russell, P., Nurse, P., 1987. Negative regulation of mitosis by wee1 , a gene encoding a protein kinase homolog. Cell 49, 559-567.

Russo, A.A., Jeffrey, P.D., Pavletich, N.P., 1996. Structural basis of cyclin-dependent kinase activation by phosphorylation. Nat. Struct. Mol. Biol. 3, 696-700.

Sabharwal, S.S., Schumacker, P.T., 2014. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? Nature Reviews Cancer 14, 709-721.

Sahai, M., & Kirson, I., 1984. Withaphysalin D, a new withaphysalin from Physalis minima Linn. var. indica. J. natural products. 47, 527-529.

Sahai, M., Neogi, p., Ray, A. B., Oshima, Y., & Hikino, H., 1982. Structures of withaperuvin-b and withaperuvin-C, withanolides of *physalis peruviana* roots. Heterocycles, 19, 37-40.

Sajeli, B., Sahai, M., Suessmuth, R., Asai, T., Hara, N., & Fujimoto, Y., 2006. Hyosgerin, a new optically active coumarinolignan, from the seeds of Hyoscyamus niger. Chemical and pharmaceutical bulletin, 54(4), 538-541

Salvesen, G.S., Dixit, V.M., 1997. Caspases: intracellular signaling by proteolysis. Cell 91, 443-446.

Sanghvi, I., Bindler, E., & Gershon, S., 1968. Pharmacology of a potential anti-Parkinson agent: tigloidine. Eur. J. Pharmacol. 4, 246-253.

Schafer, F.Q., Buettner, G.R., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Biol. Med. 30, 1191-1212.

Schlegel, J., Peters, I., Orrenius, S., Miller, D.K., Thornberry, N.A., Yamin, T.T., Nicholson, D.W., 1996. CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis. J. Biol. Chem. 271, 1841-1844.

Schmitt, E., Cimoli, G., Steyaert, A., Bertrand, R., 1998. Bcl-xL modulates apoptosis induced by anticancer drugs and delays DEVDase and DNA fragmentation-promoting activities. Exp. Cell Res. 240, 107-121.

Schumacher, H., Szekely, I., Patel, S., Fisher, D., 1973. Mitochondria: a clue to oncogenesis? The Lancet 302, 327.

Schumacker, P.T., 2006. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer cell 10, 175-176

Seol, D.W., Billiar, T.R., 1999. A caspase-9 variant missing the catalytic site is an endogenous inhibitor of apoptosis. J. Biol. Chem. 274, 2072-2076.

Shao, J., Gong, G., Trombetta, L., 2011. An evidence-based perspective of Hedyotis diffusa or Oldenlandia diffusa (spreading Hedyotis) for cancer patients, in Anonymous Evidence-Based Anticancer Materia Medica. Springer, pp. 179-192.

Sherr, C.J., 1995. D-type cyclins. Trends Biochem. Sci. 20, 187-190.

Siegel, R., Naishadham, D., Jemal, A., 2012. Cancer statistics, 2012. CA: Cancer J. Clin. 62, 10-29.

Simonian, P.L., Grillot, D.A., Nunez, G., 1997. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. Blood 90, 1208-1216.

Simonsen, H.T., Adsersen, A., Bremner, P., Heinrich, M., Wagner Smitt, U., Jaroszewski, J.W., 2004. Antifungal constituents of Melicope borbonica. Phytother. Res. 18, 542-545.

Singh, R.K., Lange, T.S., Kim, K.K., Brard, L., 2011. A coumarin derivative (RKS262) inhibits cell-cycle progression, causes pro-apoptotic signaling and cytotoxicity in ovarian cancer cells. Invest. New Drugs 29, 63-72.

Sivaprakasam, S., Karunakaran, K., Subburaya, U., Kuppusamy, S., Subashini, T., 2014. A Review on phytochemical and pharmacological profile of Hedyotis corymbosa Linn. Int. J. Pharm. Sci. Rev. Res. 26, 320-324.

Solomon, M., Glotzer, M., Lee, T., Philippe, M., Kirschner, M., 1990. Cyclin activation of p34^cdc2 Cell. 63, 1013-1024.

Somnath, D. E., Dey, A., Babu, A. S., & Aneela, S., 2014. In vitro antihelmintic activity of aqueous and methanolic extracts of oldelandia umbellata. Int. J. Pharm. Pharma. Sci. 6.

Srinivasan, A., Li, F., Wong, A., Kodandapani, L., Smidt, R.,Jr, Krebs, J.F., Fritz, L.C., Wu, J.C., Tomaselli, K.J., 1998. Bcl-xL functions downstream of caspase-8 to inhibit Fas- and tumor necrosis factor receptor 1-induced apoptosis of MCF7 breast carcinoma cells. J. Biol. Chem. 273, 4523-4529.

Stark, G.R., Taylor, W.R., 2006. Control of the G2/M transition. Mol. Biotechnol. 32, 227-248.

Stennicke, H.R., Jürgensmeier, J.M., Shin, H., Deveraux, Q., Wolf, B.B., Yang, X., Zhou, Q., Ellerby, H.M., Ellerby, L.M., Bredesen, D., 1998. Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. 273, 27084-27090.

Stewart, B., Wild, C., 2014. World cancer report 2014: World Health Organization.

Strausfeld, U., Labbe, J., Fesquet, D., Cavadore, J., Picard, A., Sadhu, K., Russell, P., Doree, M., 1991. Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein.

Szatrowski, T.P., Nathan, C.F., 1991. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 51, 794-798.

Takahashi, A., Alnemri, E.S., Lazebnik, Y.A., Fernandes-Alnemri, T., Litwack, G., Moir, R.D., Goldman, R.D., Poirier, G.G., Kaufmann, S.H., Earnshaw, W.C., 1996. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc. Natl. Acad. Sci. U. S. A. 93, 8395-8400.

Tanabe, H., Suzuki, H., Mizukami, H., Inoue, M., 2005. Double blockade of cell cycle progression by coptisine in vascular smooth muscle cells. Biochem. Pharmacol. 70, 1176-1184.

Tanaka, H., Kato, I., & Ito, K., 1985. Total synthesis of cleomiscosin B, a coumarinolignoid. Heterocycles, 23, 1991-1997.

Tanaka, T., Koike, K., Mitsunaga, K., Narita, K., Takano, S., Kamioka, A., Sase, E., Ouyang, Y., Ohmoto, T., 1995. Chromones from Harrisonia perforata. Phytochemistry 40, 1787-1790.

Taneyama, M., Yoshida, S., Kobayashi, M., & Hasegawa, M., 1983. Isolation of norbergenin from Saxifraga stolonifera. Phytochemistry. 22, 1053-1054.

Tartaglia, L.A., Ayres, T.M., Wong, G.H., Goeddel, D.V., 1993. A novel domain within the 55 kd TNF receptor signals cell death. Cell 74, 845-853.

Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S., Dixit, V.M., 1995. Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. Cell 81, 801-809.

Toyokuni, S., Okamoto, K., Yodoi, J., Hiai, H., 1995. Persistent oxidative stress in cancer. FEBS Lett. 358, 1-3.

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., Chiao, P.J., Achanta, G., Arlinghaus, R.B., Liu, J., 2006. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate. Cancer cell 10, 241-252.

Tsao, Y.P., D'Arpa, P., Liu, L.F., 1992. The involvement of active DNA synthesis in camptothecin-induced G2 arrest: altered regulation of p34cdc2/cyclin B. Cancer Res. 52, 1823-1829.

Tuma, R.S., 2008. Reactive oxygen species may have antitumor activity in metastatic melanoma. J. Natl. Cancer Inst. 100, 11-12.

Um, B.H., Lobstein, A., Weniger, B., Spiegel, C., Yice, F., Rakotoarison, O., Andriantsitohaina, R., Anton, R., 2003. New coumarins from Cedrelopsis grevei. Fitoterapia 74, 638-642.

van Diest, P.J., Michalides, R.J., Jannink, L., van der Valk, P., Peterse, H.L., de Jong, J.S., Meijer, C.J., Baak, J.P., 1997. Cyclin D1 expression in invasive breast cancer. Correlations and prognostic value. Am. J. Pathol. 150, 705-711.

Van Gurp, M., Festjens, N., van Loo, G., Saelens, X., Vandenabeele, P., 2003. Mitochondrial intermembrane proteins in cell death. Biochem. Biophys. Res. Commun. 304, 487-497.

Van Houten, B., Woshner, V., Santos, J.H., 2006. Role of mitochondrial DNA in toxic responses to oxidative stress. DNA repair 5, 145-152.

Van Meerbeeck, J. P., 2007. Evidence-based supportive management in lung cancer: MTP17-01. J. Thorac. Oncol. 2, S282-S284.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutellingsperger, C., 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J. Immunol. Methods 184, 39-51.

Vermeulen, K., Van Bockstaele, D.R., Berneman, Z.N., 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 36, 131-149.

Waga, S., Hannon, G.J., Beach, D., Stillman, B., 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369, 574-578.

Walker, N., Talanian, R., Brady, K., Dang, L., Bump, N., Ferenza, C., Franklin, S., Ghayur, T., Hackett, M., Hammill, L., 1994. Crystal structure of the cysteine protease interleukin-1β-converting enzyme: a (p20/p10) 2 homodimer. Cell 78, 343-352.

Wang, E., Wong, A., Cortopassi, G., 1997. The rate of mitochondrial mutagenesis is faster in mice than humans. Mutat. Res. Fund. Mol. M. 377, 157-166.

Waterhouse, N.J., Ricci, J., Green, D.R., 2002. And all of a sudden it's over: mitochondrial outer-membrane permeabilization in apoptosis. Biochimie 84, 113-121.

Weinberg, R.A., 1995. The retinoblastoma protein and cell cycle control. Cell 81, 323-330.

Weinert, T., 1997. A DNA damage checkpoint meets the cell cycle engine. Science 277, 1450-1451.

Wilson, K.P., Black, J.F., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., Raybuck, S.A., 1994. Structure and mechanism of interleukin-lβ converting enzyme.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G., Youle, R.J., 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. 139, 1281-1292.

Wu, T., Huang, S., Jong, T., Lai, J., Kuoh, C., 1988. Coumarins, acridone alkaloids and a flavone from Citrus grandis. Phytochemistry 27, 585-587.

Wu, T., Lin, D., Shi, L., Damu, A.G., Kuo, P., Kuo, Y., 2003. Cytotoxic anthraquinones from the stems of Rubia wallichiana Decne. Chem. Pharm. Bull. 51, 948-950.

Xiong, Y., Hannon, G., Zheng, H., Casso, D., Kobayashi, R., Beach, D., 1991. p21 is a universal inhibitor of cyclin kinases. Ried 49, 370.

Xu, M., Sheppard, K.A., Peng, C.Y., Yee, A.S., Piwnica-Worms, H., 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol. 14, 8420-8431.

Yamashiro, S., Yamakita, Y., Hosoya, H., Matsumura, F., 1991. Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis. Nature 349, 169-172.

Yan, D., Jin, C., Xiao, X., Dong, X., 2008. Antimicrobial properties of berberines alkaloids in Coptis chinensis Franch by microcalorimetry. J. Biochem. Biophys. Methods 70, 845-849.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275, 1129-1132.

Ye, X., Li, X., 2015. Isolation of coptisine with high purity from Coptis chinensis. Faming Zhuanli Shenqing. CN 104418852 A 20150318.

Yoganarasimhan, S., 2000. Medicinal plants of India. Volume 2. Tamil Nadu. Bangalore: The Author i .

Zha, J., Harada, H., Yang, E., Jockel, J., Korsmeyer, S.J., 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X L. Cell 87, 619-628.

Zhang, H. J., & CHEN, Y. G., 2006. Glycosides from *Hedyotis Diffusa*. J. Yunnan Normal U. (Natural Sciences Edition). 3, 016.

Zhang, H., Bazzill, J., Gallagher, R.J., Subramanian, C., Grogan, P.T., Day, V.W., Kindscher, K., Cohen, M.S., Timmermann, B.N., 2012. Antiproliferative withanolides from Daturawrightii. J. Nat. Prod. 76, 445-449

Zhang, J., Nishimoto, Y., Tokuda, H., Suzuki, N., Yasukawa, K., Kitdamrongtham, W., Akazawa, H., Manosroi, A., Manosroi, J., Akihisa, T., 2013. Cancer chemopreventive effect of bergenin from Peltophorum pterocarpum wood. Chem. Biodiversity 10, 1866-1875.

Zhao, D.B., Tang, Y.L., Wang, H.Q., 2005. Studies on the alkaloids from herb of Corydalis adunca. Zhongguo Zhong Yao Za Zhi 30, 1756-1757.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., Wang, X., 1997. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c–dependent activation of caspase-3. Cell 90, 405-413.

Zou, H., Li, Y., Liu, X., Wang, X., 1999. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. 274, 11549-11556.

Zurer, I., Hofseth, L.J., Cohen, Y., Xu-Welliver, M., Hussain, S.P., Harris, C.C., Rotter, V., 2004. The role of p53 in base excision repair following genotoxic stress. Carcinogenesis 25, 11-19.

Appendix

LIST OF PUBLICATIONS FROM THESIS WORK

1. **Poorna Chandra Rao**, Mohammad Ali Farboodniay Jahromi, Zahra Hosseini Jahromi, Saketh Sriram D, Srikant Viswanadha, Mahendra Sahai, Ahil Sajeli Begum "Cytotoxicity of Withasteroids: Withametelin induces cell cycle arrest at G2/M phase and mitochondria mediated apoptosis in Non-small cell lung cancer A549 cells" Tumor Biology (Accepted)

2. Senthi Mahibalan, **Poorna Chandra Rao**, Rukaiyya Khan, Ameer Basha, Ramakrishna Siddareddy, Hironori Masubuti, Yoshinori Fujimoto, Ahil Sajeli Begum. "Cytotoxic constituents of *Oldenlandia umbellata* and isolation of a new symmetrical coumarin dimer" Medicinal Chemistry Research. 2016, 1-7. 10.1007/s00044-015-1500-z

3. Rukaiyya Sirajuddin Khan, Mahibalan Senthi, **Poorna Chandra Rao**, Ameer Basha, Mallika Alvala, Dinesh Tummuri, Hironori Masubuti, Yoshinori Fujimoto,Ahil Sajeli Begum, "Antiproliferative constituents of Abutilon indicum leaves against U87MG human glioblastoma cells" Natural Product Research. 2015, 29.11, 1069-1073.

4. **Poorna Chandra Rao**, Sajeli Begum, Mahendra Sahai, Saketh Sriram D, Srikant Viswanadha "Coptisine, a tetraoxygenated protoberberine alkaloid induces cell cycle arrest at G2/M phase and ROS-dependent mitochondria mediated apoptosis in Non-small cell lung cancer A549 cells" Life Sciences. (Under revision (UR))

5. **Poorna Chandra Rao**, Ahil Sajeli Begum, Saketh Sriram, Yoshinori Fujimoto

"Antiproliferative mechanism of cedrelopsin, a dimethylallyl coumarin derivative in Non-Small Cell Lung Cancer (A549) Cells" Cancer Chemotherapy and Pharmacology (UR)

OTHER PUBLICATIONS

1. Rukaiyya Khan, Yoshinori Fujimoto, Ameer Basha, Ahil Sajeli Begum, GoverdhanamRagavendra, Poorna Chandra Rao, Yuhei Tanemura, "Attenuation of TNF- alpha secretion by L-proline-based cyclic dipeptides produced by culture broth of Pseudomonas aeruginosa" Bioorg. Med. Chem.Lett. 25 (2015) 5656-5761.

PAPERS PRESENTED AT NATIONAL/INTERNATIONAL CONFERENCES FROM THESIS

1. P C Rao, S Mahibalan, A Sajeli begum. Identification of anti colon cancer lead from *Hedyotis umbellata*. 5th ICSCC, Mumbai 19- 21st Oct 2013.
2. A.S. Begum, S. Mahibalan, P.C. Rao, S.R. Khan, Y. Fujimoto, B.S. Ramakrishna Reddy. Cancer cell proliferation effect of iridoid glucosides isolated from *Hedyotis umbellata* L. Presented in 15th Tetrahedron Symposium-Asia Edition, held at Singapore from October 28-31,2014
3. S Mahibalan, P C Rao, Rukaiyya S Khan and A Sajeli Begum. Newer cytotoxic leads from *Hedyotis umbellata* against lung and breast cancer. Presented in 66th IPC, held at Hyderabad from January 23-25, 2015.

BIOGRAPHY OF V V S P C RAO MATHAMSETTI

Mr. V V S P C Rao Mathamsetti completed his Bachelor of Pharmacy from Siddartha College of Pharmaceutical Sciences, Nagarjuna University, Vijayawada in the year 2007 and M Pharmacy in Pharmacology from Andhra University College of Pharmaceutical Sciences, Vishakhapatnam. He worked for Incozen Therapeutics Pvt Ltd, Hyderabad for 1.6 years as Research associate from 2010-2012. He then joined Birla Institute of Technology and Science, Pilani, Hyderabad Campus to pursue Ph.D under the supervision of Prof. A. Sajeli begum through Institute Fellowship. He has co-authored for four scientific publications in international journals. Also, he has presented papers at various conferences.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Ahil Sajeli Begum is currently an Associate Professor in Department of Pharmacy, Birla Institute of Technology and Science, Pilani-Hyderabad Campus. She received her B. Pharm degree (1999) from The Tamilnadu Dr. M.G.R. Medical University, Chennai and M. Pharm degree (2001) in Pharmaceutical Chemistry from Institute of Technology-Banaras Hindu University (IT-BHU), Varanasi. She was awarded with Ph. D degree (2005) by BHU for her thesis work on "Chemical Investigation of Solanaceous Plants". Prof. AS Begum is a recipient of the Deutscher Akademischer Austausch Dienst (DAAD) fellowship (2004) to pursue research at Eberhard Karls Univesrity, Tubingen, Germany. Soon after completing her Ph.D. program, she joined in Department of Pharmaceutics at IT-BHU, Varanasi as an Assistant Professor and then moved to BITS-Pilani Hyderabad in mid 2010. She has 10 years of experience in teaching and research. She has successfully completed two projects funded by University Grants Commission (UGC) -New Delhi and Council of Scientific and Industrial Research-New Delhi. She has 28 publications to her credit and authored a book chapter in "Progress in the Chemistry of Organic Natural Products" published by Springer Wien New York. Prof. AS Begum is a life time member of various scientific forums like Association of Pharmaceutical Teachers of India (APTI), Indian Pharmacy Graduates Association (IPGA) and Indian Chemical Society. She has successfully guided two Ph.D students and currently supervising two students for their doctoral thesis work.