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THESIS

EVALUATION OF NATURAL AND SYNTHETIC COMPOUNDS IN HUMAN TUMOUR CELL LINES: EFFECTS ON CELL GROWTH, CELL CYCLE AND APOPTOSIS

KANTIMA CHOOSANG

A Thesis Submitted in Partial Fulfillment of
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Fifty natural and synthetic compounds of xanthones, terpenes and flavonoids were evaluated for their capacity to inhibit the growth of three carcinoma cell lines, NCI-H460 (non-small cell lung cancer), MCF-7 (breast adenocarcinoma) and A375-C5 (melanoma). Initial elucidation of the mechanisms involved in the cell growth arrest of the most potent compounds was carried out by verifying their effect in cell cycle profile and apoptosis. The results showed that one triterpene (odoratol) and two limonoids (gedunin and cedrelone) caused cell cycle arrest. In addition, analysis of apoptosis indicated that triterpene odoratol and limonoid 6α-acetoxy-14β,15β-epoxyazadirone cause a very small increase in cell death while limonoid gedunin and particularly cedrelone, are very potent inducers of programmed cell death.

In conclusion, limonoid cedrelone is very potent by causing cell cycle arrest and apoptosis, which makes it a very interesting compound for further studies in order to investigate its full potential as antitumour agent.

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Kantima Choosang
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LIST OF ABBREVIATIONS

BAD	=	Bcl-2 antagonist of cell death protein
Bcl-2	=	B cell leukemia-2 proto-oncogene
Bid	=	BH3 interacting domain death agonist
Bcl-xL	=	Bcl-2 family member, antiapoptotic longisoform
Caspases	=	Cysteine-aspartic-acid-proteases
Cdc2	=	Cell division cycle 2 protein
Cdc 25	=	Cell division cycle 25 protein
CDK4	=	Cyclin dependent kinase 4
CDKI	=	Cyclin dependent kinase inhibitor
Chk1	=	Checkpoint kinase 1
Chk2	=	Checkpoint kinase 2
DAPI	=	4', 6-diamidino-2-phenylindole
DNA	=	Deoxyribonucleic acid
DOX	=	Doxorubicin
DR	=	Death receptor
E2F	=	Elongation factor 2
ERK	=	Extracellular signal-regulated kinase
FADD	=	Fas-associated death domain
FACS	=	Fluorescent-activated cell sorting
FCM	=	Flow cytometry
FITC	=	Fluorescein isothiocyanate
FL1	=	Fluorescence intensity, channel 1
FL2	=	Fluorescence intensity, channel 2
FL3	=	Fluorescence intensity, channel 3
FSC	=	Forward scatter
G0	=	Quiescent or resting phase of cell cycle
G1	=	Growth phase 1- cell cycle
G2	=	Growth phase 2- cell cycle
MAPK	=	Mitogen-activated protein kinase
MEK	=	MAPK/ERK kinase

LIST OF ABBREVIATIONS (Continued)

MTT	=	Methyl tetrazolium bromide
NSCLC	=	Non-small cell lung carcinoma
PI	=	Propidium iodide
PBS	=	Phosphate buffered saline
PI-3K	=	Phosphoinositde-3 kinase
PS	=	Phosphatidyl serine
RT	=	Room temperature
S	=	Synthesis phase, cell cycle
SCLC	=	Small-cell lung carcinoma
SSC	=	Sideward scatter

EVALUATION OF NATURAL AND SYNTHETIC COMPOUNDS IN HUMAN TUMOUR CELL LINES: EFFECTS ON CELL GROWTH, CELL CYCLE AND APOPTOSIS

INTRODUCTION

Cancer remains a major source of morbidity and mortality throughout the world. All forms of cancer are characterized by abnormal cell growth resulting from a relatively small number of inherited or environmentally-induced genetic mutation. Although localized cancers can often be successfully treated by surgery and/or radiation therapy, chemotherapy remains the usual treatment of choice especially for advanced or metastatic disease. Cancer cells also frequently become resistant to chemotherapy as a consequence of cellular changes that include increased expression of drug detoxifying enzymes and drug transporters, altered interactions between the drug and its target, an increased ability to repair DNA damage, drug metabolizing enzymes and defects in the cellular machinery that mediate apoptosis. Drug resistance limits the effectiveness of existing treatment options and is a major challenge faced by current research. The development of a new class of anticancer drugs with lack the toxicity of conventional chemotherapeutic agents and are unaffected by common mechanisms of chemoresistance would be a major advance in cancer treatment. Indeed, numerous bioactive compounds, normally small molecules have been improved over the years through chemical modifications by medicinal chemists.

Natural products have been the major sources of chemical diversity as starting materials for driving pharmaceutical discovery over the past century. Many natural products and synthetically modified natural product derivatives have been successfully developed for clinical use to treat human diseases in almost all therapeutic areas. In fact, plant-derived compounds have played an important role in treatment of cancers, and some of the most promising and better drugs have come up from plant sources like taxol®, camptothecin, combrestatin, vinblastine and

vincristine. These drugs have also been the major source of new drug candidates for cancer chemotherapy. Due to the increase development of drug resistance cancer cells, the need to search for novel compounds with greater effectiveness, selectivity, increased bioactivity, metabolic stability and low toxicity are necessary. Some group of plant natural compounds such as xanthones and triterpenes are very interesting for using not only as starting materials for molecular modifications as well as models for synthesis of novel compound. Xanthone scaffold can support a large number of different substituents, depending on their chemical nature and position on the aromatic rings, leading to an interesting diverse of biological activities of its derivatives. Triterpenes, concerning cyclic triterpenoid-like substances, is one of the most interesting group of compounds due to their diverse biological effect, such as anti-inflammatory activity, hepatoprotective, analgesic, antimicrobial, antimicotic, virostatic, immunomodulatory and tonic effects. In this study, the evaluation of antitumour activity of natural and synthetic compounds from xanthone and triterpene groups was carried out.

OBJECTIVES

1. To evaluate the capacity of diverse natural and synthetic compounds to inhibit the *in vitro* growth of three human tumour cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and A375-C5 (melanoma).

2. To investigate the mechanism underlying the growth activity of the most active compounds regarding their effect on the cell cycle profile of inhibitory NCI-H460 cells.

3. To investigate the effect of the most active compounds on programmed cell death of the NCI-H460 cells.

LITERATURE REVIEW

1. Cancer: the state of art

Cancer is one of the major health concern. Worldwide, the burden of this disease affects on the lives of tens of million, and it continues to grow. According to latest statistics which reported by the World Health Organization (WHO), there are 10 million new cases, 6.2 million deaths and 22.4 million people living with cancer each and every year. Given the current trends in the adoption of unhealthy lifestyles, such as smoking and drinking alcohol, the number of new cases is expected to grow by 50% over the next 15 years to reach 15 million by 2020. Almost half of the new cases of cancer are diagnosed early enough to be treated with surgery or radiotherapy. In the cases which cancer has metastasized, the method use to treat is chemotherapy. In many cases chemotherapy can effectively contributes for the cure of several cancer, such as, leukemia, lymphoma, choriocarcinoma and testicular cancers. However, 12% of people die from cancer, worldwide. Consequently, these statistics remind us that although there have been great improvements in the understanding and therapy of disease, there is an ever increasing need to find preventive and curative measures to effectively manage the disease. To deal with it however, it is necessary to understand the mechanisms of cancer development.

The greatest achievement of understanding the process of cancer development has been research in the mid-seventies that developed the concept that a malignant tumor was the result of a complex multistage carcinogenic process. A primary cancer is a tumor mass present at the site of initial mutation of a normal cell to a tumor cell. If all cells remained in the primary tumor, cancer would be of little clinical importance. Growth of the tumor would produce pressure on surrounding tissue, which would ultimately become harmful to the host, but the well-defined tumor mass could be excised by surgery in a straight forward and permanent way. However, tumor cells do not always remain at the primary site, but move away by invasion and metastasis, the spread of cells to distant sites, usually via the bloodstream, lymphatic system, or through body spaces. The most invasion usually occurs before any

metastasis takes place (Oppenheimer *et al*., 2006). Invasion in its simplest form is merely the expansion of tumor cells into surrounding tissues as a result of continuous cell division. However, active cell movement may also occurs. Cells in cancers tend to adhere poorly to each other, break away from the tumor mass, and then move away from each other. Such movement does not occur in normal tissue. Normal cells do indeed move both in culture and in the course of embryological development, but when normal adult cells in culture touch each other, they usually stop both growth and movement. However, cancer cells, at least in culture, are not controlled by cell contact, and in the body they continue to grow and move into surrounding tissues. Also, most cancer cells release proteases, which probably help them digest away surrounding tissue materials, thereby facilitating invasion (Mazzocca *et al*., 2005).

Many molecules involved in DNA repair and DNA damage signaling are thought to play an important role in maintaining genomic integrity in response to endogenous and exogenous DNA damage. DNA repair pathways mediated by these molecules are important etiological factors in human cancer. Epigenetic modifications, including DNA methylation, histone acetylation, methylation and poly-ADP-ribosylation, link chromatin remodeling and gene expression, which affect virtually every step in tumour progression. Aberrant histone modifications can cause incorrect gene activation and improper gene silencing, which can lead to cancer. However, the mechanisms underlying the control of cellular processes and initiating pathogenesis are largely unknown. Studying the functions of these genes in chromosomal stability, DNA repair, cell cycle controls, cell death pathways, chromatin remodeling and the role of environmental factors in causing specific genetic changes and also find out the novel compounds that can control all of these processes are important areas of cancer research. Therefore, understanding genetic events associated with cancer onset, progression and metastasis are fundamental to improving our abilities to successfully prevent, diagnose and treat cancer.

Lung cancer is the leading cause of cancer-related mortality worldwide which is broadly divided into two classes - non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Squamous cell carcinoma, adenocarcinoma and large cell

carcinoma are classified as NSCLC and account for 75% to 80% of all lung cancer cases. For early stage NSCLC, surgery is almost curative. Most of the patients, however, have advanced disease when they present to an oncologist and their disease is beyond surgical resection. Chemotherapy and/or radiotherapy in such patients produce less than desirable results. No chemotherapeutic regimen for lung cancer is either completely effective or has conclusively led to cure (Pathak *et al*., 2004). In addition, despite advances in treatment, the 5-year survival rate for NSCLC across all stages is presently only 16% (Boyle and Ferlay, 2005). Owing to lack of early detection methods and the asymptomatic early stage of cancer, 65% of patients are diagnosed with an advanced disease (Naruke, 2003).

It is estimated that about 90% of male lung cancer deaths and 75-80% of lung cancer deaths in the US are caused by smoking each year. Evidence in North America shows smoking has been decreasing in men in recent years, but lung cancer still remains as the leading cause of cancer death among men and the second most common cancer deaths among women (behind breast cancer). In addition to tobacco smoking, many environmental factors can contribute to lung cancer risk such as asbestos, radon, alcohol, benzene, coal and other occupation chemicals. More new cases of lung cancer are diagnosed every year despite many countries having taken preventative measures towards causes of lung cancer by banning asbestos, enforcing laws on public smoking, and restricting tobacco advertising. As a consequence, lung cancer will remain as one of the leading forms of cancer death for years to come. The challenge is, thus, to find the new effective drugs for treatment of this disease (Berman and Crump, 2008).

2. Chemotherapy in cancer treatment

The principal modes of cancer therapy are surgery, radiotherapy, chemotherapy and immunotherapy, all of which may be applied alone or in combination depend on the stage of cancer (Danesi *et al*., 2002). The treatment of cancer by chemotherapy began in 1943 using alkylating agent in patients with lymphoma and leukemia. The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. The efficacies of these drugs were later described as associated to their ability to damage DNA replicating and inhibit DNA synthesis. The discovery of many novel chemo-types showing a range of cytotoxic activities (Cragg and Newman, 2005), including the taxanes and camptothecins, from the early 1960s to the 1990s after the United States National Cancer Institute (NCI) initiated an extensive plant collection program in 1960, focused mainly in temperate regions. However, their development into clinically active agents spanned a period of some 30 years. The development of new screening technologies led to the revival of collections of plants and other organisms in 1986, with a focus on the tropical and sub-tropical regions of the world then drugs with different mechanisms of action have been developed and approved.

Anticancer drugs in general are acting on both cancer and normal cells and are therefore often toxic against rapidly-dividing normal cells, such as gastrointestinal cells and blood-generating cells. Chemotherapy drugs, are sometimes feared since patients concern of their toxic effects. Their role is to decrease and hopefully stop the growth and spread of a cancer. Unfortunately, the majority of drugs currently on the market are not specific, which leads to the many common side effects associated with cancer chemotherapy. Because the common approach of all chemotherapy is to decrease the growth rate (cell division) of the cancer cells, the side effects are seen in bodily systems that naturally have a rapid turnover of cells including skin, hair, gastrointestinal, and bone marrow. These healthy, normal cells, also end up damaged by the chemotherapy program. Because of this reason, in the last few years new drugs have been approved which exert their effect on more specific targets in the cells,

such as the small molecule inhibitors imatinib (Glivec) and erlotinib (Tarceva) which act on the tyrosine kinases and epithelial growth factor receptor (EGFR) respectively, bortezomib (Velcade) which targets the proteasome and the larger antibodies rituximab (Mabthera) used against CD20 in lymphoma, trastuzumab (Herceptin) against the Her2 receptor in breast cancer, cetuximab against EGFR and bevacizumab (Avastin) acts on the vascular endothelial growth factor receptor (VEGFR). Since these drugs have more specific targets than the classical drugs, in some cases they are likely to cause fewer side effects. However, targeting one protein is often not sufficient since the signaling pathways in normal and cancer cells are complex and inhibition of one signaling pathway may enable the cell to use another pathway to escape the drug action (Danesi *et al*., 2002; Horvath *et al*., 2004; Jordan *et al*., 2004; Marinho *et al*., 2008).

3. Mechanisms of action of anticancer agents

Carcinogenesis is a complex multistage genetic change affecting proto-oncogenes or tumor-suppressor genes, and that sequence of events has many steps for intervention, with the aim of preventing, slowing down or reversing the process. A major causative factor in the development and progression of cancer is defective apoptosis (programmed cell death). Synthesis of compounds which affect the overall process of carcinogenesis by several mechanisms, including decrease or increase in reactive oxygen species, DNA oxidation and fragmentation, regulation of heatshock-protein expressions (Jakubowicz-Gil *et al*., 2005) cell cycle arrest (Lee *et al*., 2003) modulation of survival/proliferation pathways (Gong *et al*.,2003; Spencer *et al*., 2003) microtubule interference increase in migration-inhibitory factor (MIF), and modulation of nuclear factor (Gong *et al*., 2003; Gopalakrishnan and Kong, 2008) have been developed for the past two decades. Cytotoxic drugs which are currently used for the treatment of malignant diseases such as etoposide, cytarabine, cyclophosphamide, doxorubicin and methotrexate are thought to exert their effects through inhibition of topoisomerase II (etoposide), DNA-polymerase (cytarabine), antagonization of folic acid (methotrexate), inhibition of DNA-crosslinking (cyclophosphamide) and DNA intercalation (doxorubicin). Although the primary

intracellular targets of drug action are rather distinct, it has become evident that drug-induced cytotoxicity definitively converges on a common pathway, causing apoptosis (Debatin, 1999; Kaufmann *et al*., 2000; Cohen and Flescher, 2008).

4. Mechanisms of resistance to anticancer drugs

The use of chemotherapy to treat cancer usually results in the development of broad resistance to a wide variety of drugs with different chemical structures and mechanisms of action. Multidrug resistance (MDR) is the major cause of cancer chemotherapy failure, where tumor have become resistant or were initially resistant to chemotherapy treatment. This form of resistance is mediated primarily by classical ATP driven drug efflux pumps such as the P-glycoproteins and the MRP family of proteins. The multidrug resistance (MDR) related to P-glycoprotein is inherently expressed at high levels in cancers derived from epithelial tissues such as kidney, prostate and colon (Croop *et al*., 1989; Dorai *et al*., 2004; Dean, 2005; Szakacs *et al*., 2006). The groups of anticancer drugs that are substrates of multidrug resistance associated proteins (MRP) include anthracyclines such as DOX, vinca alkaloids, and etoposide. By pumping these agents out of the tumor cells, MRP causes reduced intracellular accumulation of drugs, leading to resistance.

5. Cell cycle

The cell cycle is a complex process involved in the cell growth and proliferation, organism development, regulation of DNA damage repair, tissue hyperplasia as response to injury, including diseases such as cancer (Schafer *et al*., 1998). The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events culminating in mitosis and production of two daughter cells. The eukaryotic cell cycle is divided into four stages: G1, S, G2 and M during which the DNA content of the cell changes as it prepares to divide. The G1 and G2 phases of the cycle represent the “gaps” in the cell cycle that occur between DNA synthesis and mitosis. At the first gap, the G1 phase, the cells prepare for the process of DNA replication. The S phase is defined as the stage during which DNA

synthesis occurs and therefore have aneuploid DNA content between 2N and 4N. During the second gap, the G2 phase, the cells prepare for cell division which the cells have twice amount of DNA. The M phase is defined as the mitosis, the phase during which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells with the normal DNA content for the species (2n or diploid). In addition to G1, S, G2 and M, the term G0 is used to describe cells that have exited the cell cycle and have become quiescent. Because of the different amount of DNA content present in each phase of the cell cycle, it is possible to analyze the cell cycle distribution in a cell population of cell by measuring their nuclear DNA content (Nunez, 2001).

The progression of cell cycle in each phase is controlled by a number of cyclin-dependent kinases (CDKs) which are heterodimeric complexes composed of a catalytic kinase subunit and a regulatory cyclin subunit (Deep and Agarwal, 2008). Cyclin-dependent kinases (CDKs) and their cyclin partners are positive regulators or accelerators that induce cell cycle progression; whereas, cyclin-dependent kinase inhibitors (CKIs) that act as inhibitors to stop cell cycle progression in response to regulatory signals are important negative regulators. Cell cycle transition is an ordered, tightly-regulated process that involves multiple checkpoints that assess extracellular growth signals, cell size, and DNA integrity. This control of cell-cycle order is maintained through an intracellular "checkpoint" that monitors the integrity and completion of DNA synthesis before authorizing the initiation of mitosis.

5.1 Cell cycle transition and protein expression

As previously described, in the cell cycle, two interacting components are mentioned: (a) the “mechanical” component, which refers to DNA replication, mitosis and cytokinesis; and (b) the “regulatory” components which describe about events in G1 that control entry into the S phase and those in G2 that regulate entry into mitosis . The main progression of the cell cycle events depends primarily upon accurate chromosome replication and segregation, which is achieved by assuring that each step, is completed before the next one begins. For this ordered dependency to be

enforced, the cell has developed control mechanism called checkpoints. Damage to checkpoint mechanisms, which results in failure to control cell cycle events, can cause alterations and mutations that may lead to cancer (Vermeulen *et al.*, 2003). These cell cycle checkpoints are regulated by components of the cells cycle machinery. In cases where nuclear DNA has been damaged normal cells stop to progress through the cell cycle at one of two points: either prior to entry into S-phase or prior to entry into mitosis. Arrest at these phases will limit propagation of genetic mutations to daughter cells by giving the time for DNA repair or initiation of cell death (Rosenberg *et al.*, 2001). Cell cycle progression is governed by key regulatory proteins, which are controlled by post-translational modification.

In order for cells to complete the G1 and then enter to S phase, transition extracellular growth factors must be present for the cells to pass the first restriction point. This restriction point is a point in late G1 at which growth factors are no longer required. If adequate growth factors are not present the cell will enter G0. Throughout the transition past this initial restriction point. The series of biochemical events occur. First the protein complex cyclin D/cdk 4 is activated. This activated complex will hyperphosphorylate the protein RB. In general, the unphosphorylated RB protein prevents cells from entering S phase of cell cycle. It starts by binding to transcriptional factors called EF2. Upon hyperphosphorylation, RB will disengage from its partner E2F-1. The physical interaction of RB with E2F-1 prevents gene activation by E2F-1, a necessary step for cell cycle progression. Thus, upon hyperphosphorylation of RB, the E2F-1 gene is released and can now mediate gene activation and progression to the S phase (Nevins, 1998). The result of DNA damage which occur at G1 phase is cell cycle arrest due to the tumor suppressor gene, p53. Following DNA damage p53 levels and activity are increased. An increase in p53 can lead to an increase in the level of p21 (WAF1/CIP1) or known as cyclin-depebdent kinase inhibitor 1 which serves to inhibit the activity of the cyclin/cdk complexes. Inhibition of cyclin/cdk will prevent hyperphosphorylation of RB thus preventing E2F-1 release which has previously been shown to drive the G1 - S transition. p53 also functions as a cell cycle checkpoint protein by driving apoptosis following DNA damage (Levine, 1997).

A complete copy of the genetic material must be generated in S phase. For this to occur, DNA synthesis must begin at a defined location in the genome or an origin of replication. For DNA synthesis to be initiated and progress multiple proteins must bind to the DNA to start this process. These proteins are called the origin of replication complex (ORC). There are two ORCs involve in this phase of the cell cycle, a pre-ORC and a post-ORC. The pre-ORC binds to and prepares the DNA for replication at the end of G1 but does not allow for replication to begin. Upon entry into S phase the post-ORC complex forms and allows the cell to proceed with DNA synthesis. Regulation of the pre-ORC and post-ORC is likely responsible for the mechanism by which cells cannot re-replicate DNA during the S phase (Rosenberg *et al*., 2001). While little is known about the DNA-damage induced checkpoint controls during this phase of the cell cycle, an identified gene, ATM, a cytoplasmic protein kinase, is known to phosphorylate certain proteins such as p53 which are involved in DNA damage repair (Banin *et al.*,1998).

Following the S phase the cell enters G2 and prepares for mitosis. During mitosis the duplicated genome is divided into two daughter cells. It is important that M phase be successfully completed because errors in this process can lead to alterations in the genetic material such as loss of a chromosome (Hartwell *et al.*, 1989). There are several stages within M-phase. During the first stage cells enter mitosis; this is controlled to prevent the segregation of chromosomes that have not finished DNA synthesis. During the second stage structural changes occur that prepare the sister chromatids to separate. The next stage at which the sister chromatids separate is an important checkpoint for the cell because this process is not easily reversed. During the last stage the chromosomes decondense, reform their nuclear envelope, and the cells undergo cytokinesis to complete the cell cycle (Rosenberg *et al*., 2001). The regulation of mitosis composes of three levels. The first level is activation of the protein complex cyclin B/cdk1 which governs whether cells enter mitosis. In this level, cyclin B increase during late S and G2 phase. The newly synthesized cyclin B will bind to dephosphorylated cdk1 (Blow *et al*., 1988) and then the cdk-activating complex will localize to the nucleus. The second level of regulation is modulated by a family of kinases known as Polo-Like Kinases (PLKs). These are

localized to spindle pole bodies, kinetichores and the spindle mid-zone at different stages in mitosis, thus playing a role in spindle apparatus assembly, pairing of the sister chromatids and separation. PLKs are also involved in activation of the anaphase promoting complex (APC), also called cyclosome, is a complex of several proteins activated during mitosis to initiate anaphase. The APC cyclosome serves as the third regulatory mechanism. The APC cyclosome causes destruction of certain proteins by ubiquination. Thus the APC cyclosome controls the entry into, transition through, and exit from mitosis (Rosenberg *et al*., 2001). During mitosis a checkpoint exists to prevent the metaphase to anaphase transition until sister chromatid pairs are aligned and attached at the spindle apparatus. This checkpoint is also thought to be controlled by the p53 protein.

5.2 Cell cycle analysis by flow cytometry

Flow cytometry has been used as a useful and quick method to efficiently and reproducibly determine the relative nuclear DNA content of cells. Basically, a flow cytometer is a fluorescence microscope which analyses the signals that result from the moving of particles in a liquid stream through a beam of light.

To determine the DNA content, the cells are stained with a fluorescent dye that binds to DNA in a manner that accurately reflects the amount of DNA present. The most widely used dye is propidium iodide (PI). It is a red fluorescence which can be excited at 488 nm. However, PI has two disadvantages. The first disadvantage is that it stains all double stranded nucleic acids, therefore the making it imperative to incubate the cells with RNase to remove any RNA. The second disadvantage is that the dye is excluded by the cellular membrane therefore the cells have to be fixed or permeabilized before adding the dye. The simplest method is to fix the cells in 70% ethanol.

The stained material is then analyzed in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Cells in quiescent and G1 of cell cycle have one copy of DNA and therefore have 1X fluorescence intensity.

Cells in G2/M phase of the cell cycle have two copies of DNA and accordingly have 2X intensity. Since the cells in S phase are synthesizing DNA they have fluorescence values between the 1X and 2X cell populations.

6. Apoptosis

Apoptosis, or programmed cell death, is an important process during normal development. It defines a genetically encoded cell death program, which is morphologically and biochemically distinct from necrosis or accidental cell death. It is the process which is used to eliminate the abnormal cells. There are several instances in which cells may need to be destroyed, such as cells may become damaged or undergo some type of infection. One way to remove such cells without causing harm to other cells is through apoptosis. Dysregulation of apoptosis contributes to many diseases including autoimmunity, cancer, stroke, and also contributes to the problem of drug resistance in tumors and the acquired immunodeficiency syndrome (AIDS) (Reed, 2002; Elmore *et al*., 2007).

6.1 Morphologic features and molecular mechanisms of apoptosis

Apoptotic cells can be recognized by typical morphologic changes that are induced by molecular and biochemical events that lead to the activation of proteolytic enzymes and DNA fragmentation. Morphology change in apoptosis is characterized by the early events of cell dehydration and followed by loss of intracellular water leading to condensation of cytoplasm. The result of early events is a change in cell shape and size: the originally round cells may become elongated and generally, are smaller. In the next event, perhaps the most characteristic feature of apoptosis, is condensation of nuclear chromatin. Then the apoptotic cells detach from the surrounding tissue and form extensions (budding), the result being the formation of apoptotic bodies, which contain packed cellular organelles and lamin proteins undergo proteolytic degradation, follow by nuclear fragmentation (karyorrhexis). Finally, the apoptotic bodies are rapidly removed by phagocytic cells, such as immature dendritic cells and macrophages as well as non-phagocytic cells, comprised of multiple cell types (Ravichandran *et al*., 2007). Rapid and efficient clearance of

apoptotic cells ensures that the uninvolved surrounding cells keep cellular integrity, and prevents the leakage of cellular contents, which in turn, limits the immune response to antigens from the dying cells. Thus, a characteristic feature of apoptosis is a relative lack of an inflammatory response. In contrast, cells may undergo another form of cell death, necrosis, swell and rupture. The released intracellular contents from necrosed cells can damage surrounding cells and often causes inflammation (Compton *et al*., 1992; Oltvai and Korsmeyer, 1994; Wyllie *et al*., 1992). Upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive changes occur in the cell. A family of proteins known as caspases mediate most events that culminate in the apoptotic phenotype. The caspases are a family of proteins that belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes which can activate other caspases, establishing a "caspase cascade". The activation of caspase-3 is a central and specific event in the process of apoptosis. This caspase is a major effector or executioner protease, which cleaves several intracellular target proteins, and ultimately leads to cell death. The activation of effector caspases in apoptotic cells can be induced by two signaling pathways, the extrinsic (receptor-mediated) and the intrinsic (mitochondria-mediated) signaling pathways (Leist and Jaattella, 2001).

The extrinsic pathway is triggered by death receptor engagement (CD95R, TRAILR, TNFR) followed by the formation of the death-inducing-signaling-complex (DISC), which initiates a signaling cascade mediated by caspase-8 activation. Caspase-8 activation leads to directly induce caspase-3 and stimulates the release of cytochrome C from mitochondria. Caspase-3 activation leads to the degradation of cellular proteins necessary to maintain cell survival and integrity. One major regulator of this pathway at the DISC level is c-FLIP (Antonsson *et al*., 2000; Ashkenazi *et al*., 2002).

The intrinsic (Bcl-2 inhibitable or mitochondrial) pathway of apoptosis functions in response to various types of intracellular stress including growth factor withdrawal, DNA damage, unfolding stresses in the endoplasmic reticulum and death receptor stimulation. In response to apoptotic stimuli several proteins, such as

cytochrome C, are released from the intermembrane space of mitochondria into the cytoplasm (independently of caspase-8 activation). Release of cytochrome C from mitochondria, which believed to be modulated by bcl-2, appears to be one of the earliest events of apoptosis, triggering activity of caspases and other downstream apoptotic effectors. Once cytochrome C has been released into the cytosol it is able to interact with a protein called Apaf-1. This leads to the recruitment of pro-caspase 9 to form a multi-protein complex with cytochrome C and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase 9 which, in turn, activates pro-caspase-3. This death pathway is largely controlled by the pro-apoptotic (e.g. Bax, Bad, Bid and Bak) and anti-apoptotic (e.g. Bcl-2 and Bcl-xL) Bcl-2 family proteins. Caspase-8 activated by the extrinsic death signal may crosslink with the intrinsic pathways by inducing cleavage of Bid, which, in turn, induces the translocation of the pro-apoptotic Bcl-2 family proteins Bax and/or Bak to the mitochondrial membrane (Denault *et al*., 2002).

6.2 Caspases and chromatin breakdown

One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The caspases play an important role in this process by activating DNases, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus (Chen *et al*., 2002). This processes is explained below.

(1) Inactivation of enzymes involved in DNA repair

The enzyme poly (ADP-ribose) polymerase, or PARP, was one of the first proteins identified as a substrate for caspases. PARP is involved in the repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3.

(2) Breakdown of structural nuclear proteins

Lamins are intra-nuclear proteins that maintain the shape of the nucleus and mediate interactions between chromatin and the nuclear membrane.

Degradation of lamins by caspase 6 results in the chromatin condensation and nuclear fragmentation commonly observed in apoptotic cells.

(3) Fragmentation of DNA

Rapid fragmentation of the nuclear DNA follows caspases activation. The fragmentation of DNA into nucleosomal units - as may be seen in DNA laddering assays - is caused by an enzyme known as CAD, or caspase activated DNase. Normally CAD exists as an inactive complex with ICAD (inhibitor of CAD). During apoptosis, ICAD is cleaved by caspases, such as caspase 3, to release CAD.

6.3 Regulation of apoptosis

Since all cells are believed to have the capacity to undergo apoptosis, and its dysregulation is linked to the pathogenesis of many diseases, multiple mechanisms have evolved to regulate apoptosis. The most important of these are the proteins in the Bcl-2 family and the Inhibitor of Apoptosis Protein (IAP) family (Dean *et al*., 2007; Youle and Strasser, 2008).

Bcl-2 was originally discovered in B-cell follicular lymphomas. However, in contrast to some other oncogenes, it was found to inhibit cell death rather than promote cell proliferation. Later, it became clear that Bcl-2 family members, besides their role in cancer, are essential for a wide range of actions in developmentally programmed cell death, tissue turnover and host defense. Classically, the Bcl-2 family is divided into 3 classes: those that inhibit apoptosis (e.g., Bcl-2 and Bcl-xL); those that promote apoptosis (e.g., Bax, Bak and Bok); and finally the BH3 only proteins (e.g., Bad, Bid, Bim, Noxa and Puma). These last ones have a conserved BH3 domain that binds and regulates the antiapoptotic Bcl-2 proteins in order to promote apoptosis. The BH3 only proteins are located mainly in the cytoplasm, but after activation - mostly via posttranslational modifications - they are translocated to the mitochondria. This leads to activation of the pro-apoptotic proteins Bax and Bak, which are finally responsible for the permeabilization of the outer mitochondrial membrane. BH3 - only proteins thus sense signals that induce apoptosis, passing on

the information to the core Bcl-2 family members, which then promote or interfere with cell death (Youle and Strasser, 2008).

6.4 Detection of apoptosis by flow cytometry

Cells undergoing apoptosis display typical changes in their morphological and physical properties (cell shrinkage, rapid blebbing of the plasma membrane, condensation of chromatin and DNA fragmentation) which are the final results of a complex biochemical cascade of events (Compton *et al*., 1992). Because of these typical changes in apoptosis, they have been markers used to identify this kind of cell death biochemically, by microscopy or cytometry. A series of methods have been proposed for measuring apoptotic cell death through evaluation of light scattering such as flow cytometry and fluorescence microscopy. In particular, flow cytometry can be used to follow the percentage of apoptotic cells present in a cell population over the course of an experiment.

6.5 Measurement of cellular DNA content

Activation of endonuclease(s) preferentially cleaving DNA between the nucleosomes is another characteristic event of apoptosis. The products of DNA degradation are nucleosomal and oligonucleosomal DNA fragments (180 bp and multiplicity of 180 bp) which generate a characteristic "ladder" pattern during agarose gel electrophoresis. This DNA fragment feature provided the basis for development of a flow cytometry assay to identify apoptotic cells. In this approach, the cellular DNA content is measured after cell permeabilization with detergents or prefixation with precipitating fixatives such as ethanol, methanol, or acetone. The highly degraded DNA is not fully preserved within apoptotic cells after permeabilization or alcohol fixation; this fraction of DNA leaks out during subsequent cell rinsing and staining. As a consequence, apoptotic cells contain fractional DNA content and therefore following staining of cellular DNA, can be recognized as the cells with deficit in DNA content often defined as "sub-G_1" peak subpopulation. This means that

after staining with a quantitative DNA-binding dye, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak (Nunez, 2001).

6.6 Analysis of apoptosis by Annexin V- FITC / PI

The loss of transport function and the loss of the structural integrity of the plasma membrane are the main features used to distinguish dead from live cells. The assays for cell viability detection have been developed based on changes in properties of the plasma membrane. In normal cells, plasma membrane phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane. The external surface of the lipid bilayer composes of phosphatidylcholine and sphingomyelin while the inner surface also contains phosphatidylserine. During apoptosis, the loss of phospholipid asymmetry occurs, leading to exposure of phosphatidylserine on the outside of the plasma membrane. In general, though not always, this is an early event in apoptosis and is thought to be a signal to neighboring cells that a cell is ready to be phagocytosed. The anticoagulant annexin V is a binding protein that preferentially binds to negatively charged phospholipids such as phosphatidylserine. It can be used to detect apoptotic cells. By conjugating fluorescein (FITC) to annexin V, it has been possible to use such a marker to identify apoptotic cells by flow cytometry. For this staining method it is essential to add a dead cell discrimination dye like propidium iodide (PI) or 7-amino-actinomycin D to the stained cells, because late apoptotic or necrotic cells also externalize phosphatidylserine and can be distinguished from the early apoptotic cells by presenting both fluorescence signals. During apoptosis the cells become reactive with annexin V after the onset of chromatin condensation but prior to the loss of the plasma membrane ability to exclude PI (Vernes *et al.,* 1995). Therefore, by staining cells with a combination of annexin V - FITC and PI, it is possible to detect nonapoptotic live cells (annexin V negative/PI negative), early apoptotic cells (annexin V positive, PI negative) and late apoptotic or necrotic cells (annexin V positive, PI positive) by flow cytometry (Ormerod *et al.,* 1992).

7. Flow cytometry (FCM)

FCM began in the 1960's with the combination of emerging technologies: microscopy and electronics. The first commercialized flow cytometers were developed in the late 1960's by Van Dilla and Dittrich and G¨ohde (Shapiro and Leif, 2003). In 1976, Herzenberg reported on the first instrument to combine fluorescence flow cytometry with cell sorting, commercialized as the FACS (fluorescence-activated cell sorting) by Becton- Dickinson. In the decades to come, flow cytometry continued to adapt newer technologies and advancements; today, flow cytometers are capable of measuring up to 18 fluorescence signals with the aid of recently developed quantum dots (Chattopadhyay *et al.,* 2006).

Typical FCM machines are capable of measuring four to six fluorescence signals using two lasers. Cells, which have previously been stained with fluorescent conjugated antibodies (e.g., anti-CD5-PE for quantifying murine T cells or propidium iodide (PI) for quantifying DNA content or for cell cycle analysis) are introduced in the fluidics system of the flow cytometer. Hydrodynamic forces align the cells in tandem past the light sources (i.e., the two lasers) at rates of up to 70,000 cells per second. Upon contact with the laser beams, the cells will scatter some of the light and also absorb it and then re-emit it. The scattered light is measured by at least two detectors: the forward scatter (FSC) detector measures the amount of light diffracted in the forward direction and this is proportional to cell size. The side scatter (SSC) detector measures the amount of light reflected by the cells and it is proportional to cell granularity or internal complexity. Light being absorbed and then re-emitted at lower frequencies is detected by an additional set of photomultiplier tubes (PMT), each of which is configured to measure a particular range of wavelengths with the use of filters. The fluorochrome-antibody pairs are designed to bind to particular proteins on the cell surface. When light makes contact with a cell, the fluorochrome absorbs the light and then re-emits the light (fluoresces) at lower frequency. The amount of fluorescence detected is proportional to the amount of protein in the cell (Shapiro, 2004).

8. Analysis of FCM data by FlowJo software

Flow cytometry data is typically manually analyzed using commercial software such as FlowJo (Tree Star Inc., Ashland, Oregon). FlowJo is an interactive software with advanced data visualization capabilities. The data is loaded into the workspace and visualizes the data in 2-dimensional scatter plots. Cell populations are identified visually in the plots and cell populations are labelled and selected by drawing a boundary around them, which is referred as gating. This gate is then used to filter the population for subsequent analysis. When analyzing many files, the gates are usually determined for one file (usually a control) or taken from a template and then these gates are applied to the rest of the corresponding files. For the NCI-H460 cell analysis referred here, one gating templates was determined for PI staining. The first step of the analysis consisted of filtering out debris. Debris events are easily identified by their relative lower FSC and SSC values. Once cells have been gated, the next steps consist of classifying each cells with the intensity of DNA content. The identification of dead cells or dying cells is commonly accomplished with the aid of a viability marker, in this case PI. PI is a non-permanent dye that can penetrate the membrane of dying or dead cells, intercalating into DNA or RNA molecules (for this reason it can also be used for cell cycle analysis or quantifying DNA content) (Shapiro and Leif, 2003).

9. Compounds tested : chemistry

9.1 Xanthone derivatives

Xanthones or xanthen-9H-ones are secondary metabolites found in some higher plant families such as *Gentianaceae*, *Guttiferae*, *Moraceae* and *Polygalaceae*, fungi and lichens (Peres *et al.*, 2000; Vieira and Kijjoa, 2005), and they comprise an important class of oxygenated heterocycles. The xanthone nucleus is known as 9-xanthenone or dibenzo-γ-pyrone. Many natural compounds with xanthone scaffold as well as their synthetic derivatives have been investigated. Some of natural and synthetic xanthones have shown their broad spectrum of biological activities namely

antiallergic, anti-inflammatory, anti-tumor, antimycobacterial, cardiovascular and neuropharmacological effects (Gales and Damas, 2005; Pinto *et al*., 2005; Vieira and Kijjoa, 2005; Souza and Pinto, 2005; Suksamrarn *et al*., 2006). The chemical structure of xanthone nucleus is numbered according to a biosynthetic convention and IUPAC rules (international union of pure and applied chemistry). Carbons 1-4 are assigned to the acetate-derived ring A, and carbons 5-8 to the shikimate-derived ring B (Appendix Figure A1) (Peres *et al.,* 2000).

The xanthones can be divided into five different major groups: simple oxygenated xanthones, xanthone glycosides, prenylated and related xanthones, xanthonolignoids, and miscellaneous xanthones. The xanthone structure is a very interesting scaffold for different groups leading to a large variety of pharmacological activities which have been reported, especially inhibitors of growth of a variety of tumor cell lines. Several studies have been designed to examine the anticancer activities of xanthones isolated from mangosteen-fruit pericarp (Pedraza-Chaverri *et al*., 2008). Hepatocellular carcinoma (Ho *et al.,* 2002), SKBR3 human breast cancer (Moongkarndi *et al*., 2004) and human leukemia cell lines have been used. Ho *et al*. (2002) found that garcinone E has a potent cytotoxic effect on hepatocellular carcinoma cell lines.

The natural C-glucoside xanthone mangiferin has been reported in various parts of mango leaves, fruits, stem bark, heartwood and roots (Scartezzini and Speroni, 2000). Mangifirin, shows strong antioxidant, anti lipid peroxidation, immunomodulation, cardiotonic, hypotensive, wound healing, antidegenerative and antidiabetic activities (Shah *et al*., 2010). Mangiferin is a xanthone which is some of the most potent antioxidants known; it is thought to be more potent than both vitamin C or vitamin E and is sometimes unofficially referred to as “super antioxidants”. Another promising antileukemic agent is psorospermin, from *Psorospermun febrifugum* (Pinto *et al.,* 2005).

As xanthones from natural origin are relatively limited in type and position of the substituents imposed by the biosynthetic pathways, the syntheses of new

compounds leading to enlarge the possibilities of having different nature and positions of the substituents on the xanthonic nucleus will allow the researchers to rationalize and characterize the structure features that are important to their activity. Based on these considerations a synthesized series of mono-, di- and trioxygenated simple xanthones were investigated in Center of Medicinal Chemistry of the university of Porto, Portugal (CEQUIMED-UP) to find their potential anti tumor and immunomodulatory activity (Pedro *et al*., 2002). It is important to highlight that some synthetic xanthones reached clinical trials for antitumour agents. One of them, is DMXAA (5,6-dimethylxanthenon-4-acetic acid) acting by antivascular action. Another compound, belongs to thioxanthone family, is an aminoalkylderivative, named SR271425 (Lockhart *et al*., 2009) for activity in leukaemia and lymphoma. In summary, investigations of many naturally occurring xanthones as well as their synthetic derivatives, suggest that this family of compounds would be interesting as a source of candidates for cancer therapy (Saraiva *et al*., 2003; Raquel *et al*., 2009; Palmeira *et al*., 2010). In the present study, xanthone derivatives (TX, XC1, XC3, XC5, 1,2P1, 3,6CIAC, 36P1, XEA-S, XEL-L, XEL-D, XEVOL-L, XEVOL-D, XEGOL-1L, XEGOL-2D, XEGOL-2L) indicated in Appendix Table A1 were screened for cytotoxicity against three human tumor cell lines which are non small cell lung cancer, breast cancer and melanoma.

9.2 Flavonoids derivatives as potent anti-cancer agents

Flavonoids are polyphenolic compounds which are fundamental components of the human diet present as constituents of flowering plants, particularly of food plants. They are phenyl substituted chromones (benzopyran derivatives) consisting of a 15-carbon basic skeleton (C6-C3-C6) (Appendix Figure A2) composed of a chroman (C6-C3) nucleus (the benzo ring A and the heterocyclic ring C), also shared by the tocopherols, with a phenyl (the aromatic ring B) substitution usually at the 2-position (Kandaswami *et al*., 2005). Several plants and spices containing flavonoid derivatives have found application as disease preventive and therapeutic agents in traditional medicine in Asia for thousands of years. Epidemiological investigations and human clinical trials indicate that flavonoids have

important effects on cancer chemoprevention and chemotherapy. Many mechanisms of action such as antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, reduction of oxidative damage and reversal of multidrug resistance or a combination of these mechanisms have been identified (Pedro *et al*., 2006, Rudolf *et al*., 2007; Hatti *et al*., 2009). Most of the biological activities are associated with the presence of hydroxyl and prenyl groups in the flavonoid ring system. According to the carbon structure and the substituents, this family of compounds can be subdivided in flavanols, anthocyanidins, flavones, flavanones, and chalcones (Kale *et al*., 2008). In this study, several derivative of flavonoids were studied , according Appendix table A1 (FP13, FP4, G3, G5).

9.3 Sulfated small molecules

Another group of sulfated compounds, namely derivatives of xanthones, flavonoids, as well as of gallic acid and salicin were tested. These compounds are interesting considering the high polarity and the aqueous solubility associated. Some of these compounds are also being studied at CEQUIMED as anticoagulants and antifungal agents. For this study the following substances are listed at Appendix Table A1: ERS, US, SS, DS, MS, SBS and AGS.

9.4 Terpenes

Terpenes (isoprenoids) are small molecules that consist of repeating units of a molecular moiety called isoprene. Isoprene or 2-methyl-1,3-butadiene, is a common organic compound with the formula $CH_2=C(CH_3)CH=CH_2$. Terpenes play many important roles in the plant kingdom from deterring insect predation, protection from environmental stresses and as chemical raw materials for more complex molecules, like cannabinoids. Many plant terpenes act synergistically with other terpenes and some serve to either catalyze or inhibit formation of other compounds within a plant. They are not only the largest group of plant natural products, comprising at least 30,000 compounds (Connolly and Hill, 1991), but also contain the widest assortment of structural types. Hundreds of different monoterpene (C10)

(Dewick, 1999), sesquiterpene (C15) (Fraga, 2006), diterpene (C20) (Hanson, 2000) and triterpene (C30) (Connolly and Hill, 2005) carbon skeletons are known. Natural products chemists have long marveled at the structural diversity of terpenes and speculated on its biosynthetic basis. Terpenes are derived biosynthetically from units of isoprene, which has the molecular formula C_5H_8. The basic molecular formulae of terpenes are multiples of that, $(C_5H_8)_n$ where n is the number of linked isoprene units. This is called the *isoprene rule* or the *C5 rule*.Some of terpene such as monoterpenes are isolated from the essential oils of plants elicit multiple chemotherapeutic and chemopreventive effects in diverse models of cancer (Gould *et al*., 1997; Degenhardt *et al*., 2009), preventing the carcinogenic process at both the initiation and promotion/progression stages. In addition, monoterpenes are effective in treating early and advanced cancers. Monoterpenes such as limonene and perillyl alcohol have been shown to avoid mammary, liver, lung, and other cancers. These compounds have also been used to treat a variety of rodent cancers, including breast and pancreatic carcinomas.Anticancer activities associated with regressing mammary tumors include the induction of cytostasis and apoptosis upon monoterpene application. This antitumor activity correlates with the differential expression of growth and apoptotic genes necessary for tumor proliferation (Ariazi *et al*., 1999). According Appendix Table A1 the following substances belonging to terpenes group were tested, Limonoid gedunin, limonoid cedrelone, limonoid 6α-acetoxy and triterpene odoratol.

MATERIALS AND METHODS

1. Chemicals

RPMI-1640, fetal bovine serum (FBS), l-glutamine and trypsin were supplied from Gibco Invitrogen Co. (Scotland, UK). Trypan blue, dimethyl sulfoxide (DMSO) penicillin, propidium iodide (PI), RNase, streptomycin, sulforhodamine B (SRB), Triton X-100 and trypan blue were from Sigma–Aldrich Co. (Saint Louis, USA). Sodium dodecyl sulfate (SDS), Trichloroacetic acid (TCA), methanol, Tris-base and RNase were sourced from Merck (Darmstadt, Germany).

2. Compounds

Natural and synthetic compounds as shown in Appendix Table A1 were used in this study. All compounds were generously supplied by Centro de Quimica Medicinal Universidade de Porto (CEQUIMED-UP). Stock solutions of compounds were prepared in DMSO and kept at -20 ^{o}C. Appropiate dilutions of compounds were freshly prepared just prior to each assay.

3. Cell culture

The following human cell lines were used: MCF-7 (breast carcinoma), A375 C5 (melanoma) and NCI-H460 (non small cell lung cancer). MCF-7 and A375-C5 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) while NCI-H460 cell line was provided by the National Cancer Institute (NCI, Bethesda, USA). Cells, growing in monolayer, were routinely maintained in RPMI-1640 medium supplemented with 5% heat-inactivated FBS and 2 mM glutamine at 37°C, in a humidified atmosphere containing 5% CO_2. All experiments were carried out in cells with more than 90% viability as assessed with trypan blue exclusion assay. For this assay, cells were stained with 0.2% trypan blue and viable and non-viable cells were counted in a hemocytometer. Cells were *Mycoplasma* free which confirmed by DAPI staining.

4. Tumour cell growth assay

The effect of the synthetic compounds on the growth of human tumour cell lines was evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) in the "In vitro Anticancer Drug Discovery Screen" that uses the protein-binding dye sulforhodamine B to assess cell growth (Skehan *et al*., 1990). Exponentially growing cells were obtained by plating 5,000 cells/well for MCF-7, 7,500 cells/well for A375-C5 and 5,000 cells/well for NCI-H460, followed by 24 h incubation. Cells were then treated with five serial concentrations of synthetic compounds: 150 µM, 75 µM, 37.5 µM, 18.75 µM and 9.38 µM.

Following this treatment period, adherent cells were fixed *in situ* with 50% TCA, washed with distilled water and stained with 0.4% SRB (Sigma–Aldrich Co., Saint Louis, USA). The bound stain was washed with 1% acetic acid (Sigma), solubilized in 10 mM Tris (pH 10.5) and the absorbance was measured at 492 nm in a microplate reader (Bio-tek Instruments Inc.,Powerwave XS, Wincoski, USA). For each cell line a dose-response curve was plot between compound concentration and % cell growth inhibition which was calculated as described by Monks *el al.* (1991). The concentration of compounds that inhibited 50% of the net cell growth (GI_{50}) was read from a dose- response curve.

$$\% \text{ control cell growth} = \frac{\text{mean ODsample at 48h - mean ODsample at day0}}{\text{mean ODneg control at 48h - mean ODsample at day0}}$$

$$\% \text{ cell growth inhibition} = 100 - \% \text{ control cell growth}$$

5. Trypan blue exclusion assay

The compounds with GI_{50} below 12 µM were selected for further analysis. The assay was carried out in NCI-H460 cells, since they were one of the most sensitive cells to these compounds. The tumour cells were plated into 6-well plates (1.5×10^5 cells/well) and maintained for 24 h. Exponential tumour cells growing were treated with complete medium (control), the compound solvent (DMSO) or with the selected compounds at their respective GI_{50} concentration for 48 h. After treatment,

attached cells were released by trypsinization and mixed with non-adherent cells. Cell suspension was diluted 1:1 with trypan blue and pipetted up and down several times to ensure a uniform suspension. Viable cells were counted using hemacytometer.

6. Cell cycle analysis

NCI-H460 cells were plated at 1.5 x 10^5 cells/well in 6-well plates, left to adhere for 24h and then treated with the GI_{50} concentration of selected compounds for 24h and 48h. After treatment, attached cells were released by trypsinization and mixed with non-adherent cells. An aliquot was used for cell counting and determining viability by the Trypan Blue exclusion assay. The remaining cells were centrifuged at 228 g for 5 min, washed twice with PBS, fixed with 70% ice-cold ethanol and kept at 4 ^{o}C from overnight to a maximum of 1 week . Fixed cells were then centrifuged at 2,800 g for 10 min at 4 ºC and resuspended in the DNA staining solution containing 5 µg/ml propidium iodide and 0.1 mg/ml RNase in PBS. DNA cellular content was analyzed with a Coulter Epic XL-MCL flow cytometer (Beckman-Coulter-Electronics, Hialeah, FL) with a 15 mW air cooled argon laser turned to single line emission at 488 nm.

Data was acquired in a listmode data file, gated to 50,000 events in cell cycle, using the XL SYSTEM II™ software (Beckman-Coulter) included in the system. Cell cycle was analysed using the Flowjo software program (Flowcytometry analysis software, Tree Star, Inc.) with an activated gate to eliminate aggregates (FL2-A/FL2-W). At least three independent experiments were carried out and results are presented as the average and standard error (SE) of these experiments.

7. Analysis of apoptosis by Annexin V – FITC / PI

NCI-H460 cells were cultured and determined their viability as described before. The remaining cells were centrifuged at 228 g for 5 min, washed in PBS, and resuspended with 3 ml of fresh growth medium. Cell suspensions were incubated for 30 min to allow cells to recover from the incubation with trypsin (hence reducing the

number of false positives) and apoptosis assayed by the Human Annexin V-FITC Apoptosis Kit (Bender MedSystems, Vienna, Austria). Briefly, cells were centrifuged at 228 g for 5 min and resuspended in 400 µl Annexin buffer. Then, 195 µl of cell suspension were taken and mixed with 5 µl of annexin V-FTIC, incubated at room temperature for 10 min, 10 µl of propidium iodide (20 µg/ml) added, incubated for 1-2 min and placed on ice until analysis by flow cytometry. In addition, the remaining cells in Annexin buffer were kept on ice for checking if compounds had auto-fluorescence.

8. Screening for *Mycoplasma*

All cell lines were screened for *Mycoplasma* using DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) staining. The fluorescent DAPI dye (Sigma, D-8417, Deisenhofen, Germany) was dissolved in water to make the 1 mg/ml stock. The working solution was freshly prepared by diluting the stock DAPI into 1 µg/ml with deionized water. Cells were transferred to slides and were fixed in methanol for 30 min at -20 ^{o}C. The slide was rinsed once with the working solution, incubated with the working solution at 37 ^{o}C for 15 min, then rinsed once with methanol. The slides were mounted with glycerol and examined under a fluorescence microscope with 340/380 nm excitation filter and LP 430 nm barrier filter (Olympus, BX50, Japan).

RESULTS AND DISCUSSION

Results

1. Tumour cell growth assay

The i*n vitro* growth inhibitory effect of fifty natural and synthetic compounds was evaluated in three human tumour cell lines (A375-C5, NCI-H460 and MCF-7). The growth inhibitory 50 concentration (GI_{50}) of forty-six synthetic compounds against the three cell lines are shown in Table 1. Fifteen compounds presented moderate GI_{50} between 13-50 μM and eleven compounds presented GI_{50} values between 51-150 μM. Eighteen of fifty compounds presented GI_{50} values above 150 μM in all cell lines. However, two compounds show GI_{50} below 150 μM for A375 and NCI-H460 but above 150 μM for MCF-7. In addition, the GI_{50} concentration of four natural compounds (limonoid gedunin, limonoid cedrelone, limonoid 6α-acetoxy-14β,15β-epoxyazadirone and tirucallane triterpene odoratol) was previously determined by a collaborator of CEQUIMED-UP, using the same protocol, are shown in Table 2. All of these natural compounds showed strong cell growth inhibitory activity, presenting their GI_{50} values below 12 μM. Results presented in Table 1 and 2 are the Mean ± SD of at least 3 independent experiments. The chemical structures of compounds are shown in the Appendix Table A1 and the example GI_{50} graphs of synthetic compounds are shown in the Appendix Figure A3-A62. The compounds that showed GI_{50} below 12 μM were selected for further analysis of their effect on cell cycle profile, apoptosis and trypan blue exclusion assay.

Table 1 GI_{50} values of forty six synthetic compounds in A375-C5, MCF-7 and NCI-H460 cells.

Compounds	A375-C5	MCF-7	NCI-H460
FP13	27.67±2.52	23±5.57	24±4.36
FP4	27±3.46	18.33±2.52	16.67±4.73
G3	55±1.0	55±8.89	50±1.7
TX127	>150	>150	>150
TXOH	15.33±1.53	23.33±2.52	21±2.65
TX104	>150	>150	>150
TXOME	>150	>150	>150
TXTREO	73±9.52	111±20.3	73±11.46
TX53	60.33±3.21	92.67±4.51	89.67±4.16
TXISO	27.33±9.24	48±5.29	38.67±7.64
TX62	>150	>150	>150
XEA-S	116.67±20.82	>150	78±10.44
XEA-L	69.33±9.29	78.67±7.37	63.33±11.02
XEA-D	63.67±4.73	72.33±7.09	66.33±5.03
XEVOL-L	>150	>150	>150
XEVOL-D	88±7.00	>150	123.33±11.5
XEGOL-1L	37.33±3.06	52.67±4.04	42±0
XEGOL-2D	42±2.65	53±2	40.33±3.79
XEGOL-2L	36.67±5.03	54±7.55	42.67±3.06
TXA3	>150	>150	>150
TXA4	>150	>150	>150

Table 1 (Continued)

Compounds	A375-C5	MCF-7	NCI-H460
AGS	>150	>150	>150
DS	>150	>150	>150
SS	>150	>150	>150
MS	>150	>150	>150
SBS	>150	>150	>150
US	>150	>150	>150
ERS	>150	>150	>150
TXOH1H	34.0±11.13	39.0±6.2	28.7±3.8
TX15	33.0±4.6	33.0±4.0	27.3±4.7
TX34	13.3±0.6	15.0±1.7	13.3±1.2
TX48	20±3.6	28.3±1.2	22.7±1.5
TX79	>150	>150	>150
TX86	54.8±16.3	70.8±12.0	61.3±7.1
TX87	>150	>150	>150
TX96	34.0±7.0	34.3±5.1	35.0±9.0
TX105	81.7±4.9	85.0±1.7	58.3±12.4
TX128	55.0±2.6	65.7±2.5	59.3±11.2
TX14L	24.3±5.7	34.0±5.3	39.3±11.2
TX10Me4M	>150	>150	>150
XC1	>150	>150	>150
XC3	>150	>150	>150

Table 1 (Continued)

Compounds	A375-C5	MCF-7	NCI-H460
XC5	102.3±6.8	106.7±5.8	126.7±11.5
3,6P1	25.0±3.6	25.3±2.3	25.0±1.7
1,2P1	71.7±4.6	66.0±2.6	62.3±4.5
3,6 ClAC	>150	>150	>150

Table 2 GI_{50} values of four natural compounds in A375-C5, MCF-7 and NCI-H460 cells.

Compounds	A375-C5	MCF-7	NCI-H460
Limonoid 6α-acetoxy-14β,15β-epoxyazadirone	9.27±0.37	11.67±1.20	10.63±0.68
Limonoid cedrelone	2.60±0.21	4.20±0.73	2.77±0.07
Limonoid gedunin	8.80±0.92	9.06±0.43	8.36±0.84
Tirucallane triterpene odoratol	11.67±0.67	10.50±0.50	11.67±1.20

2. Trypan blue exclusion assay

Limonoid gedunin, limonoid cedrelone, limonoid 6α-acetoxy and triterpene odoratol were further studied of their effect on NCI-H460 cells viability using trypan blue exclusion assay. The proportion of viable cells was determined with viability of DMSO control cells set at 100% (Table 3). In this assay, the GI_{50} concentration of each compound was used. The results demonstrated that the number of NCI-H460 cells was strongly reduced close to 50% after treatment the cells with their GI_{50} of each compound. The most decrease was found in limonoid cedrelone compared with the DMSO control cells (33 % of cell number in compound treated as versus 100 % of DMSO control cells) (Figure 1). Limonoid gedunin, tirucallane triterpene odoratol and limonoid 6α-acetoxy-14β,15β-epoxyazadirone also caused inhibition to NCI-H460 cells, ie. the percentage of cell number following treatment with each compound was 43.50, 50.70 and 61.24, respectively.

Table 3 Mean and percentage of cell viability from the trypan blue exclusion assay of NCI-H460 treated with four selected compounds.

Compounds	Cell viability	% of viable cells
DMSO	$2.09x10^6 \pm 3.85$	100
Limonoid 6α-acetoxy-14β,15β-epoxyazadirone	$1.28x10^6 \pm 3.09$	61.24
Limonoid cedrelone	$6.90x10^5 \pm 0.51$	33.00
Limonoid gedunin	$9.09x10^5 \pm 1.68$	43.50
Tirucallane triterpene odoratol	$1.06x10^6 \pm 3.21$	50.70

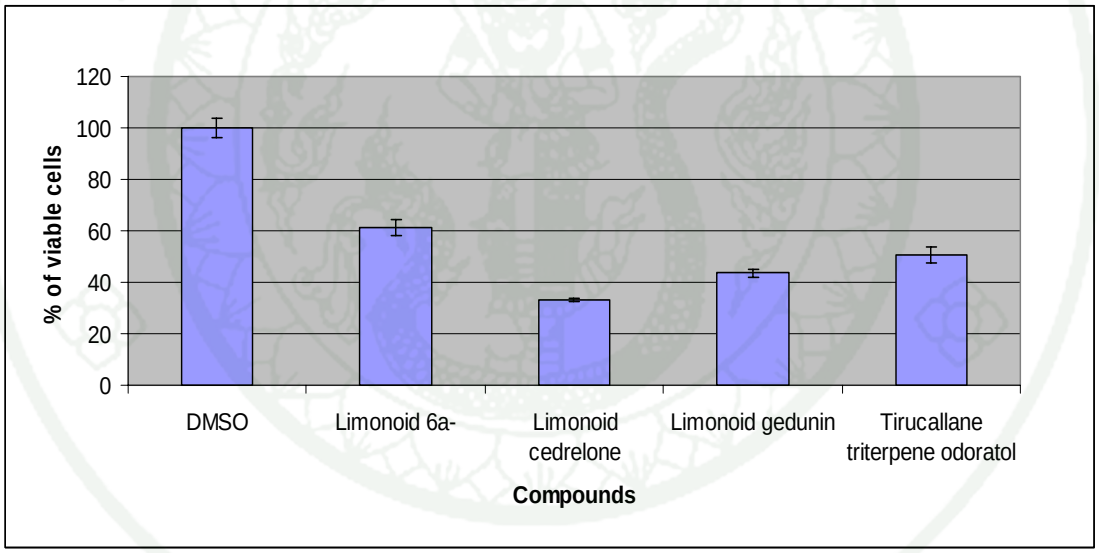


Figure 1 Control of the cellular effect of the GI_{50} concentration of the four selected compounds. Effect of four selected compounds on NCI-H460 viable cell number versus viable cell number of untreated cells. Each point represents the mean ± standard deviation of three independent experiments.

3. Evaluation of the effect of selected compounds in the cell cycle distribution of NCI-H460 cell line after 48 h treatment

The inhibitory growth results clearly demonstrated that all four compounds, limonoid 6α-acetoxy-14β,15β-epoxyazadirone, limonoid cedrelone, limonoid gedunin and tirucallane triterpene odoratol had profound effect on NCI-H460 cell growth. In order to elucidate the mechanism underlying the growth inhibitory activity, the compounds were assayed for their effects on the cell cycle progression, therefore the untreated and compound - treated NCI-H460 cells were subjected to flow cytometry analysis.

3.1 Effect of DMSO on cell cycle of NCI-H460 cells

In order to rule out the possibility that DMSO (which was used as the solvent to dissolve the compounds) was not involved in the cell cycle profile alteration, it was used as control. Statistical comparisons were performed using Student's t-test to determine the significance of the effects. Flow cytometry analysis of the untreated cells, indicated 48.73, 28.29 and 22.75 % of cells were distributed among G0/G1, S and G2/M phase, respectively (Figure 2A). After exposure of NCI-H460 to DMSO, cell cycle profile was not different from the profile of the untreated cells (Table 4). The percentage of cell distribution after treating the cells to DMSO were 48.26, 29.07 and 20.77 % in G0/G1, S and G2/M phase, respectively (Figure 2B).

Table 4 Cell cycle distribution after treating NCI-H460 with DMSO for 48 h.

Compounds	G1	S	G2/M	SubG1
Control	48.73±0.012	28.29±0.01	22.75±2.09	0
DMSO	48.26±1.47	29.07±0.71	20.77±0.37	0
t-test (p-value)	0.694	0.745	0.045	0.076

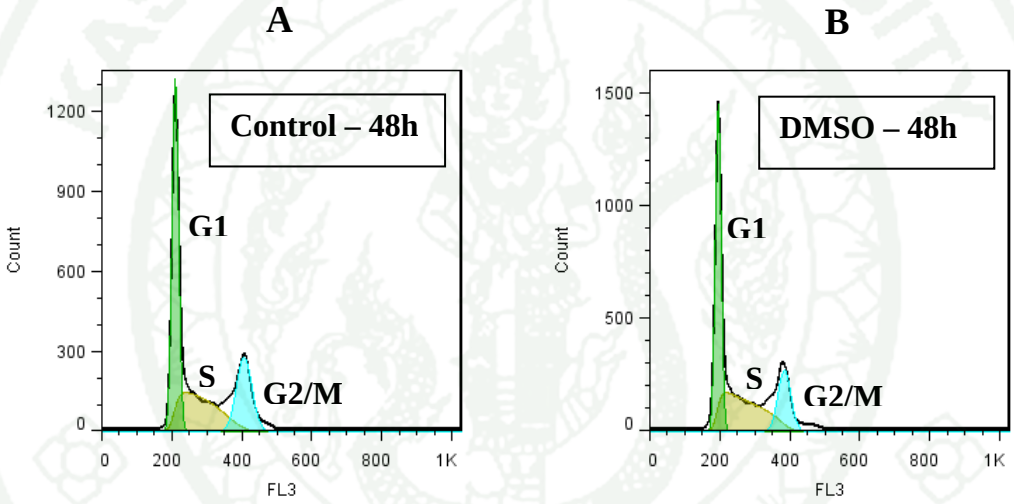


Figure 2 Effect of DMSO on cell cycle distribution of NCI-H460 cells. Cells were incubated with growth media alone (control) [**A**], DMSO (solvent control) [**B**] for 48 h. The cell cycle distributions were evaluated by PI staining and flow cytometric analysis.

3.2 Effect of limonoid 6α-acetoxy-14β,15β-epoxyazadirone on cell cycle of NCI-H460 cells

Treating NCI-H460 cells with limonoid 6α-acetoxy-14β,15β-epoxyazadirone resulted in the decrease of viable cells as shown in Figure 1. The results showed that after exposure to limonoid 6α-acetoxy-14β,15β-epoxyazadirone, cell distribution became 48.5, 25.31 and 23.38% in G0/G1, S and G2/M phase, respectively. The differences between untreated and limonoid 6α-acetoxy-14β,15β-epoxyazadirone treated cells were not significant with p-value of 0.733 (G1), 0.281 (S) and 0.560 (G2/M) (Figure 3, Table 5).

3.3 Effect of limonoid cedrelone on cell cycle of NCI-H460 cells

To examine whether the block in cell growth caused by limonoid cedrelone was due to an arrest in a specific stage of the cell cycle, the cell cycle profile after 48 h of limonoid cedrelone treatment was analyzed and is shown in Figure 4B. An S and G2/M phase of NCI-H460 cell arrest which induced by limonoid cedrelone was observed. After treating of cells with this compound, the percent cell distribution was increased in S phase to 39.58 %, G2/M to 43.47 % compared to 28.9 % and 22.75 % in the control cells, respectively. The statistical analysis demonstrated the significant increase of G2/M phase with p-value < 0.05 (Table 6). In contrast, there was no significant increase in the S phase. The increase of G2/M and S phase was accompanied by the decrease of G1 phase of 20.47 compared to 48.73 in the control cells. In addition, an increase of subG1 of about 10.7 % was observed.

Table 5 Cell cycle distribution after treating NCI-H460 with limonoid 6α-acetoxy-14β,15β-epoxyazadirone for 48 h.

Compounds	G1	S	G2/M	SubG1
Control	48.73±0.012	28.29±0.01	22.75±2.09	0
Limonoid 6α–	48.53±3.01	25.31±1.41	23.38±1.77	0
t-test (p-value)	0.733	0.281	0.560	0.402

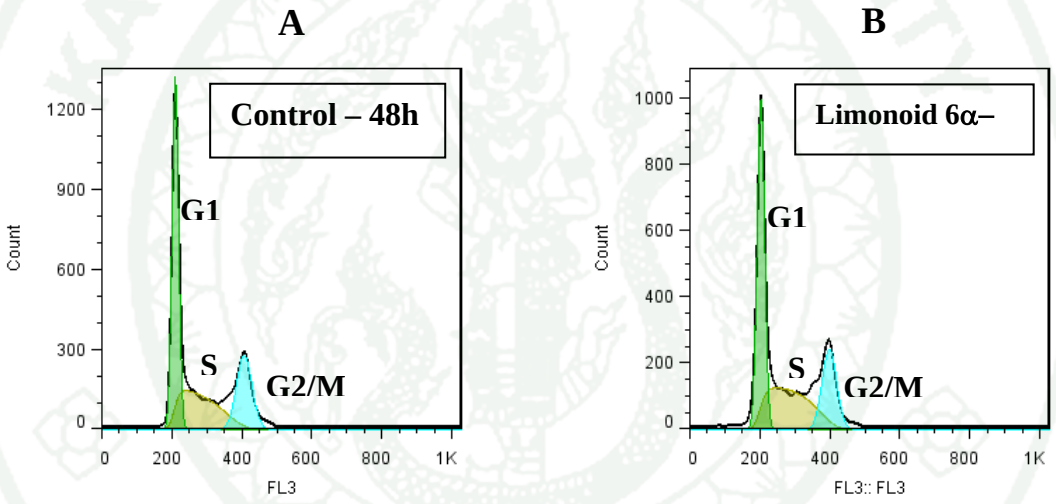


Figure 3 Effect of the limonoid 6α-acetoxy-14β,15β-epoxyazadirone on cell cycle distribution of NCI-H460 cells. Cells were treated with growth media alone (control) [**A**], of limonoid 6α-acetoxy-14β,15β-epoxyazadirone [**B**] for 48 h. The cell cycle distributions were evaluated by PI staining and flow cytometric analysis.

Table 6 Cell cycle distribution after treating NCI-H460 with limonoid cedrelone for 48 h.

Compounds	G1	S	G2/M	SubG1
Control	48.73±0.012	28.29±0.01	22.75±2.09	0
Limonoid cedrelone	20.47±1.97	39.58±2.78	43.47±1.43	10.71±1.76
t-test (p-value)	0.012	0.154	0.000	0.024

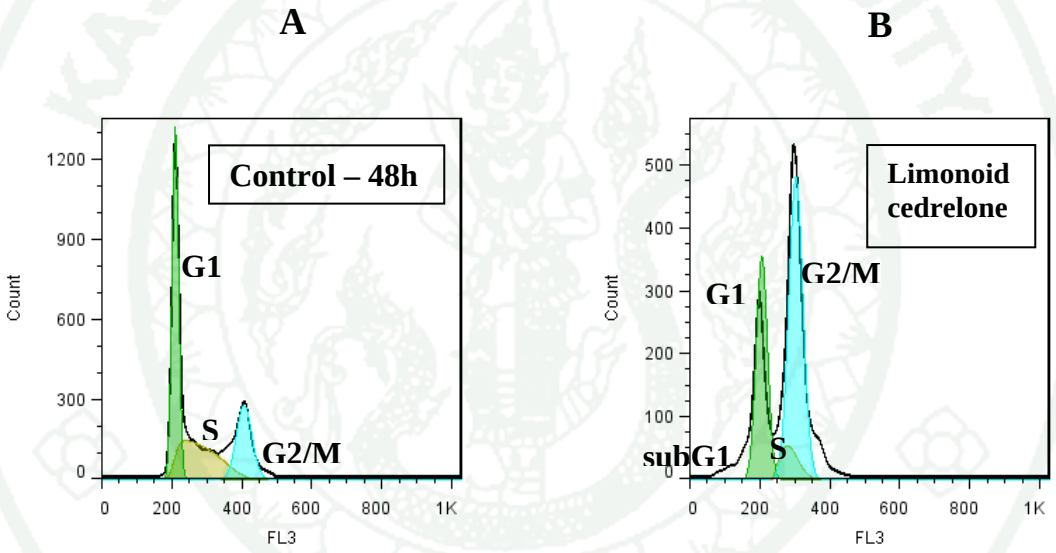


Figure 4 Effect of the limonoid cedrelone on cell cycle distribution of NCI-H460 cells. Cells were treated with growth media alone (control) [**A**], limonoid cedrelone [**B**] for 48 h. The cell cycle distributions were evaluated by PI staining and flow cytometric analysis.

3.4 Effect of limonoid gedunin on cell cycle of NCI-H460 cells

Analysis of cell distribution by flow cytometry after 48h treatment with limonoid gedunin demonstrated the accumulation of NCI-H460 cells in S phase. The percentage increase in S phase from 28.29 % (untreated cells) to 59.92 % (after 48 h exposure) was observed. The increase of S phase was accompanied by the loss of cells in G1 phase (from 48.73 % in control to 19.55 %). The statistical analysis of cell distribution demonstrated the significant increase of S phase with p-value 0.003 and significant decrease of cells in G1 phase with p-value of 0.002. In addition, the increase of subG1 about 7.95 % was observed . All of the results are shown in Figure 5 and Table 7.

3.5 Effect of tirucallane triterpene odoratol on cell cycle of NCI-H460 cells

As shown in Figure 6 and Table 8, concomitant with growth inhibitory effect, tirucallane triterpene odoratol induced a cell cycle arrest in G1 phase of cell cycle, with a more pronounced effect being in the presence of the other compounds used in this study . After treatment of NCI-H460 with tirucallane triterpene odoratol, the percent cell cycle distribution increased in G1 phase to 70.57 % compared to 48.73 % in the control cells. In addition, the increase of subG1 about 9.2 % was observed.

Table 7 Cell cycle distribution after treating NCI-H460 with limonoid gedunin for 48 h.

Compounds	G1	S	G2/M	SubG1
Control	48.73±0.012	28.29±0.01	22.75±2.09	0
Limonoid gedunin	19.55±1.26	59.92±0.30	18.20±0.46	7.95±0.83
t-test (p-value)	0.002	0.003	0.227	0.240

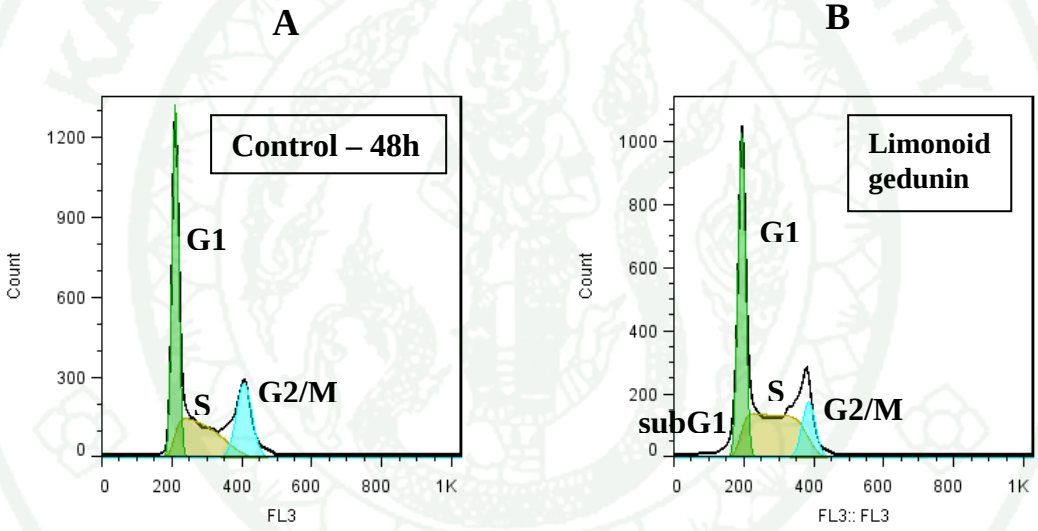


Figure 5 Effect of the limonoid gedunin on cell cycle distribution of NCI-H460 cells. Cells were treated with growth media alone (control) [**A**], limonoid gedunin [**B**] for 48 h. The cell cycle distributions were evaluated by PI staining and flow cytometric analysis

Table 8 Cell cycle distribution after treating NCI-H460 with tirucallane triterpene odoratol for 48 h.

Compounds	G1	S	G2/M	SubG1
Control	48.73±0.012	28.29±0.01	22.75±2.09	0
Triterpene odoratol	70.57±3.16	13.34±2.75	10.89±1.31	9.2±72.69
t-test (p-value)	0.009	0.024	0.022	0.040

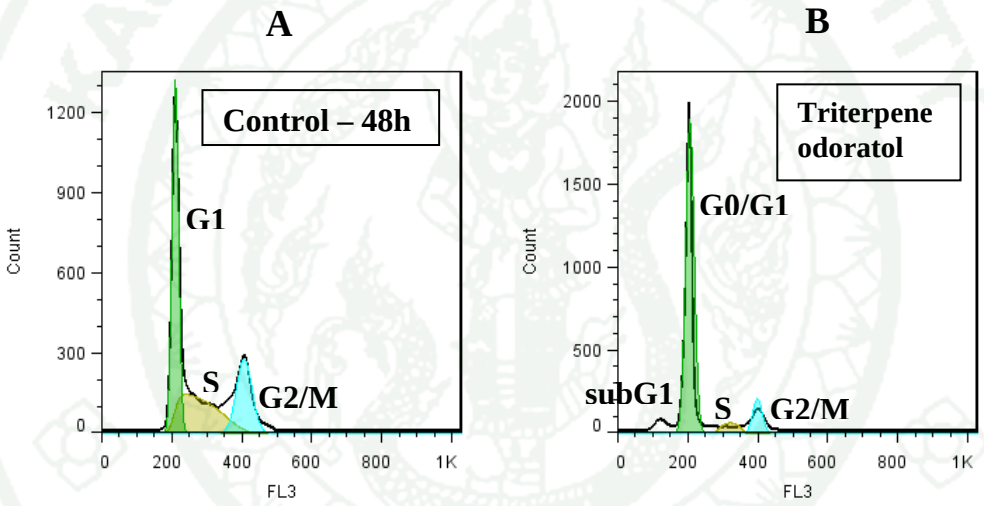


Figure 6 Effect of the tirucallane triterpene odoratol on cell cycle distribution of NCI-H460 cells. Cells were treated with growth media alone (control) [**A**], tirucallane triterpene odoratol [**B**] for 48 h. The cell cycle distributions were evaluated by PI staining and flow cytometric analysis.

4. Evaluation of the effect of selected compounds in cell cycle distribution of NCI-H460 cell line after 24 h treatment

Tirucallane triterpene odoratol was the compound that more drastically altered the normal cell cycle distribution of this cell line. It was therefore selected for additional experiments performed after 24 h of incubation with the compounds. After treating of NCI-H460 with tirucallane triterpene odoratol, the percent cell distribution was increased in G1 phase to 72.64 % compared to 40.15 % in the control cells. In addition, the increase of subG1 about 6.19 % was observed. All of the results are shown in Table 9.

Table 9 Cell cycle distribution of NCI-H460 cells following 24 h treatment with growth media alone (control), DMSO or with tirucallane triterpene odoratol. Results are the Mean ± SE of three independent experiments.

Phase	Control	DMSO	Tirucallane triterpene odoratol
G1	40.15±1.71	40.71±2.36	72.64±1.53
S	35.56±3.72	37.42±2.08	11.61±1.83
G2/M	22.27±4.98	21.87±4.98	8.54±1.35
SubG1	0.283±0.52	0.45±1.00	6.19±1.56

5. Induction of apoptosis by anticancer compounds and apoptosis assessment by annexin-V-FITC/PI staining

Table 10 showed the results of early and late apoptosis after NCI-H460 cells were stained with annexin V-FITC and PI. Figure 7 showed the example of FACS histogram with dual parameters including annexin V-FITC and PI. In the untreated NCI-H460 cells, 5.2 ± 0.9 % of cells were annexin V-positive/PI-negative (early apoptosis), 7.3 ± 2.0% of cells were both annexin V- and PI-positive (late apoptosis). After the cells were treated with limonoid 6α-acetoxy-14β,15β-epoxyazadirone, 7.3 ± 0.7% were annexin V-positive/PI negative and 7.9 ± 2.9 % of cells were double-positive (Figure 7C). Comparing the results to the control, there was no significant increase of apoptosis with p-value 0.423 and this apoptosis results correlated to the result of cell cycle being treated with this compound.

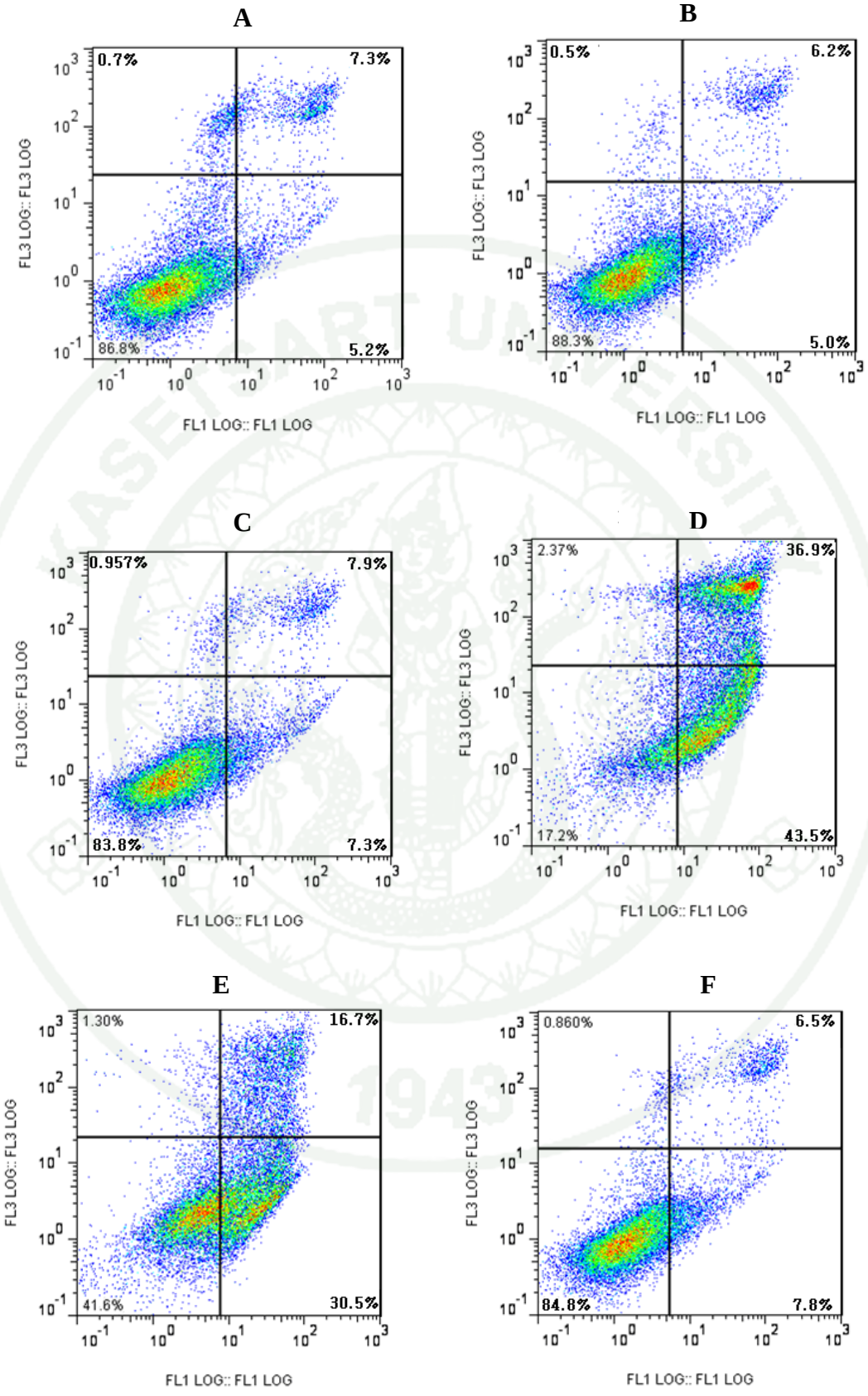
The highest increase of early and late apoptosis in NCI-H460 was found after being treated with limonoid cedrelone. The annexinV positive/PI-negative and double-positive cells were increased to 43.5 ± 6.2 % and 36.9 ± 8.6 %, respectively, with the p-value of 0.0001 (Figure 7D). In limonoid gedunin treated NCI-H460 cells, the annexinV positive/PI-negative and double-positive cells were increased to 30.5 ± 3.2 % and 16.7 ± 4.2 %, respectively (Figure 7E). In addition, the annexinV positive/PI negative were increased to 7.8 ± 1.2 % and the double-positive cells were increased to 6.5 ± 1.9 % after treating NCI-H460 cells with tirucallane triterpene odoratol. A significant increase was found when compared to the results of the control cells having the p-value of 0.005.

The maximum apoptosis was observed after NCI-H460 cells were treated with limonoid cedrelone which showed 80 % of cell death.

Table 10 Induction of apoptosis in NCI-H460 cells following 48 h treatment with the GI_{50} of different compounds, assayed by Annexin V-FITC/PI staining. Results are the average of at least 3 independent experiments ± SD.

Compounds	% Cells in early apoptosis (AnexxinV+/PI-)	% Cells in late apoptosis (AnexxinV+/PI+)	Total % of cells undergoing apoptosis	p-value (compare to control)
Control	5.2 ± 0.9	7.3 ± 2.0	12.6 ± 2.6	
DMSO	5.0 ± 1.3	6.2 ± 1.8	11.2 ± 1.2	0.589
Limonoid 6α-acetoxy	7.3 ± 0.7	7.9 ± 2.9	15.2 ± 2.6	0.423
Limonoid cedrelone	43.5 ± 6.2	36.9 ± 8.6	80.3 ± 4.5	0.000
Limonoid gedunin	30.5 ± 3.2	16.7 ± 4.2	47.2 ± 3.7	0.003
Tirucallane triterpene odoratol	7.8 ± 1.2	6.5 ± 1.9	14.3 ± 2.8	0.005

Figure 7 Flow cytometric analysis of phosphatidylserine externalization (annexin V binding) and cell membrane integrity (PI staining) of NCI-H460 control cell [**A**] DMSO [**B**] cells were treated with limonoid 6α-acetoxy-14β,15β-epoxyazadirone [**C**] limonoid cedrelone [**D**] limonoid gedunin [**E**] and tirucallane triterpene odoratol [**F**] for 48 h. The dual parametric dot plots combining annexin V-FITC and PI fluorescence show the viable cell population in the lower left quadrant (annexinV–/PI–), the early apoptotic cells in the lower right quadrant (annexin V+/ PI–), and the late apoptotic cells in the upper right quadrant (annexin V+/PI+). Results are representative of three independent experiments.



Discussion

Nature is an important source of new biological molecules for drug discovery. Modification of natural molecules using chemical synthesis may improve the activity of them and provide a chance to get more effective molecules for drug development. It is known that source of chemotherapeutic agents for cancer treatment, is from nature. The chemotherapeutic agents including etoposide, camptothecin, vincristine, paclitaxel (Taxol), 5-fluorouracil and doxorubicin have been observed to kill cancer cells by inducing apoptosis (Kaufmann and Earnshaw, 2000; Johnstone *et al.*, 2002). Among them, the chemoterapic agents that alter the cell cycle have been the most interest, since cell cycle regulation is the basic mechanism underlying cell development such as proliferation, differentiation or acquire death (Dobashi *et al.*, 2003).

The central and novel finding in the present study is the identification of *in vitro* anti-cancer efficacy of compounds against human non small lung cancer (NCI-H460), breast carcinoma (MCF-7) and melanoma (A375-C5). The natural derivative compounds from terpene xanthone and flovonoids showed the capacity to inhibit the growth of all three human tomour cell lines by SRB assay. Four terpenes which were limoniod cedrelone, limonoid gedunin, limonoid 6α-acetoxy-14β,15β-epoxyazadirone and tirucallane triterpene odoratol, showed strong cell growth inhibitory activity, presenting their GI_{50} values below 12 μM for all three cell lines. Interestingly, the limonoid cedrelone was found to be not only the most active growth inhibitor but also slightly more selective for NCI-H460 (GI50 = 2.77 ± 0.07 μM) and A375-C5 (2.60 ± 0.21 μM) cell lines than on MCF-7 (4.20 ± 0.73). Although the capacity of limonoid cedrelone, to inhibit the growth of human cells have not been previously reported, this compound is known to demonstrate several other activities including insecticide (Koul *et al.*, 1992), antifeedant (Gopalakrishanan *et al.*, 2008), and antifungal activity (Govindachari *et al.*, 2000). Although its growth inhibitory effect was *ca* two folds less than that of limonoid cedrelone, the structurally related limonoid 6α-acetoxy-14β,15β-epoxyazadirone also exhibited the strong growth inhibitory activity against all three cancer cell lines. Interestingly, Maneerat (2008)

have found that 6α-acetoxyepoxyazadione, isolated from *Chisocheton siamensis* and which differs from (limonoid 6α-acetoxy-14β,15β-epoxyazadirone) only in the presence of the carbonyl group on C-16 of the former, was inactive against oral human epidermal carcinoma (KB), small cell lung cancer (NCI-H187) and breast cancer (MCF-7).

In addition, four triterpene used in this study are regarded as having the ability to induce the cell cycle profile alteration resulted in apoptosis cell death. Cell cycle represents a series of integrated events that allow the cell to grow and proliferate. The elements responsible for driving the cell cycle from one phase to the next are a series of protein kinases and phosphatases that activate and deactivate each other. The essential parts of the cell cycle machinery are a cyclins and cyclin-dependent kinase (CDKs) and even some of inhibitors of cyclins (CKIs) (Yuan *et al*., 2007). The cyclin dependent kinases (Cdks) are responsible for phosphorylating various substrates critical to cell-cycle progression. Until now, nine CDK have been identified and, of these, five are active during cell cycle, i.e. during G1 (CDK2, CDK4 and CDK6), S (CDK2), G2 and M (CDK1). The levels of the Cdks are invariant throughout the cell cycle, but their activities are modulated by their interaction with another set of proteins called cyclins, whose levels fluctuate. The point in the cell cycle at which the various cyclins are expressed at the highest level is somewhat cell-line dependent and can vary dramatically between transformed and non-transformed cells. In general, however, the cyclin Ds are associated with Gl, cyclin Es with the G1 to S transition, cyclin As with S phase as well as with the G2 to M transition, and cyclin Bs with the G2 to M transition. The presence of a Cdk-cyclin complex does not ensure activity, however, as the Cdk-cyclin complexes can be inhibited by the Cdk inhibitors (CDIs) (Hung *et al*., 1996). All of them can in turn be modulated by diverse intracellular growth cues (Vermeulen *et al*., 2003; Deng *et al*., 2004).

Cell cycle checkpoints are available to allow cellular repair when cells risk to damage, including DNA damage, as well as to dissipate exogenous cellular stress signals. In most cases, checkpoints may result in the activation of program cell death signaling, if the cellular damages are too serious to be properly repaired. Cell cycle

checkpoints may function to ensure that cells have time for DNA repair, whereas apoptotic cell death may function to eliminate irreparable or unrepaired damaged cells. Both cell cycle checkpoint and apoptosis are the important key roles in cell development and represent a set of potential targets for chemotherapeutic agents. In this study tirucallane triterpene odoratol at the GI_{50} concentration of 11.67 μM, inhibited of NCI-H460 cell growth, with cells accumulating in G1 phase of cell cycle. The inhibition of cell growth associated with the arrest of G1 phase may occur due to deregulation of several key proteins in cell cycle progression such as the increased expression of cell cycle inhibitor proteins like p53, p21 and p16. All of these proteins are the inhibitor of Cdk2 and cyclin E (Li *et al*., 1994; Wu *et al*., 2006). As mentioned previously, CDKs are the protein that involve in cell cycle progression, it provides a mean for the cells to move from one phase to another (G1→ S or G2 → M). The induction of p21, a CKI, results in CDK inhibition and cell cycle arrest, preventing the replication of damaged DNA (Ko and Prives, 1996). In contrast, if a cell continues to cycle with DNA damaged-intact, the apoptosis is triggered and the cell will go to apoptosis cell death. Another mechanism behind the G1 phase arrest has been reported by Yuan (2007) about principally involved a decrease in expression of cyclin D3 and phosphorylated retinoblastoma (Rb) protein. G1 cell cycle arrest due to an inhibition of CDK-cyclin kinase activity such as Cip1/p21 and kip1/p27 has been studied (Yim *et al*., 2005). These proteins are in the group of CDKIs which are tumor suppressor proteins. They down-regulated the cell cycle progression by binding with active CDK-cyclin complexes and thereby inhibiting kinase activity.

The cell cycle profile of cells treated with different compounds or with control (DMSO) were calculated. In present study, NCI-H460 exposure to limonoid cedrelone and limonoid gedunin were shown S phase arrest. The GI_{50} concentration of 2.77 and 9.36 μM were used for limonoid cedrelone and limonoid gedunin, respectively. In normal cell cycle, the cell progresses into S phase due to the induction of the Cdk-G1 cyclin complexes with a complicated assembly of DNA polymerases, primases, helicases, topoisomerases and accessory factors duplicate the genome (Hung *et al*., 1996). Since replication of eukaryotic DNA is strictly controlled so that each sequence is replicated once and only once during the S-phase,

there must be mechanism for S-phase arrest in case exogenous damage triggers cells to neither go forward nor retreat to G1 status. In fact, S-phase arrest has been observed in mammalian cells with prolonged arrest at the G1/S boundary (Borel *et al.*, 2002), Rb(+/+) mouse embryo fibroblasts treated with cisplatin, etoposide or mitomycin (Knudsen *et al.*, 2000), human melanocytes treated with thymidine dinucleotides (Pedeux *et al.*, 1998), and human cancer SAOS-2 cells transduced with the p21 gene (Ogryzko *et al.*, 1997). Overexpression of p21 resulted in S-phase arrest has been confirmed. P21 acts as an inhibitor of CDKs, directly inhibits CDK2, CDK3, CDK4, and CDK6 activity (Harper *et al.,* 1993; Gartel and Tyner, 2002). In addition, overexpression of p21 usually leads to G1 or G2 arrest by inhibiting CDK activity. P21 can also directly inhibit DNA synthesis by binding to proliferating cell nuclear antigen (PCNA) (Chen *et al*., 1995). The expression of p21 could be regulated at the transcriptional, post-transcriptional, or post-translational levels by p53-dependent and-independent mechanisms (El-Deiry *et al.,* 1993; Gartel and Tyner, 1999).

In this study, the induction of S-phase cell cycle arrest concurrently with and increase sub-G1 which consequently increase apoptosis cell death. It can be inferred that the S-phase accumulation caused by all of compounds is lethal, and these cells are dying during the apoptotic processes. The S phase checkpoint arrest suggests the possibility that DNA synthesis was blocked by the compounds in NCI-H460 cells. It may cross-link with DNA directly or interact with DNA polymerase to inhibit DNA synthesis (Liu *et al*., 2008). The results was confirmed by trypan blue exclusion assay and flow cytometry analysis after the cells were stained by AnnexinV/PI. The S phase arrest that occurred after the cancer cells were treated by anticancer drugs due to ERK activation and p21 induction was reported (Spencer *et al*., 2003; Zhu *et al*., 2004). ERK or extracellular signal-regulated protein kinase is the protein that plays vital roles in cell growth and division and can be activated by a variety of extracellular stimuli (Johnson and Lapadat, 2002), including various oncolytic agents and DNA-damaging agents (Chen *et al*., 1997; Bacus *et al*., 2001; Seidman *et al*., 2001; Tang *et al*., 2002). Its activation can lead to activation of a variety of targets. For example, treatment of NIH3T3 cells with etoposide (VP16), an inhibitor of

topoisomerase II, led to slight activation of ERK1, S-phase arrest, and induction of p21 independent of p53 (Chen *et al*., 1997). The significant accumulation of NCI-H460 cells in the S phase by the compounds tested led to the conclusion that more investigation is necessary in order to understand the mechanism associated to the cell cycle arrest.

The accumulation in G2/M phase of cell cycle with sub-sequence accumulation in sub-G1 phase of cell cycle was observed after NCI-H460 cells were treated with limonoid cedrelone. G2 is a second gap phase in which the cell prepares for division. The cell cycle arrest at G2 phase prevents the cells entry into mitosis and indicate the damage DNA which was occurred in the cells (Vermeulen *et al*., 2003). In addition, G2/M arrest prevents microtubule formation (Liepins *et al*., 1994), affects DNA replication and DNA repair mechanism. The important mechanism that induces G2 accumulation is the induction of inhibitory phosphorylations on CDK by DNA damage and alterations in the level of cyclins/CDKs together with elevation of CDK inhibitor such as p21 and p27. Besides induction of inhibitory phosphorylations on CDK, p53 may play a role in the regulation of G2/M checkpoint by increase transcription of p21 and stratifin, a protein that normally sequesters cyclin B1-Cdc2 complexes in cytoplasm or through the induction of apoptosis (Yonish-Rouach *et al*., 1991; Shaw *et al*.,1992). The accumulation in G2/M with sub-sequence accumulation in subG1 suggested the sequential events of cell cycle arrest at G2/M phase followed by apoptosis. The apoptosis evoked by limonoid cedrelone also confirmed by AnnexinV-FITC/PI double staining method.

As shown in the cell cycle analysis, the subG1 population was increased after the cells were treated with all of these four compounds, which has been suggested to be apoptotic DNA. Apoptosis is an important biological mechanism that contributes to the maintenance of the integrity of the multi-cellular organism, which is dependent on the expression of cell-intrinsic and extrinsic suicide mechanisms. Cells exposed to anticancer drugs display apoptotic alterations, such as cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation (Fisher, 1994). The transverse redistribution of plasma membrane phosphotidylserine (PS) is the hallmark

of early apoptotic cell. The human vascular anticoagulant, annexin V, is a 35-36 kDa Ca2+ dependent phospholipids binding protein that has a high affinity for PS, and shows minimal binding to phosphatidylcholine and sphingomyeline. Changes in PS asymmetry, which is analyzed by measuring annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. Annexin V labeled with FITC (green fluorescence) can identify and quantitate apoptotic cells on a single-cell basis by flow cytometry. Staining cells simultaneously with Annexin V–FITC and the non-vital dye propidium iodide (red fluorescence) allows (bivariate analysis) the discrimination of intact cells (Annexin V-FITC negative, PI negative), early apoptotic (Annexin V-FITC positive, PI negative) and late apoptotic or necrotic cells (Annexin V-FITC positive, PI positive). In this study, early apoptotic cells was found higher in NCI-H460 treated with limonoid cedrelone, limonoid gedunin and tirucallane triterpene odoratol. In contrast, late apoptotic cells were observed higher after treating the cells with 6α-acetoxy-14β,15β-epoxyazadirone.

There are two major pathways in apoptosis that have been elucidated to date. One is the mitochondrial pathway in which cytochrome C is released into the cytosol, where it binds to apoptotic protease activating factor-1 (Apaf-1) to activate procaspase-9. Subsequently, caspase-9 induces procaspase-3 to become active caspase-3. It ultimately caused the death of the cell (Belmokhtar *et al*., 2003; Launay *et al*., 2005). Another apoptosis pathway is the ligation of death receptors such as Fas and TNFR1 and subsequent activation of caspase-8 to trigger apoptosis. Caspases are important regulators in the induction of cell progressing apoptosis (Baliga and Kumar, 2003). Therefore, further study should focus on the enzymatic activities of caspase-3, -8, and -9 presented in the NCI-H460 after treatment with various concentrations of these compounds. Thus the detailed mechanism requires further investigation.

In addition to these four natural compounds, forty-six synthetic compounds from xanthone derivatives, flavone derivatives, sulfate small molecules and terpenes were also evaluated for their capacity to inhibit the *in vitro* growth of three human

cell lines, MCF-7, A375-C5 and NCI-H460, using SRB assay. Twelve synthetic compounds from xanthone derivatives (3,6P1, TH14L, THX34, TX48, TX96, TX15, TXOH15, XEGOL-1L, XEGOL-2L, XEGOL-2D, TXISO and TXOH) and two flavone derivatives (FP13 and FP14) presented moderate GI_{50} between 13-50 μM against three cell lines. Eighteen synthetic compounds were found to be inactive against all three cell lines at the highest concentration tested (GI_{50} > 150 μM). Among forty-six synthetic compounds tested the xanthone derivatives, TX34, was found to have the best inhibitory activity against all three cell lines (GI_{50} = 13.3±0.6 for A375-C5, GI_{50} = 15.0±1.7 for MCF-7 and GI_{50} = 13.3±1.2 for NCI-H460). These GI_{50} values indicated that the TX34 also has interesting anti tumour activity. Thus, the further studies to elucidate the mechanism underlying this growth inhibitory is needed.

CONCLUSION AND RECOMMENDATION

From the experimental results and discussion, it could be concluded that three of four novel compounds, inhibited NCI-H460 cell growth by the mechanism that induced cell arrest at G1, G2/M or S phase resulting in apoptosis cell death while one compound, limonoid 6α-acetoxy-14β,15β-epoxyazadirone, did not affect cell cycle profile of this cell. Limonoid cedrelone is very potent by causing cell cycle arrest and apoptosis, which makes it a very interesting compound for further studies in order to investigate its full potential as antitumour agent. However, the further investigation should be done to find out the molecular mechanism of these compounds. At present, there is not only an increased effort for the isolation of bioactive molecules from medicinal plants but also a strong advance of synthetic methods for modifying structures to obtain more effective compounds. Determination of molecular structures and molecular mechanisms are equally important for providing the evidence of their efficacy which could potentially lead to the pharmaceutical development of synthetic or semi-synthetic drugs.

In summary, the overall goals concerning novel anticancer drugs are going through the characterization of signaling pathway mediating cell growth inhibition, cell cycle arrest and apoptosis. The hallmarks molecular mechanism of apoptosis such as caspase activation, death receptors and the mitochondrial apoptotic pathway-related molecules including Fas ligands, cytochrome C and Bcl-2 family proteins which are strongly associated with the signal transduction pathway of apoptosis should be further studied. In addition, the cell cycle regulatory proteins such as cyclins, cyclin-dependent kinases (Cdk) are still interesting molecules for further analysis.

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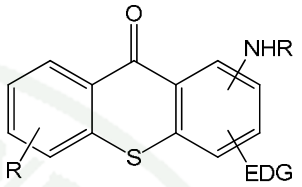
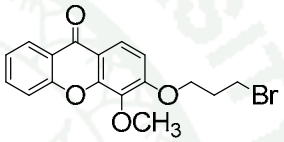
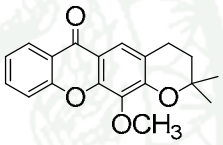
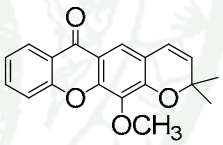
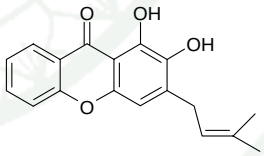
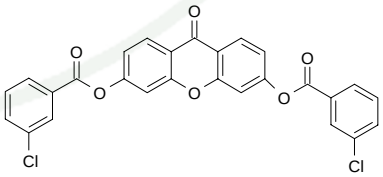
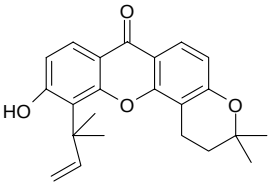
APPENDICES

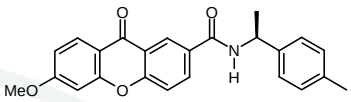
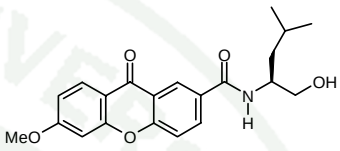
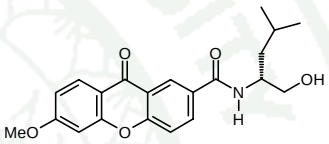
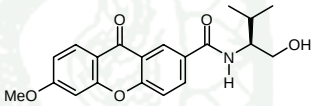
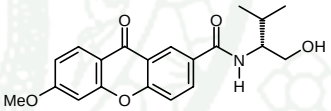
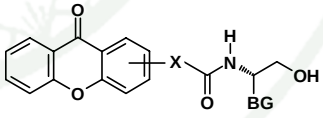
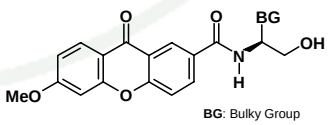
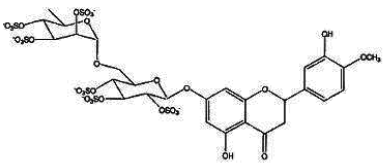
Appendix A

Compounds

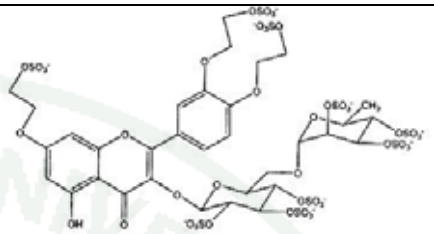
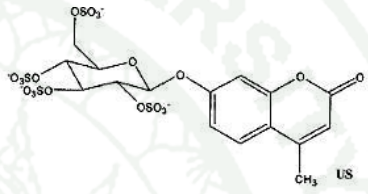
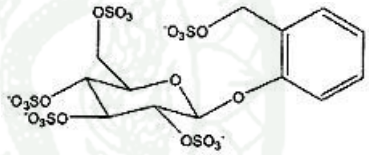
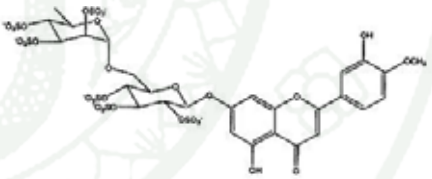
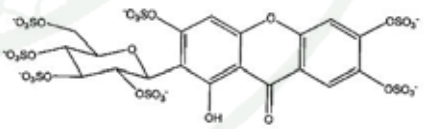
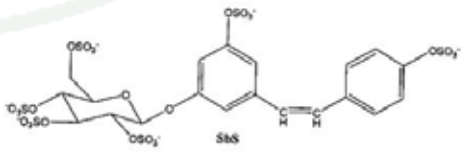
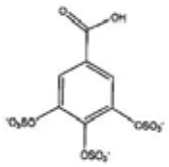
Appendix Table A1 Compounds in the present study.

Compounds	Name	Structure
TX	Tioxanthones **(under confidentiality)**	 THEY EDG=electron donor group
XC1	3-[(3'-Bromopropyl)oxy]-4-methoxy-9H-xanthen-9-one	
XC3	3,4-Dihydro-12-methoxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one	
XC5	12-methoxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one	
1,2 P1	1,2-dihydroxi-3-(3-methylbut-2-en-1-yl)-9H-xanthen-9-one	
3,6-ClAC	9-oxo-9H-xanthene-3,6-diyl bis(3-chlorobenzoate)	
3,6 P1	10-hydroxy-3,3-dimethyl-11-(2-methylbut-3-en-2-yl)-2,3-dihydropyranol[2,3-c]xanthen-7(1H)-one	

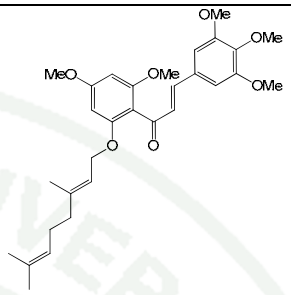
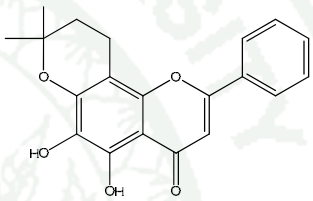
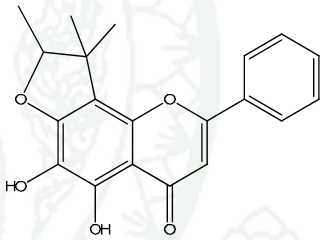
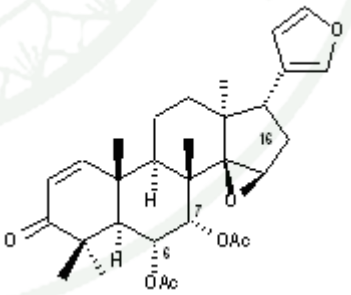
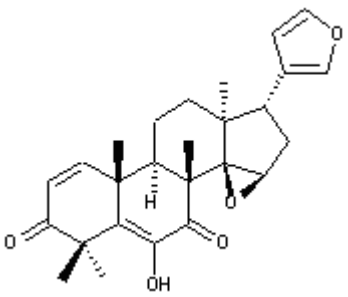
Appendix Table A1 (Continued)

Compounds	Name	Structure
XEA-S	**(under confidentiality)**	
XEL-L	**(under confidentiality)**	
XEL-D	**(under confidentiality)**	
XEVOL-L	**(under confidentiality)**	
XEVOL-D	**(under confidentiality)**	
XEGOL-1L	**(under confidentiality)**	 **X**: Chemical Bridge **BG**: Bulky Group
XEGOL-2D and XEGOL-2L	**(under confidentiality)**	 **BG**: Bulky Group
HS	**Hesperedin hexa-sulphate**	

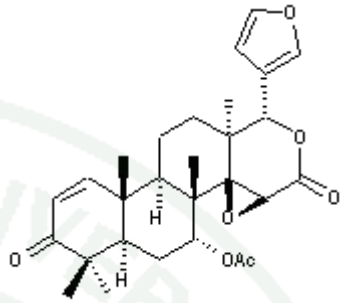
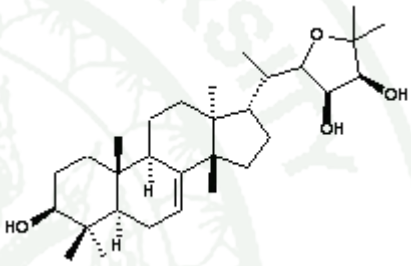
Appendix Table A1 (Continued)

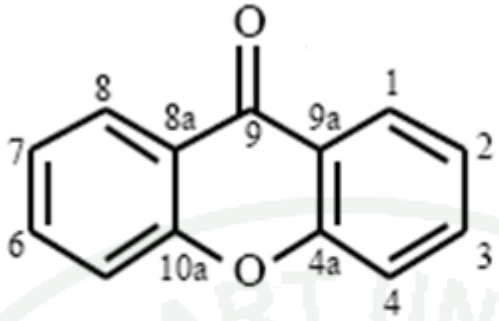
Compounds	Name	Structure
ERS	Tri-hydroxyethylrutin nona-sulphate	
US	4-methylumbeliferyl-β-D-glucopyranoside persulphate	
SS	Salicin persulphate	
DS	Diosmin hexasulphate	
MS	Mangiferin persulphate	
SBS	3-4'-5-trihydroxystilbene-3-b-D glucopyranoside persulphate	
AGS	Gallic acid persulphate	

Appendix Table A1 (Continued)

Compound	Name	Structure
FP13	(E)-1-(2-((E)-3,7-dimethylocta-2,6-dienyloxy)-4,6-dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one	
FP4	**5,6**-dihydroxy-8,8-dimethyl-2-phenyl-8,10-dihydro-4H-pyrano[2,3-h]chromen-4-one	
G3	5,6-dihydroxy-8,9,9-trimethyl-2-phenyl-8,9-dihydro-4H-furo[2,3-h]chromen-4-one	
Compound 28	Limonoid 6α-acetoxy-14β,15β –epoxyazadirone	
Compound 30	Limonoid cedrelone	

Appendix Table A1 (Continued)

Compound	Name	Structure
Compound 35	Limonoid gedunin	
Compound 75	tirucallane triterpene odoradol	

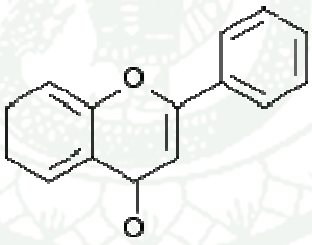


Compound name: 9H-Xanthen-9-one

Synonym: Xanthone

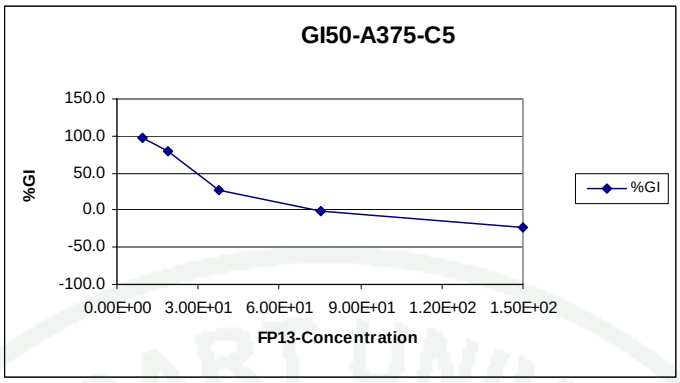
Appendix Figure A1 Xanthone structure.

Source: Peres *et al*. (2000)

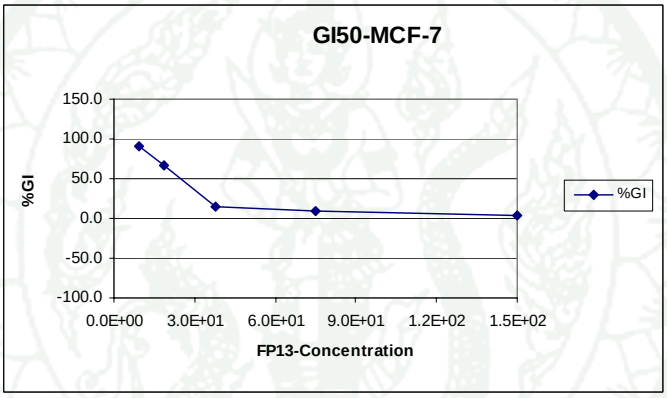


Appendix Figure A2 Chemical structure of flavone backbone.

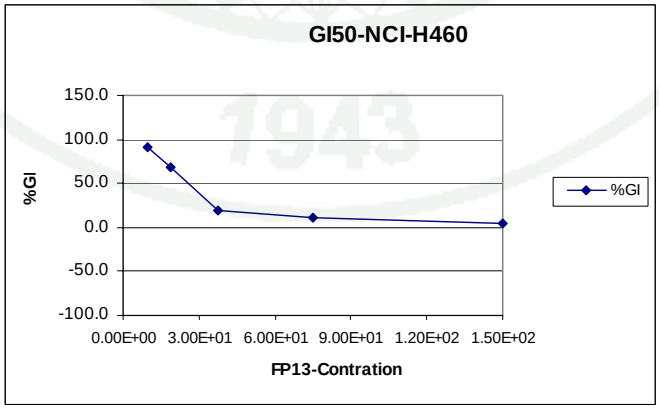
Source: Kandaswami *et al*. (2005)



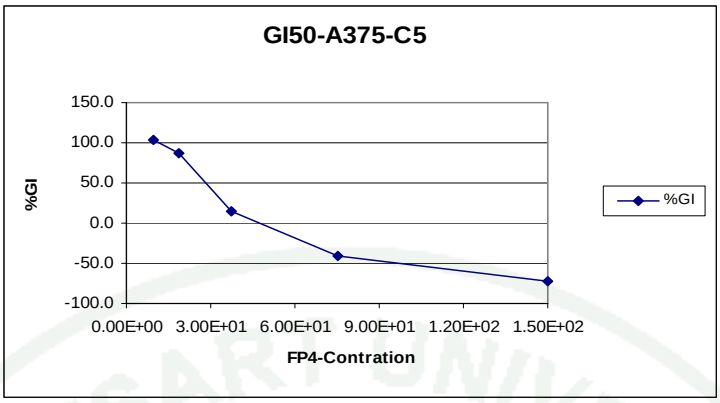
Appendix Figure A3 GI_{50} graph of FP13 in A375-C5.



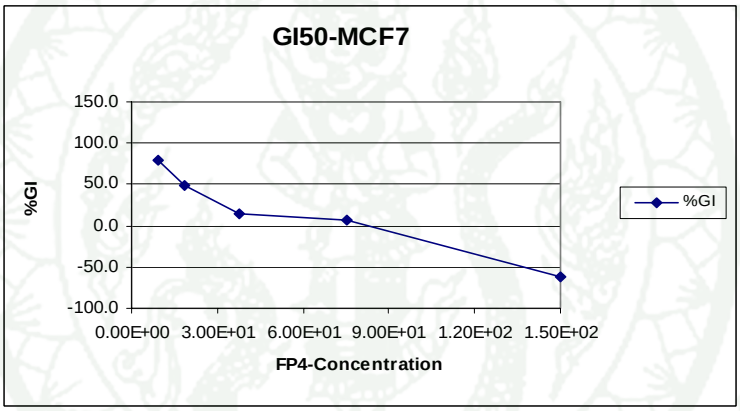
Appendix Figure A4 GI_{50} graph of FP13 in MCF-7.



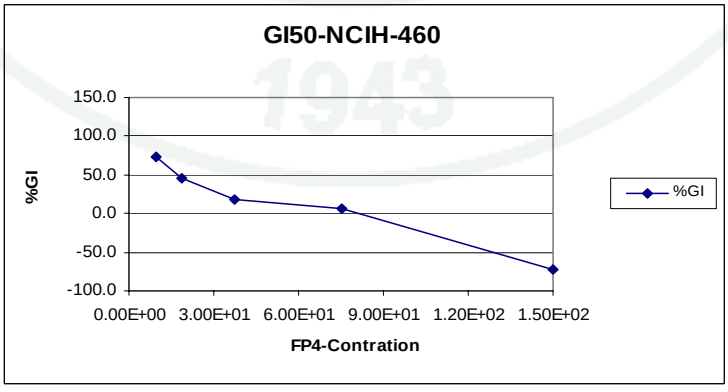
Appendix Figure A5 GI_{50} graph of FP13 in NCI-H460.



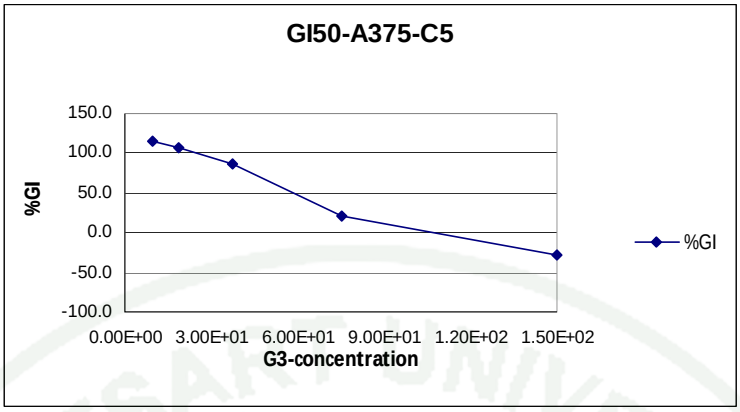
Appendix Figure A6 GI_{50} graph of FP4 in A375-C5.



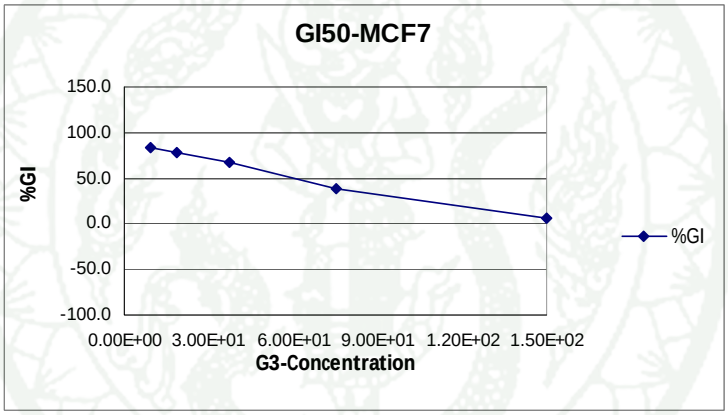
Appendix Figure A7 GI_{50} graph of FP4 in MCF-7.



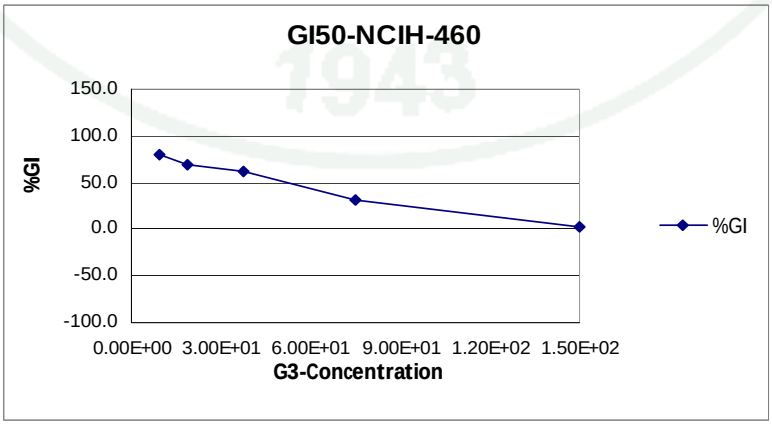
Appendix Figure A8 GI_{50} graph of FP4 in NCI-H460.



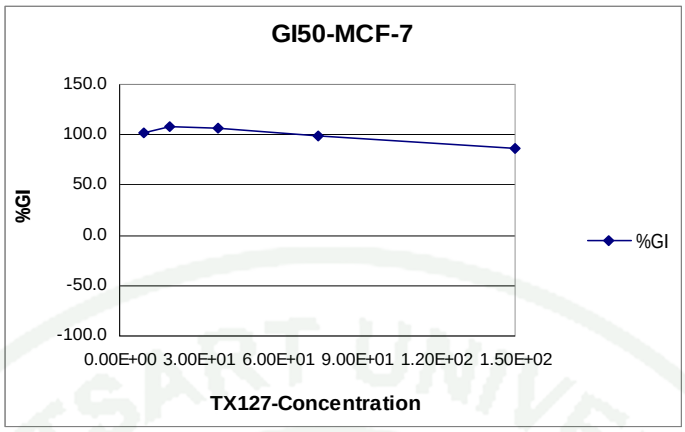
Appendix Figure A9 GI_{50} graph of G3 in A375-C5.



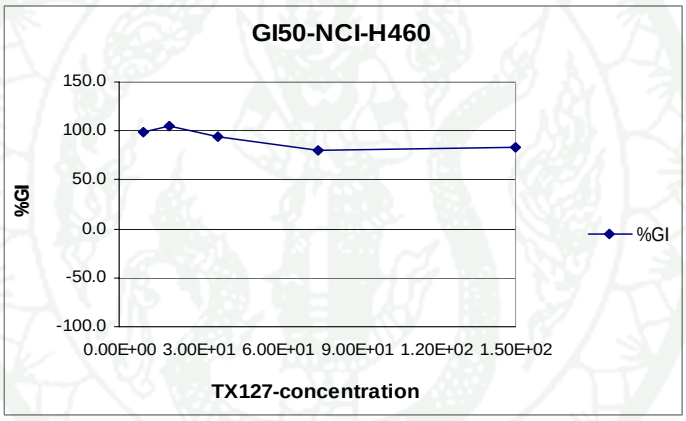
Appendix Figure A10 GI_{50} graph of G3 in MCF-7.



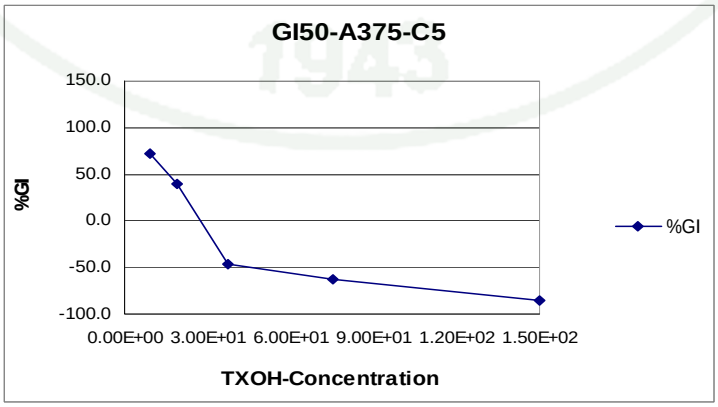
Appendix Figure A11 GI_{50} graph of G3 in NCI-H460.



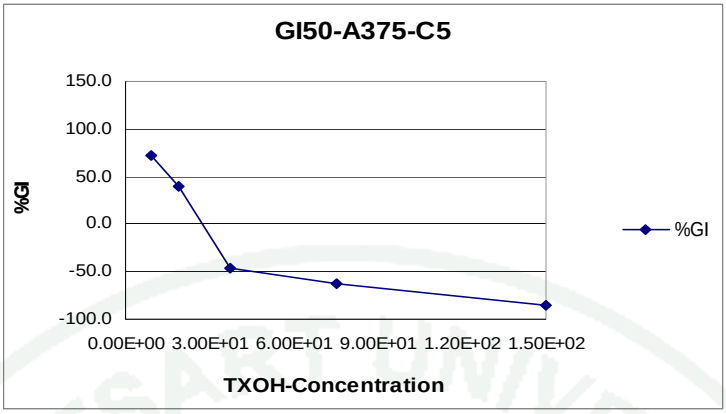
Appendix Figure A12 GI_{50} graph of TX127 in A375-C5.



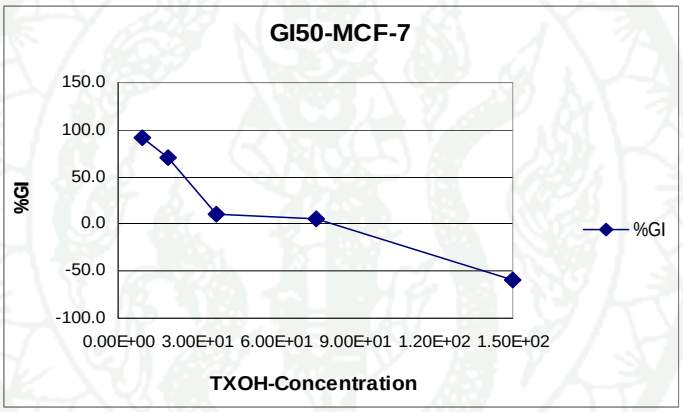
Appendix Figure A13 GI_{50} graph of TX127 in MCF-7.



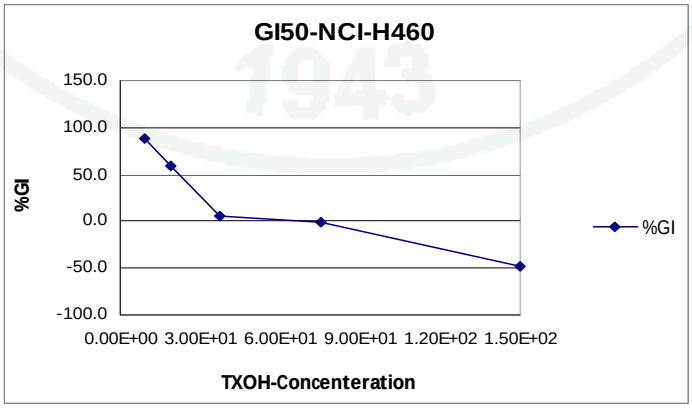
Appendix Figure A14 GI_{50} graph of TX127 in NCI-H460.



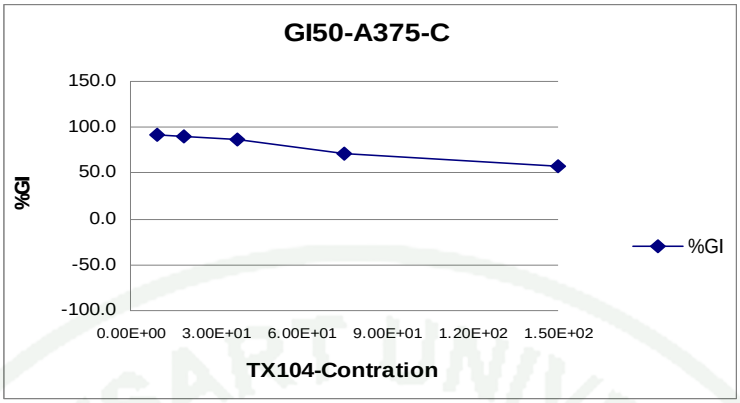
Appendix Figure A15 GI_{50} graph of TXOH in A375-C5.



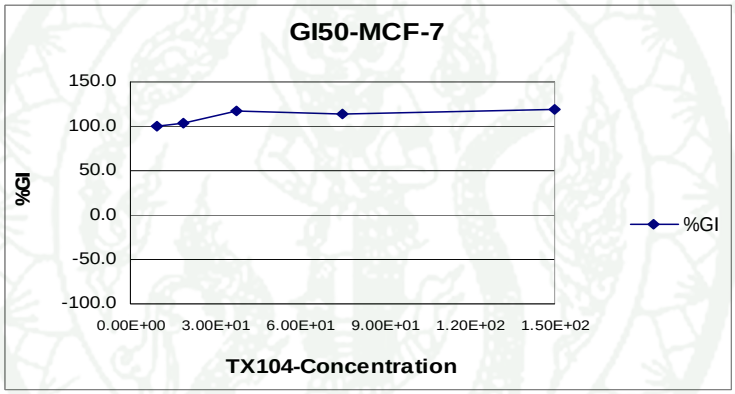
Appendix Figure A16 GI_{50} graph of TXOH in MCF-7.



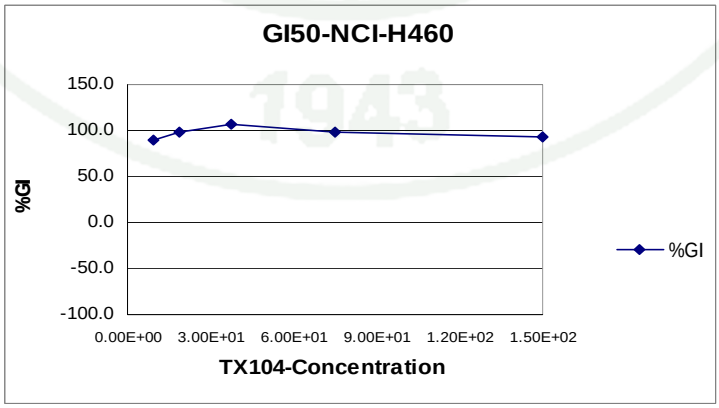
Appendix Figure A17 GI_{50} graph of TXOH in NCI-H460.



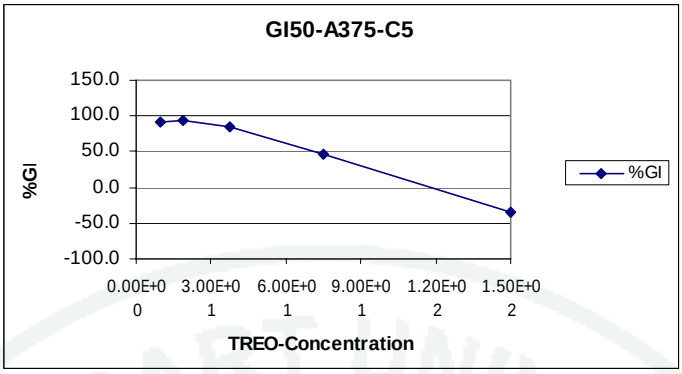
Appendix Figure A18 GI_{50} graph of TX104 in A375-C5.



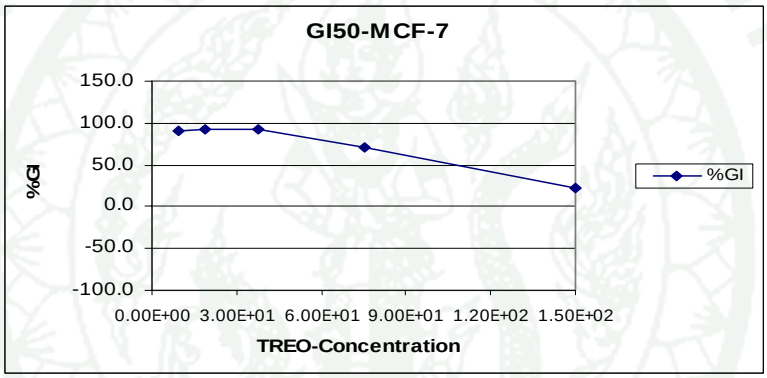
Appendix Figure A19 GI_{50} graph of TX104 in MCF-7.



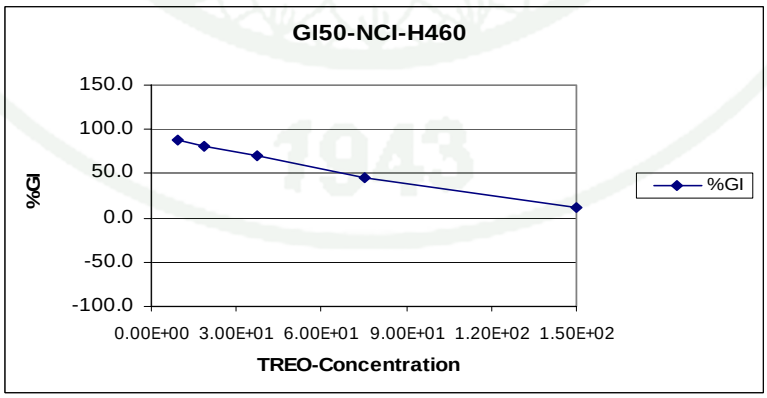
Appendix Figure A20 GI_{50} graph of TX104 in NCI-H460.



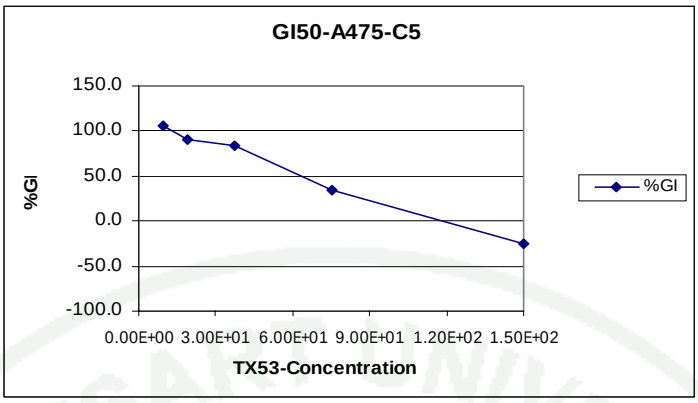
Appendix Figure A21 GI_{50} graph of TXTREO in A375-C5.



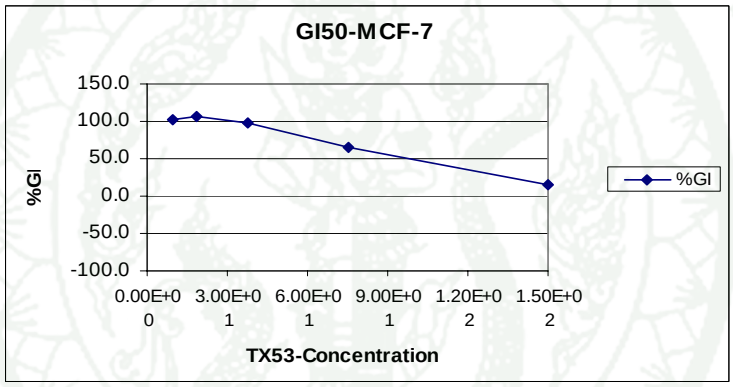
Appendix Figure A22 GI_{50} graph of TXTREO in MCF-7.



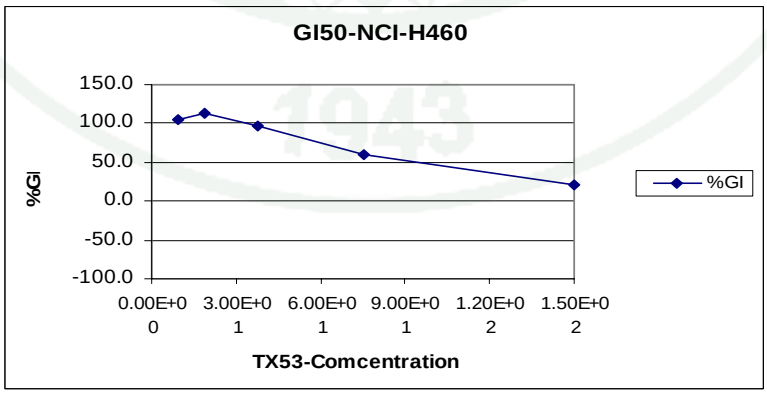
Appendix Figure A23 GI_{50} graph of TXTREO in NCI-H460.



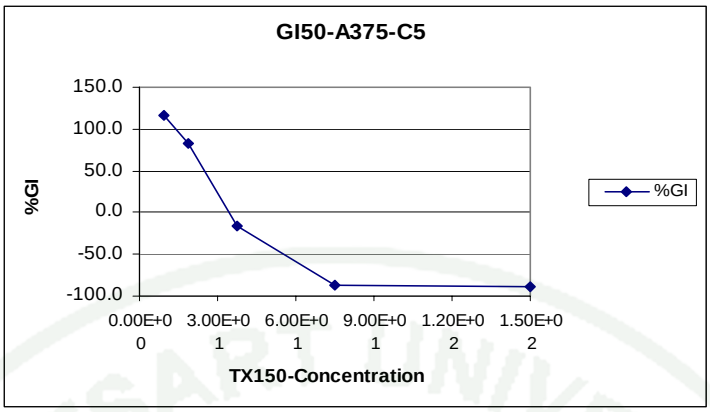
Appendix Figure A24 GI_{50} graph of TX53 in A375-C5.



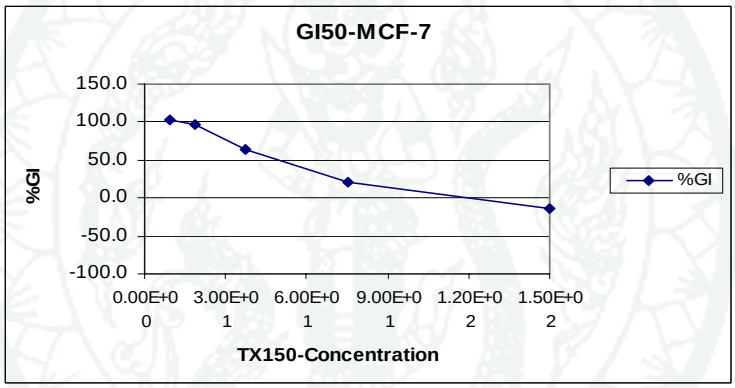
Appendix Figure A25 GI_{50} graph of TX53 in MCF-7.



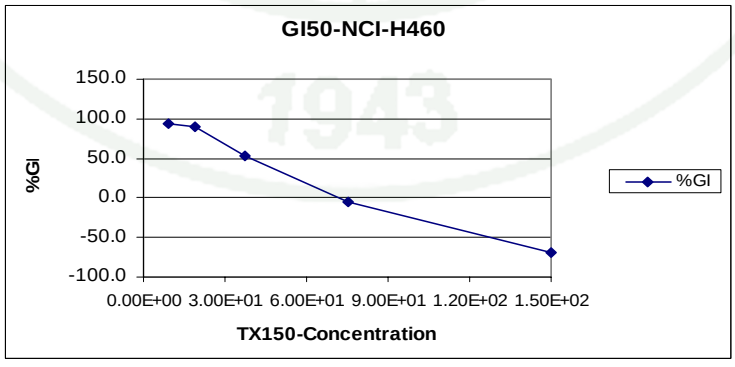
Appendix Figure A26 GI_{50} graph of TX53 in NCI-H460.



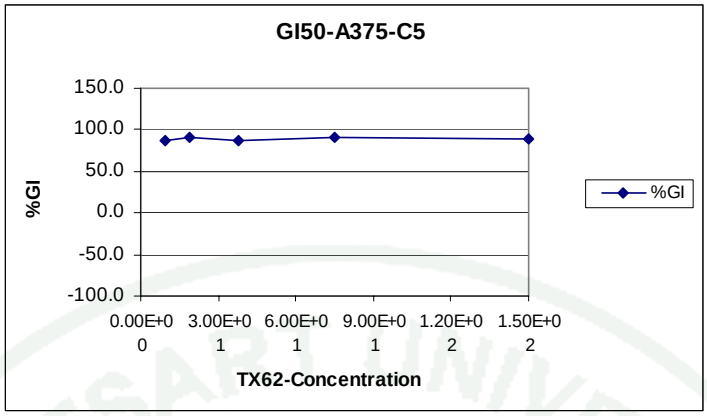
Appendix Figure A27 GI_{50} graph of TX150 in A375-C5.



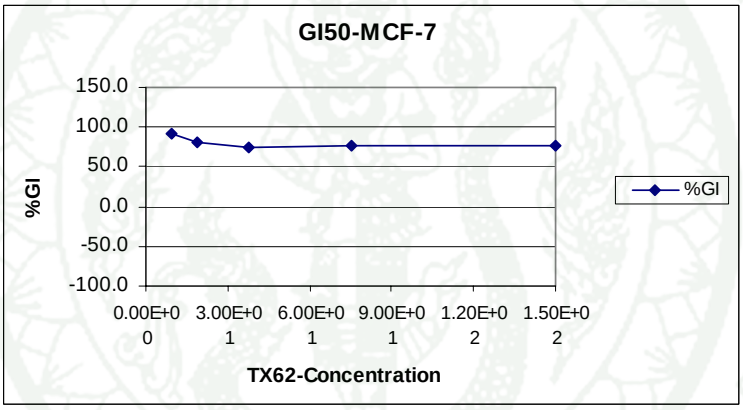
Appendix Figure A28 GI_{50} graph of TX150 in MCF-7.



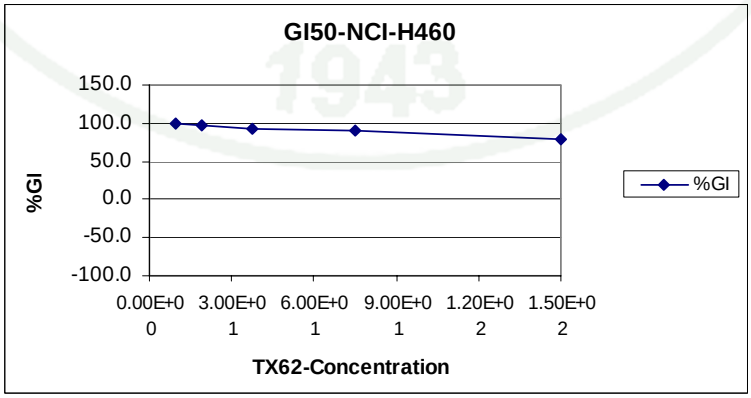
Appendix Figure A29 GI_{50} graph of TX150 in NCI-H460.



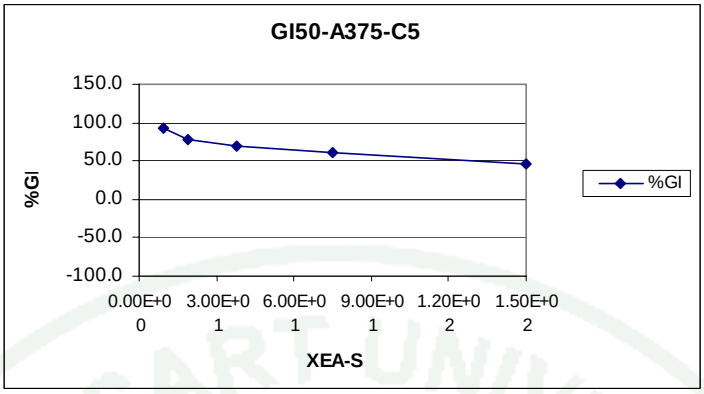
Appendix Figure A30 GI_{50} graph of TX62 in A375-C5.



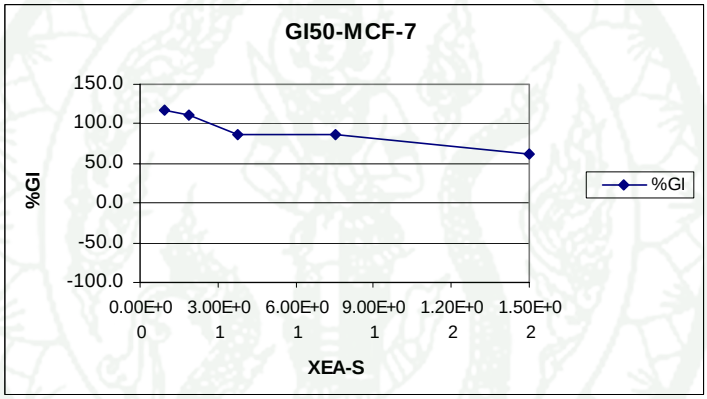
Appendix Figure A31 GI_{50} graph of TX62 in MCF-7.



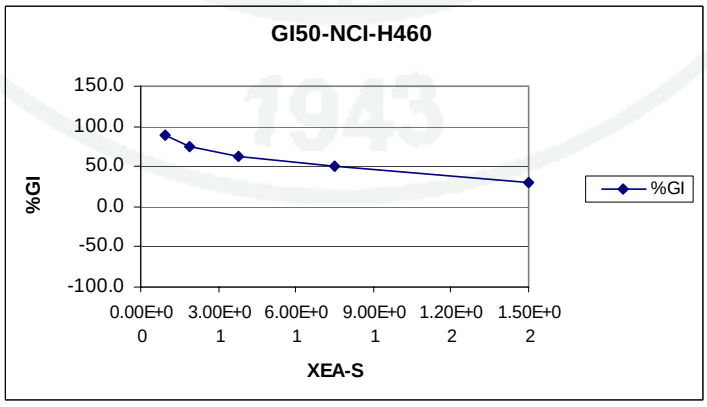
Appendix Figure A32 GI_{50} graph of TX62 in NCI-H460.



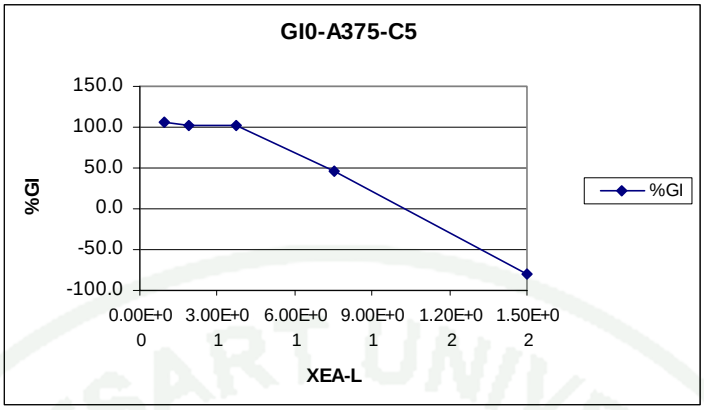
Appendix Figure A33 GI_{50} graph of XEA-S in A375-C5.



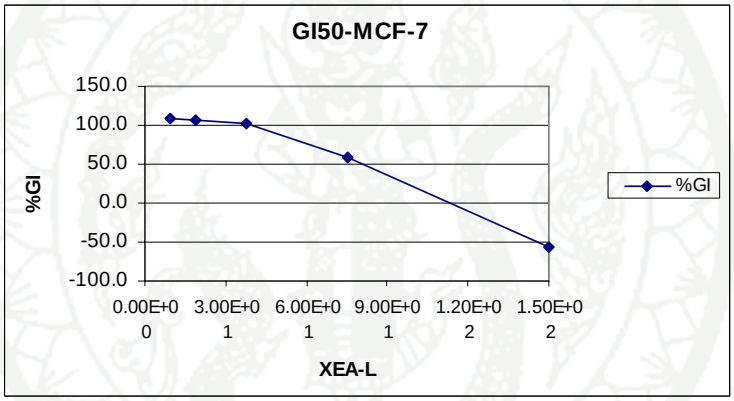
Appendix Figure A34 GI_{50} graph of XEA-S in MCF-7.



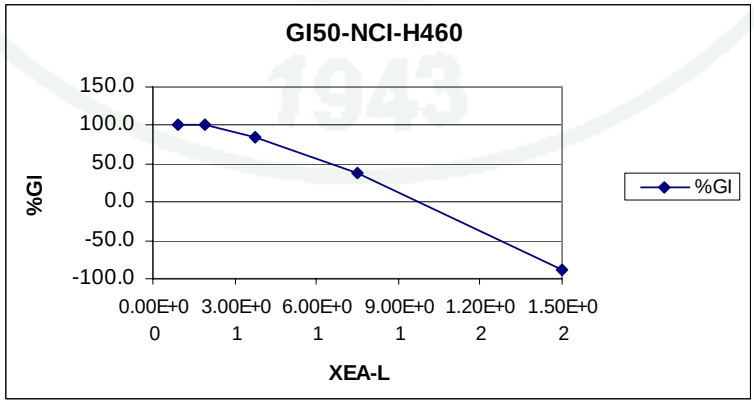
Appendix Figure A35 GI_{50} graph of XEA-S in NCI-H460.



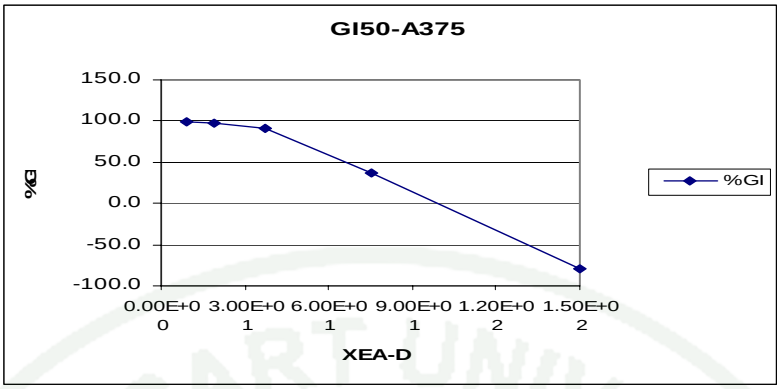
Appendix Figure A36 GI_{50} graph of XEA-L in A375-C5.



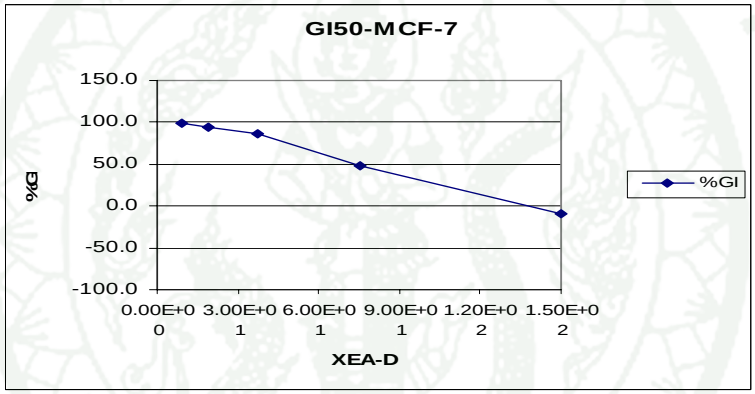
Appendix Figure A37 GI_{50} graph of XEA-L in MCF-7.



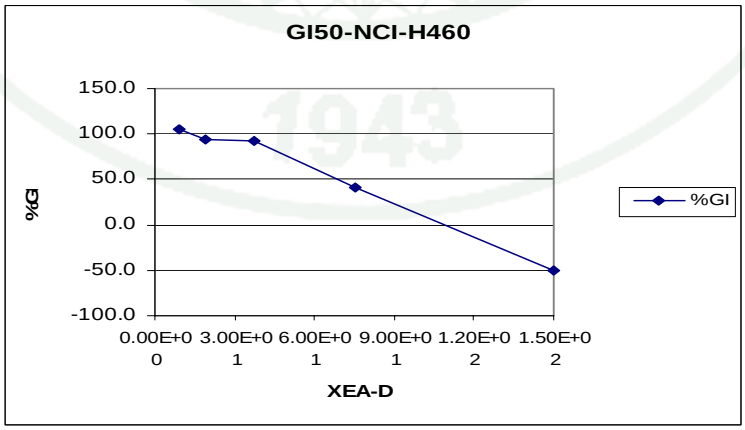
Appendix Figure A38 GI_{50} graph of XEA-L in NCI-H460.



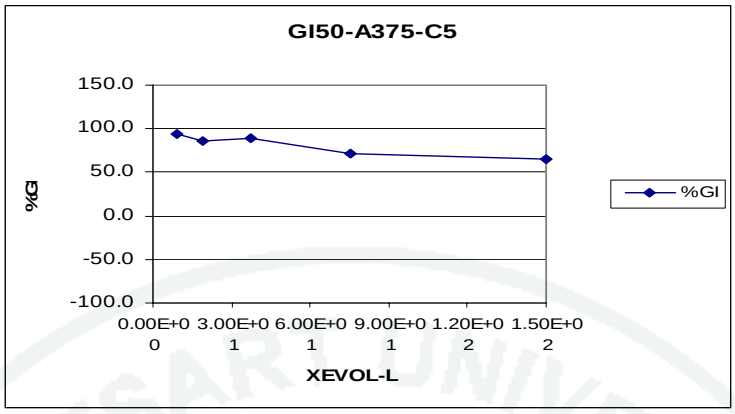
Appendix Figure A39 GI_{50} graph of XEA-D in A375-C5.



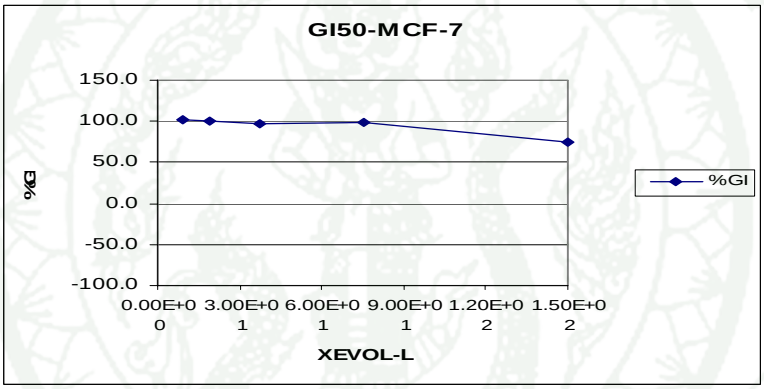
Appendix Figure A40 GI_{50} graph of XEA-D in MCF-7.



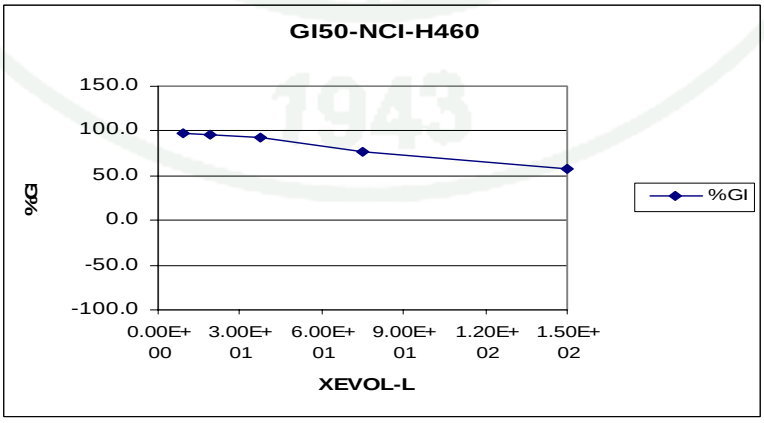
Appendix Figure A41 GI_{50} graph of XEA-D in NCI-H460.



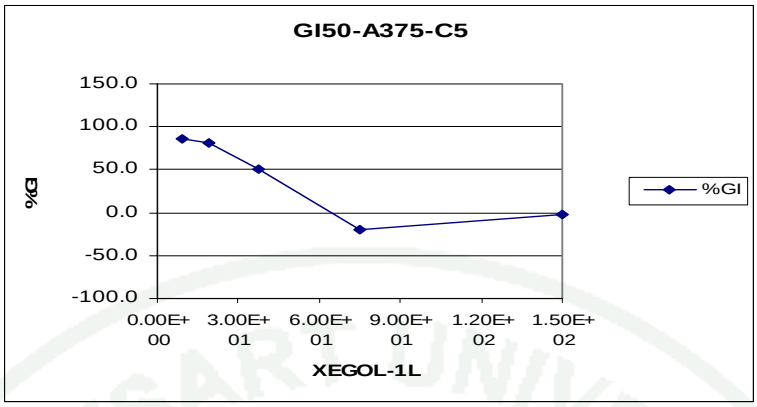
Appendix Figure A42 GI_{50} graph of XEVOL-L in A375-C5.



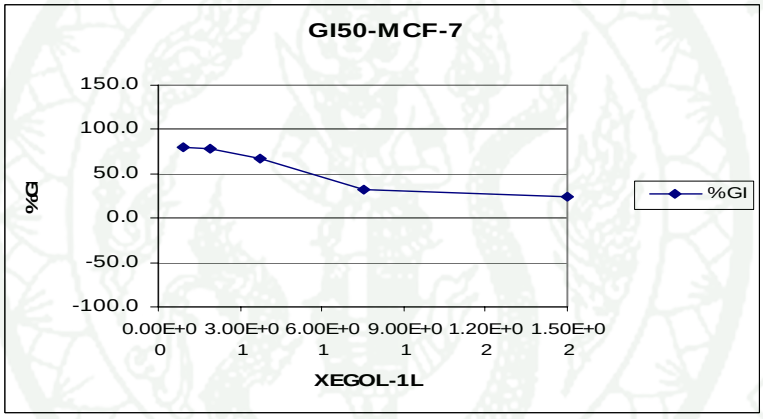
Appendix Figure A43 GI_{50} graph of XEVOL-L in MCF-7.



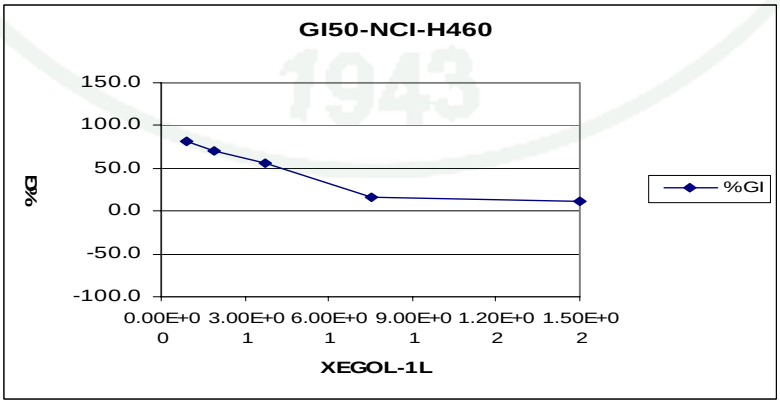
Appendix Figure A44 GI_{50} graph of XEVOL-L in NCI-H460.



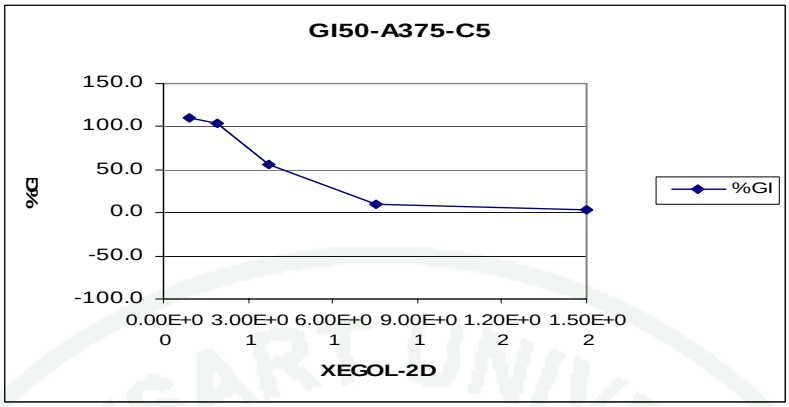
Appendix Figure A45 GI_{50} graph of XEGOL-1L in A375-C5.



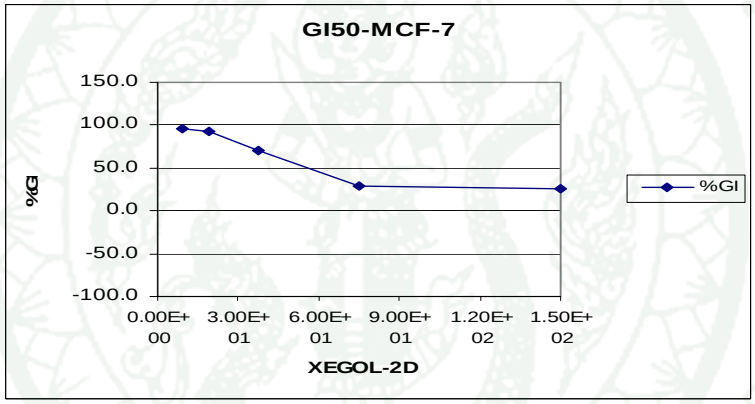
Appendix Figure A46 GI_{50} graph of XEGOL-1L in MCF-7.



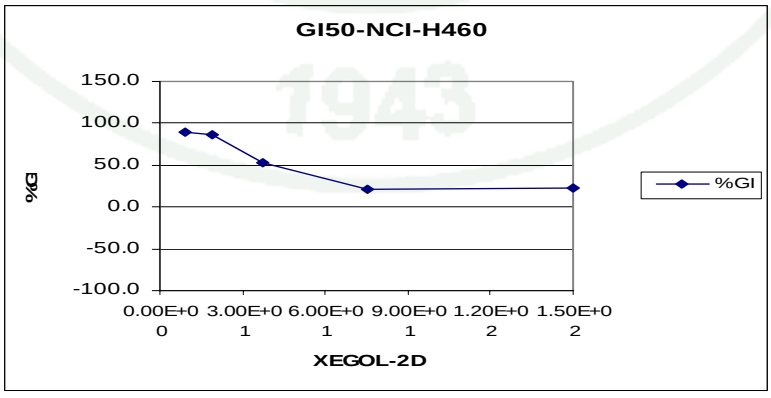
Appendix Figure A47 GI_{50} graph of XEGOL-1L in NCI-H460.



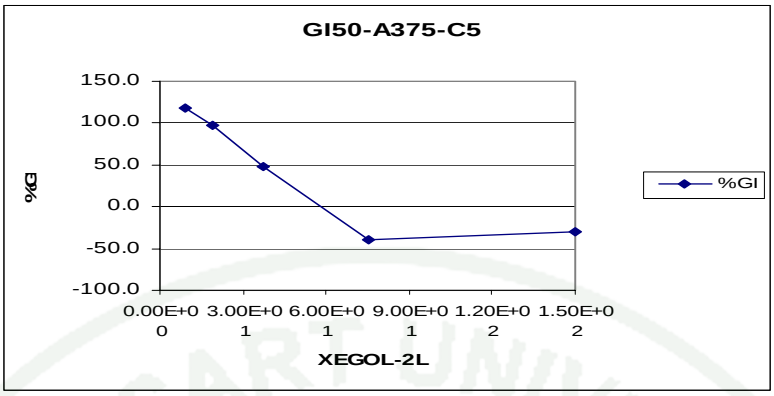
Appendix Figure A48 GI_{50} graph of XEGOL-2D in A375-C5.



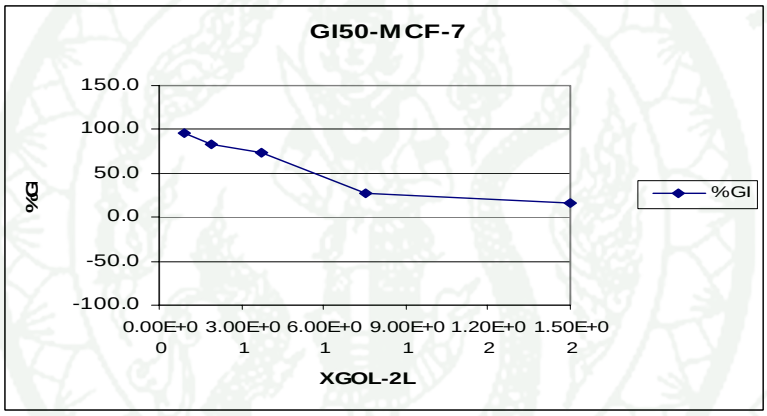
Appendix Figure A49 GI_{50} graph of XEGOL-2D in MCF-7.



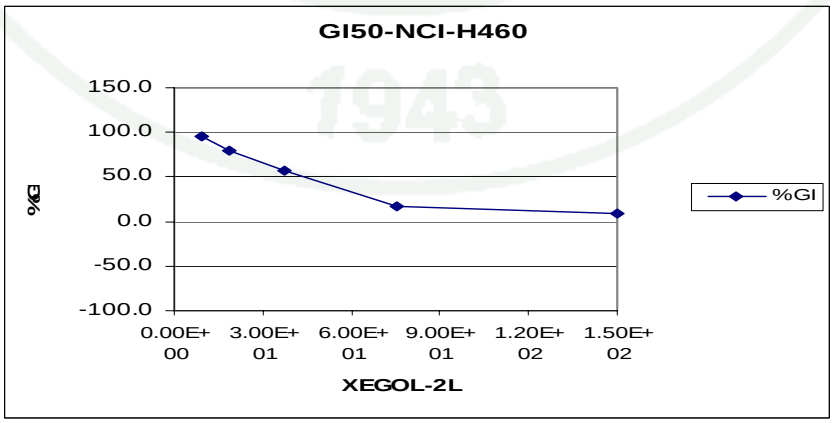
Appendix Figure A50 GI_{50} graph of XEGOL-2D in NCI-H460.



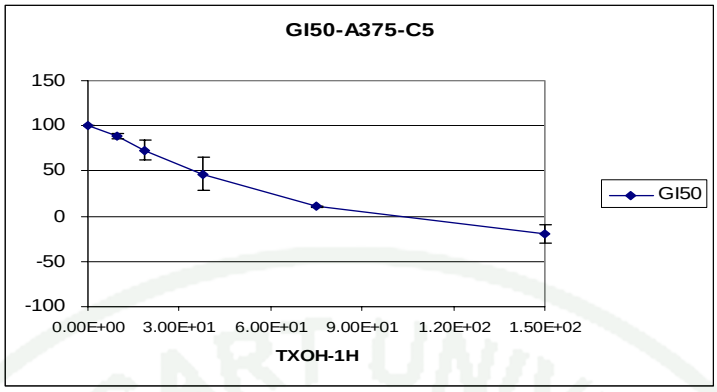
Appendix Figure A51 GI_{50} graph of XEGOL-2L in A375-C5.



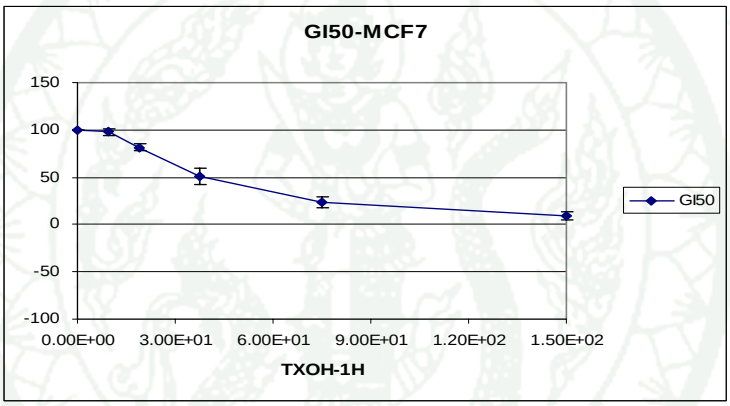
Appendix Figure A52 GI_{50} graph of XEGOL-2L in MCF-7.



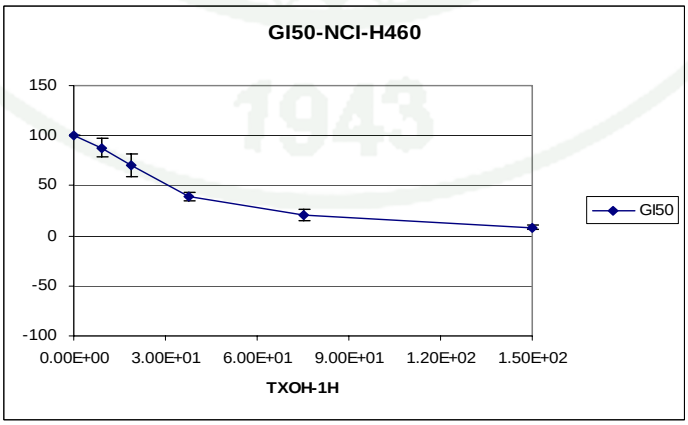
Appendix Figure A53 GI_{50} graph of XEGOL-2L in NCI-H460.



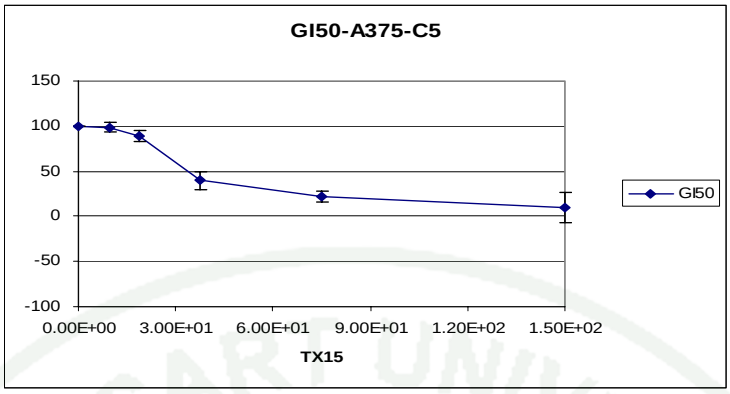
Appendix Figure A54 GI_{50} graph of TXOH-1H in A375-C5.



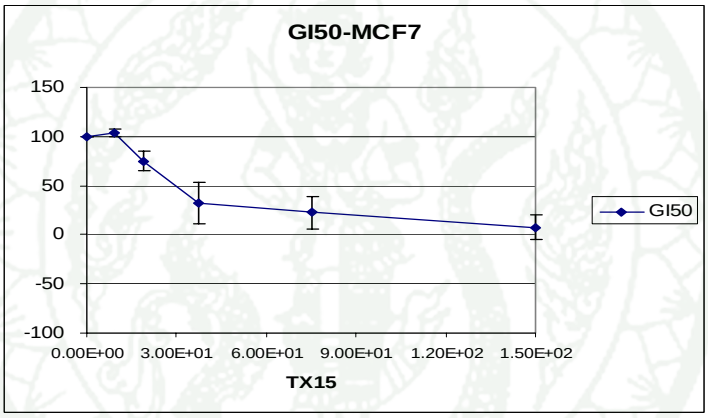
Appendix Figure A55 GI_{50} graph of TXOH-1H in MCF-7.



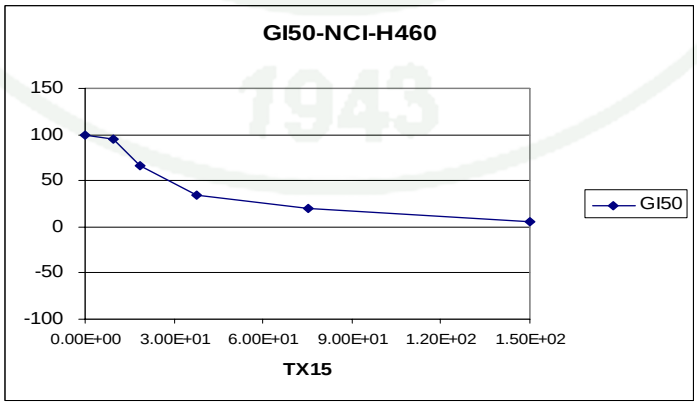
Appendix Figure A56 GI_{50} graph of TXOH-1H in NCI-H460.



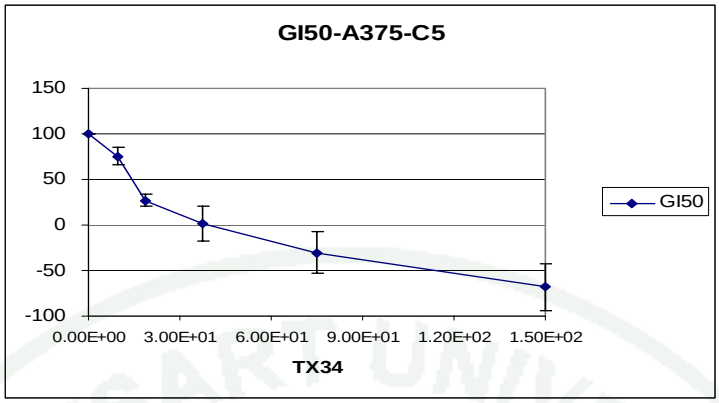
Appendix Figure A57 GI_{50} graph of TX15 in A375-C5.



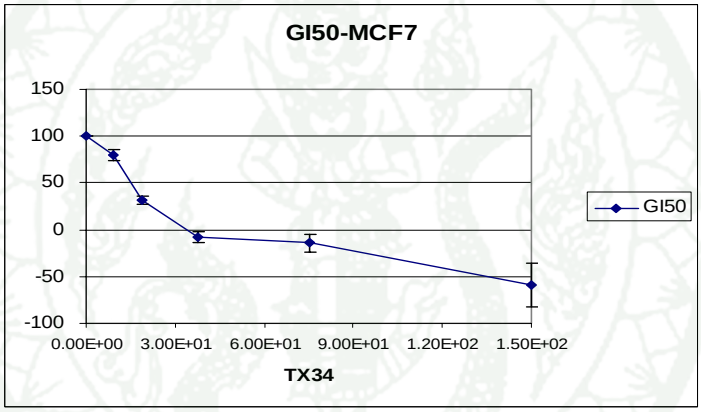
Appendix Figure A58 GI_{50} graph of TX15 in MCF-7.



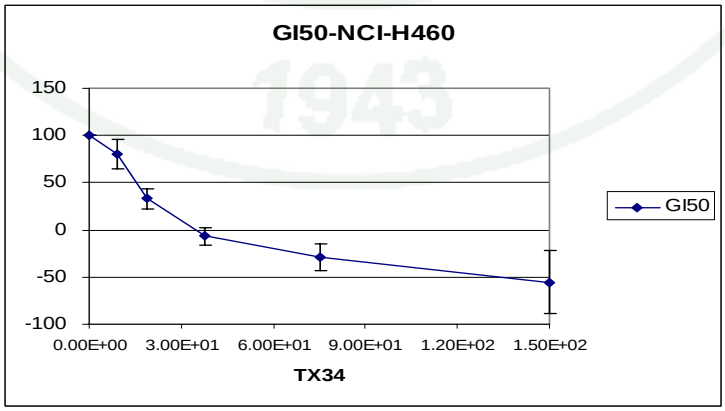
Appendix Figure A59 GI_{50} graph of TX15 in NCI-H460.



Appendix Figure A60 GI_{50} graph of TX34 in A375-C5.



Appendix Figure A61 GI_{50} graph of TX34 in MCF-7.



Appendix Figure A62 GI_{50} graph of TX34 in NCI-H460.

Appendix B

Apoptosis results

Appendix Table B1 Apoptosis of NCI-H460 cells after exposure to all four compounds.

Replicate 1	Control	DMSO	Limonoid cedrelone	Limonoid 6α	Limonoid gedunin	Tirucallane triterpene odoradol
FL1-/FL3+ Late apoptotic	0.22	0.09	3.09	2.52	0.89	3.1
FL1+/FL3+ Live Cells	5.46	7	35.48	8.63	17.21	5.71
FL1-/FL3- Early apoptotic	89.04	84.82	14.08	80.65	47.15	82.13
FL1+/FL3- Population subset	4.28	4.79	45.92	6.6	31.96	6.14
	79.46	84.11	44.47	79.79	45.83	65.02

Appendix Table B2 Apoptosis of NCI-H460 cells after exposure to all four compounds.

Replicate 2	Control	DMSO	Limonoid 6α	Limonoid cedrelone	Limonoid gedunin	Tirucallane triterpene odoradol
FL1-/FL3+ Late apoptotic	0.17	0.63	0.66	1.91	1.72	1.4
FL1+/FL3+ Live Cells	8	6.64	11.27	47.45	17.67	9
FL1-/FL3- Early apoptotic	83.38	88.02	81.27	10.59	55.48	82.88
FL1+/FL3- Population subset	5.88	4.72	6.78	40.05	26.13	6.73
	79.46	84.11	65.02	30.77	44.47	79.79

Appendix Table B3 Apoptosis of NCI-H460 cells after exposure to all four compounds.

Replicate 3	Control	DMSO	Limonoid cedrelone	Limonoid 6α	Limonoid gedunin	Tirucallane triterpene odoradol
FL1-/FL3+ Late apoptotic	2.21	1.23	4.55	1.01	4.65	0.18
FL1+/FL3+ Live Cells	8.96	8.21	37.17	8.58	22.87	9.26
FL1-/FL3- Early apoptotic	87.56	21.29	84.01	42.53	85.03	87.56
FL1+/FL3- Population	3.43	3	36.99	6.4	29.95	5.52
subset	80.78	83.14	34.01	86.13	36.7	82.19

Appendix Table B4 Apoptosis of NCI-H460 cells after exposure to all four compounds.

Replicate 4	Control	DMSO	Limonoid cedrelone	Limonoid 6α	Limonoid gedunin	Tirucallane triterpene odoradol
FL1-/FL3+ Late apoptotic	3.26	1.02	2.12	1.04	1.43	0.85
FL1+/FL3+ Live Cells	6.09	4	38.88	4.56	16.97	4.65
FL1-/FL3- Early apoptotic	86.13	88.33	16.07	85.76	40.35	84.56
FL1+/FL3- Population	4.52	6.69	42.92	8.65	41.25	9.94
subset	74.18	85.43	25.07	79.77	38.89	81.72

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