ผลของพิโนสโทรบินจากกระชายเหลือง *Boesenbergia pandurata*
ต่อแอกทิวิตีของไคเนส **Wee1** ในเซลล์ไลน์มะเร็งบางชนิด

นายจุมพล โสภณาภรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
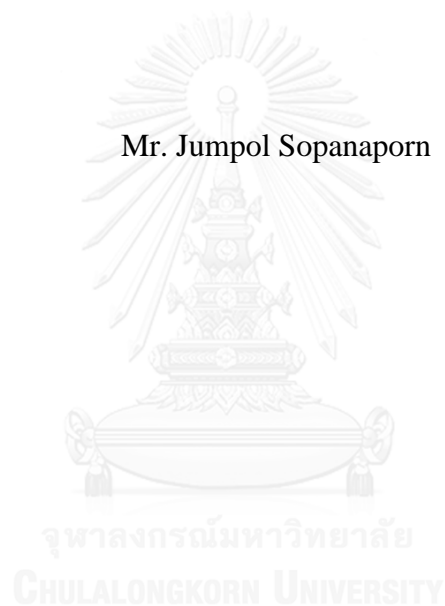สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECT OF PINOSTROBIN FROM FINGERROOT *Boesenbergia pandurata* ON
WEE1 KINASE ACTIVITY IN SOME CANCER CELL LINES

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Industrial Microbiology

Department of Microbiology

Faculty of Science

Chulalongkorn University

Academic Year 2015

Thesis Title	EFFECT OF PINOSTROBIN FROM FINGERROOT *Boesenbergia pandurata* ON WEE1 KINASE ACTIVITY IN SOME CANCER CELL LINES
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จุมพล โสภณาภรณ์ : ผลของพิโนสโทรบินจากกระชายเหลือง *Boesenbergia pandurata*ต่อแอกทิวิตีของไคเนส Wee1 ในเซลล์ไลน์มะเร็งบางชนิด (EFFECT OF PINOSTROBIN FROM FINGERROOT *Boesenbergia pandurata* ON WEE1 KINASE ACTIVITY IN SOME CANCER CELL LINES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.ชุลี ยมภักดี, 117 หน้า.

Fingerroot *Boesenbergia pandurata* หรือ กระชายเหลือง จัดอยู่ในวงศ์ Zingiberaceae จากงานวิจัยก่อนหน้า ได้แยกสารบริสุทธิ์จากกระชายเหลือง คือ พิโนสโทรบิน โดยพบว่ามีฤทธิ์ในการยับยั้งวิถีการส่งสัญญาณแคลเซียมในยีสต์ *Saccharomyces cerevisiae* สายพันธุ์กลายที่ขาดยีน *ZDS1* (Δ*zds1*) จากการวิเคราะห์หาระดับโปรตีนด้วยวิธีเวสเทิร์นบลอต พบว่า พิโนสโทรบินมีผลไปลดระดับและกิจกรรมของไคเนส Swe1ในวิถีการส่งสัญญาณแคลเซียมในยีสต์ ทั้งนี้ พบว่ายีน *SWE1* ในยีสต์ *S. cerevisiae* มีหน้าที่เช่นเดียวกันกับยีน *WEE1* ในมนุษย์ จุดประสงค์ของงานวิจัยนี้คือเพื่อศึกษาฤทธิ์ต้านการเพิ่มจำนวนเซลล์ของพิโนสโทรบินต่อเซลล์ไลน์มะเร็งบางชนิดและผลของพิโนสโทรบินที่มีต่อแอกทิวิตีของไคเนส Wee1 ในเซลล์ไลน์ชนิด Jurkat T-cell (เซลล์ไลน์มะเร็งเม็ดเลือดขาวชนิดลิมโฟไซท์), BT474 (เซลล์ไลน์มะเร็งเต้านม) และ KATO III (เซลล์ไลน์มะเร็งกระเพาะอาหาร) จากผลการทดลองพบว่าพิโนสโทรบินมีฤทธิ์ความเป็นพิษแบบเรื้อรังในการต้านการเพิ่มจำนวนของเซลล์ไลน์ Jurkat T-cell, BT474 และ KATO III โดยวิธี MTT assay มีค่า IC$_{50}$ เท่ากับ 51.2 ± 1.61, 61.9 ± 1.15 และ 24.7 ± 4.5 ไมโครโมลาร์ตามลำดับ ในทางตรงกันข้ามพิโนสโทรบิน ไม่มีความเป็นพิษทั้งต่อเซลล์เม็ดเลือดแดงและเซลล์เม็ดเลือดขาวของคนปกติ จากการวิเคราะห์หาระดับโปรตีนด้วยวิธีเวสเทิร์นบลอต พบว่า พิโนสโทรบินสามารถลดระดับและแอกทิวิตีของไคเนส Wee1 (ระดับของ P-Cdc2) ทั้งในเซลล์ไลน์ Jurkat T-cell, BT474 และ KATO III นอกจากการลดแอกทิวิตีของไคเนส Wee1 โดยพิโนสโทรบินแล้ว พิโนสโทรบินยังไปกดการแสดงออกของยีน *WEE1* โดยเฉพาะอย่างยิ่งในเซลล์มะเร็งชนิด Jurkat T-cell เมื่อวัดด้วยวิธี quantitative real-time PCR นอกจากนี้พิโนส-โทรบินยังมีผลต่อเซลล์ไลน์ที่แตกต่างกัน โดยไปเพิ่มประชากรเซลล์ในระยะ subG1 ในเซลล์ไลน์ Jurkat T-cell และ BT474 โดยที่ไม่มีผลในการหยุดหรือชะลอระยะเซลล์ในระยะใดระยะหนึ่ง ในขณะที่สามารถหยุดวัฏจักรการแบ่งเซลล์อยู่ที่ระยะ G2/M ในเซลล์ไลน์ KATO III อีกทั้งยังเหนี่ยวนำให้ให้เซลล์เกิดการตายแบบอะพ็อพโตซิสเมื่อย้อมด้วยสี Annexin V ในเซลล์ไลน์ทั้งสามที่ไวต่อพิโนสโทรบิน ที่น่าสนใจเป็นอย่างยิ่งคือพิโนสโทรบินยังช่วยเพิ่มประสิทธิภาพในการต้านมะเร็งเมื่อใช้ร่วมกับยาที่มีฤทธิ์ทำให้ดีเอ็นเอเกิดความเสียหายได้แก่ gemcitabine และ camptothecin โดยส่งผลแบบเสริมกันในการเหนี่ยวนำให้เซลล์เกิดการตายแบบอะพ็อพโตซิส

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต ....
สาขาวิชา	จุลชีววิทยาทางอุตสาหกรรม	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
ปีการศึกษา	2558	

5571944023 : MAJOR INDUSTRIAL MICROBIOLOGY

KEYWORDS: WEE1 INHIBITOR / PINOSTROBIN / ANTICANCER / CELL CYCLE / APOPTOSIS / JURKAT T-CELL / BT474 / KATO III

JUMPOL SOPANAPORN: EFFECT OF PINOSTROBIN FROM FINGERROOT *Boesenbergia pandurata* ON WEE1 KINASE ACTIVITY IN SOME CANCER CELL LINES. ADVISOR: ASST. PROF. CHULEE YOMPAKDEE, Ph.D., 117 pp.

Fingerroot *Boesenbergia pandurata* or Krachai lueng (in Thai), is classified in Family Zingiberaceae. Previous study, pinostrobin a pure compound was isolated from *B. pandurata* and identified as an inhibitor of calcium signaling pathway in *ZDS1* null mutant yeast *Saccharomyces cerevisiae* ($\Delta zds1$). Western blot analysis showed that, pinostrobin decreased the expression level and activity of Swe1 in the yeast calcium signaling pathway. *SWE1* in *S. cerevisiae* is an ortholog gene of *WEE1* in human. The aim of this research was to determine the anti-proliferative activity of pinostrobin on many human cancer cell lines and the effect of pinostrobin on level and activity of Wee1 kinase in Jurkat T-cell (Human acute T cell leukemia), BT474 (Human breast carcinoma cell line) and KATO III (Human stomach carcinoma cell line). The results revealed that pinostrobin showed chronic cytotoxicity effect against Jurkat T-cell, BT474 and KATO III as determined by MTT viability assay at IC_{50} values of 51.2 ± 1.61 μM, 61.9 ± 1.15 μM and 24.7 ± 4.5 μM, respectively. On the other hand, pinostrobin did not show cytotoxic effect to normal human red blood cells and white blood cells. Western blot analysis showed that pinostrobin could down regulate the level of Wee1 kinase as well as Wee1 kinase activity (P-Cdc2 level) in Jurkat T-cell, BT474 and KATO III. Consistent with down-regulation of Wee1 kinase activity, pinostrobin also down-regulated *WEE1* expression as revealed by quantitative real-time PCR especially in Jurkat T-cells. The pinostrobin treatment affected the sensitive cancer cell lines differently. The treatment caused increase in sub G1 of Jurkat T-cell and BT474. However, the profile of cell cycle progression did not affect, while arrested KATO III in G2/M phase. In addition, pinostrobin induced apoptotic cell death by Annexin V staining in the pinostrobin sensitive cell lines. Interestingly, pinostrobin synergistically enhanced the antitumor efficacy of various DNA-damaging agents including gemcitabine and camptothecin on apoptotic cells death induction.

Department: Microbiology   Student's Signature _____

Field of Study: Industrial Microbiology   Advisor's Signature _____

Academic Year: 2015

ACKNOWLEDGEMENTS

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CHAPTER I
INTRODUCTION

Cancer is a leading cause of death in human and increasingly important factor in the global burden of disease in the decades to come. The approximate number of new cancer patients each year is expected to increase from 10 million in 2000 to 15 million by 2020 (World Health Organization, 2015). The most common causes and factors for cancer are such as sun or UV exposure, ionizing radiation, exposure to chemicals and other carcinogens. Biological causes are infection of certain viruses and bacteria, certain hormones or family history of cancer. Chemotherapy is the use of drug to kill the cancer cells by inhibiting the cancer cells from proliferation and metastasis to other part of the body. Treatment of cancer cells by chemotherapy is highly effective. However, it often results in side effects such pain, mouth and throat sores, blood disorders or nervous system, depend on each individual patient and type of cancer.

Currently, there are many researches on biological activities of compounds isolated from medicinal plants for therapy of various diseases. Modern medicines though effective many problems such as undesired side effects, rapidly evolving drug resistance, and the lack of effective drugs against many existing to disease, as well as emerging diseases. Scientists and researchers, are studying on development of the novel screening systems to increase opportunities to find a new medicinal compounds from natural products so as to further develop to therapeutic drugs.

Calcium ions (Ca^{2+}) are important signaling molecules. Once they enter the cytoplasm they exert allosteric regulatory effects on many enzymes and proteins. Ca^{2+} signaling play key roles in the regulation of diverse cellular processes, such as T-cell activation, secretion, muscle contraction and neurotransmitter release (Clapham, 1995). In case the calcium signaling has defected, it affected various cellular functions and led to symptoms such as muscular dystrophy, heart disease, alzheimer's disease, type II diabetes and various types of the cancer (Parkash and Asotra, 2010). Small-molecule inhibitors of Ca^{2+} signaling pathway in human are of great medical importance. Since Ca^{2+} signaling in mammalian cells plays pivotal roles in the regulation of diverse cellular processes, including T-cell activation, secretion, motility and apoptosis (Clapham, 1995). The main protein player in Ca^{2+} signaling pathway is calcineurin, a

protein phosphatase involved in activation of the nuclear factor of activated T cell (NF-AT) which is a transcription factor required for the expression of cytokine genes in T cells (Im and Rao, 2004). Calcineurin inhibitors, FK506 and cyclosporin A, are widely used as potent immunosuppressants (Barten et al., 2003). In addition, several cell-cycle inhibitors showed potential as anti-cancer agents (Crews and Shotwell, 2003). Many small-molecule inhibitors elicit their physiological effects through the common mechanisms from yeast to mammalian cells by acting on the evolutionally conserved target molecules. Thus, searching for inhibitors in the Ca^{2+}-signaling pathways in yeast could be one of the powerful means for develop to a novel methodologies for the drug screening and isolating new bioactive compounds (Shitamukai et al., 2000).

Wangkangwan et al. (2009) has demonstrated that the crude extract from fingerroot *B. pandurata* exerted the strongest biological activity on Ca^{2+}-signaling pathways in *S. cerevisiae* using the Δ*zds1* proliferation assay. Δ*zds1*-assay activity guided fractionation led to obtain a pure active compound, pinostrobin which also showed inhibitor of Ca^{2+}-signaling pathways in yeast.

There are high conservation of genes involved in various important biological processes from yeasts to humans (Barrientos, 2003). Among those, *SWE1* in *S. cerevisiae* is an ortholog gene of *WEE1* in human. Wee1 is a tyrosine kinase (Russell and Nurse, 1987) that plays role on regulating the transition between the G2 and M phases in human. Wee1 negatively regulates entry into mitosis by phosphorylating the Tyr15 residue of Cyclin Dependent Kinase 1(CDK1, also known as CDC2), thus inactivating the CDK1/cyclin B complex, arresting the cell cycle. Overexpression of Wee1 has previously been reported in various cancers such as osteosarcoma, glioblastoma and breast cancer (Iorns et al., 2009, Mir et al., 2010, PosthumaDeBoer et al., 2011).

There were several reports on anti-proliferative effect of pinostrobin on various human cancer cell lines. Smolarz et al. (2006) revealed that pinostrobin isolated from *Polygonum lapathifolium* can induce apoptosis on Jurkat and HL60 cell lines. Ashidi et al. (2007) showed that pinostrobin from *Cajanus cajan* (L.) Mill sp. (Leguminosae) exhibited significant inhibition in a cell proliferation assay in human CCRF-CEM leukaemia cells and generated reactive oxygen species (ROS) in the ROS assay. Furthermore, Le Bail et al. (2000) found that pinostrobin possess anti-aromatase

activity and decreased the growth of MCF-7 cells induced by dehydroepiandrosterone sulfate and estradiol. However, the molecular mechanism of pinostrobin on anti-proliferation in some cancer cell lines has not yet been elucidated.

The aims of this research was determined molecular mechanism of pinostrobin on anti-proliferation and evaluated its role on the affected cancer cell lines.

1.1 Research methodologies

1.1.1 To study the effect of the pinostrobin on the anti-proliferation of human cancer cell lines (Jurkat T-cell, BT474 and KATO III) by MTT proliferation assay.

1.1.2 To determine the cytotoxic effect of pinostrobin on normal white blood cells by MTT Proliferation assay and red blood cells by hemolytic activity assay.

1.1.3 To examine the effect of pinostrobin on level and activity of Wee1 kinase in human cancer cell lines by Western blot analysis.

1.1.4 To investigate the effect of pinostrobin on cell cycle progression in the affected cancer cell lines by flow cytometry.

1.1.5 To evaluate the effect of pinostrobin on induction of apoptotic cell death in the affected cancer cell lines by flow cytometry.

1.1.6 To investigate the effect of pinostrobin treatment in combination with some DNA-damaging agents on anti-proliferative activity, cell cycle progression and induction of apoptotic cell death.

1.2 Expected Outcomes

- Understand the molecular mechanism of pinostrobin on its anti-proliferative activity against various human cancer cell lines.

CHAPTER II
LITERATURE REVIEWS

2.1 Overview of cancer

Cancer is a major public health problem worldwide. World Health Organization (WHO) reported that in 2011, there were new cancer cases about 12.7 million and 7.6 million or 13.6% for the deceased population from new cases. The five most common cancers in the world include lung, breast, colon, stomach and prostate cancer. New cases of 12.7 million classified into 1.6 million of lung cancer, 1.2 million of colon cancer, 0.99 million of stomach cancer and 0.9 million of prostate cancer. While 7.6 million of deceased were comprised of 1.37 million of lung cancer, 7.36 hundred thousand of stomach cancer, 6.95 hundred thousand of liver cancer, 6.08 hundred thousand of colon cancer and 4.58 hundred thousand of breast cancer. WHO extrapolated that in 2030 new cases of cancer is predicted at 21.4 million and death to about 13 million.

National Cancer Institute in Thailand (2015) reported that, the five most common cancer in male are bronchus and lung, colon and rectum, liver and bile ducts, lip and oral, cavity and esophagus. While in female, the five most common cancer are breast, cervix uteri, colon and rectum, bronchus and lung and liver and bile ducts (Fig. 2.1). Furthermore the table 2.1 shows the number of new cancer patients by age group and sex in 2012.

Table 2.1 Number of new cancer patient by age group and sex in 2012

Age group	Male		Female		Total	
	No.	%	No.	%	No.	%
0 – 4	9	0.23	11	0.28	20	0.51
5 – 9	7	0.18	5	0.13	12	0.31
10 – 14	10	0.26	6	0.15	16	0.41
15 – 19	13	0.33	6	0.15	19	0.49
20 – 24	14	0.36	13	0.33	27	0.69
25 – 29	17	0.43	32	0.82	49	1.25
30 – 34	32	0.82	72	1.84	104	2.66
35 – 39	48	1.23	133	3.40	181	4.62
40 – 44	82	2.09	249	6.36	331	8.45
45 – 49	135	3.45	343	8.76	478	12.20
50 – 54	191	4.88	359	9.17	550	14.04
55 – 59	229	5.85	354	9.04	583	14.88
60 – 64	221	5.64	280	7.15	501	12.79
64 – 69	186	4.75	213	5.44	399	10.19
70 – 74	165	4.21	153	3.91	318	8.12
75 +	195	4.98	134	3.42	329	8.40
Total	**1,554**	**39.67**	**2,363**	**60.33**	**3,917**	**100**

(National Cancer Institute in Thailand, 2015)

A)
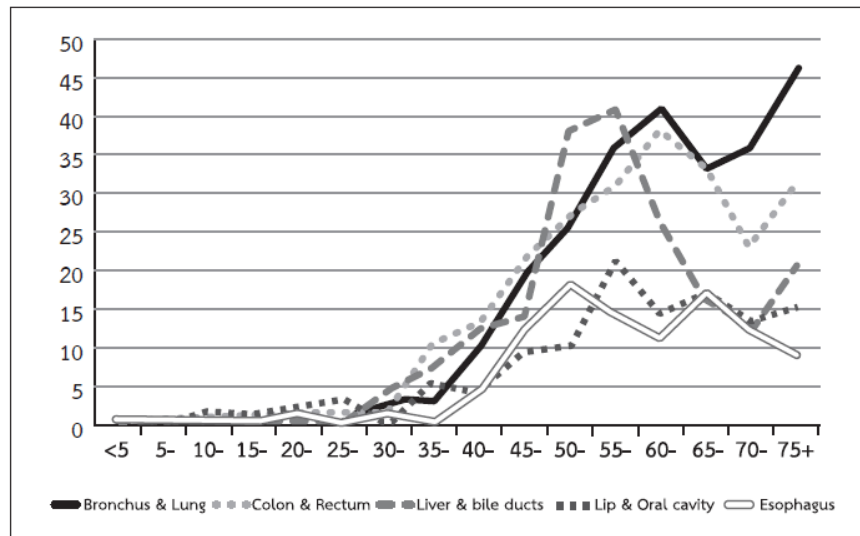
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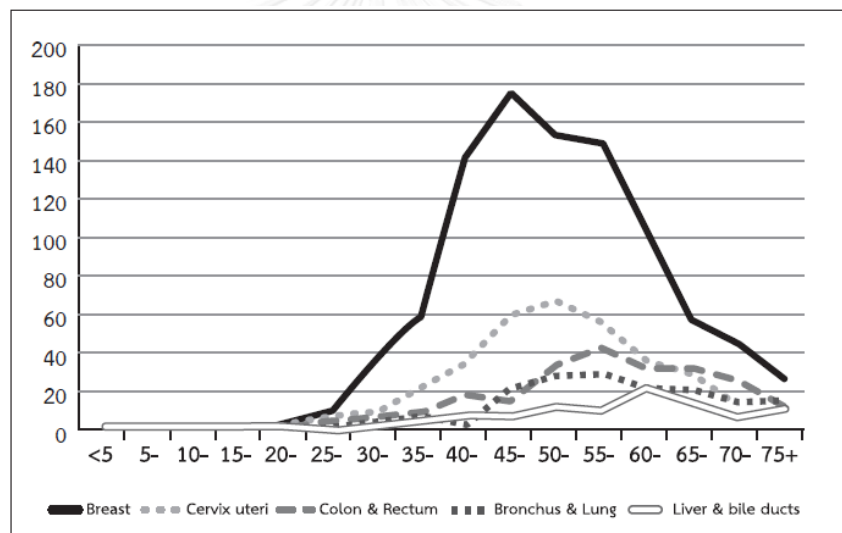
Figure 2.1 The leading site of cancer by sex in male A) and female B) in 2012 (National Cancer Institute in Thailand, 2015)

2.2 Causes of cancer

Cancer is a general name for abnormal cell, when cell in a part of the body start to grow out of the control. The underlying mechanism of cancer is DNA in the cell was damaged from oxidative damage, hydrolytic damage, free radicals or DNA strand breaks. In a healthy cell, cell can repairs DNA damage or die in cause of fail to repair. But the cancer cell, cannot repair the DNA damage and thus ignore the signal to stop

dividing cell or processes such as programmed cell death, or apoptosis. Therefor cancer cell can multiply and spread to the other part of the body. Risk factors for cancer are such as

1. Genetic factors. Many researches revealed that some genes overexpression were associated with proliferation of cancer cells. *HIWI* gene for example is member of *PIWI* gene family was found overexpressed in esophageal squamous cell carcinoma, seminomas and grastic cancer (Qiao et al., 2002, Liu et al., 2006, He et al., 2009). Matrix metalloproteinase-13 (*MMP-13* gene) was significantly up regulated in the steatotic liver, colorectal cancer and oesophageal cancer (Etoh et al., 2000, Yamada et al., 2010, Said et al., 2014, Mendonsa et al., 2015). Human epidermal growth factor receptor 2 (*HER2* gene) is a member of the epidermal growth factor receptor family by tyrosine kinase activity. *HER2* gene is overexpressed in breast cancer (Press et al., 1993, Shou et al., 2004).

2. Infection with a variety of viruses, bacteria and parasites. Das et al. reported that BK virus (BKV) was presented as a cofactor in the cause of prostate cancers (Das et al., 2004). JC virus (JCV), has been suggested to play a role on oncogenesis in the colon and central nervous system (Maginnis and Atwood, 2009). Bacterial strains are associated with various type of cancers such as *Fusobacterium nucleatum,* which has emphasized potential pathogenic relevance of the colonization of malignant tissues and leading to colorectal cancer (Kostic et al., 2013, Rubinstein et al., 2013). *Helicobacter pylori*, can infect and colonizes at stomach and cause gastric adenocarcinoma (Correa and Piazuelo, 2011). *Chlamydia pneumonia* was reported to be related with increased risk for lung cancer (Littman et al., 2005, Chaturvedi et al., 2010).

3. Carcinogen is a substance, radionuclide, or radiation. It is an agent directly leading to cause of cancer. Carcinogen might damage the genome or disrupt the metabolic processes in to the cell. Previous research showed that many of carcinogen such as alcohol, aflatoxin, tamoxifen or 2-napthylamine can induce the normal cell to become cancer cell. Table 2.2 shows the list of carcinogens and the cancer they cause.

Table 2.2 List of carcinogen and the cancer they cause

Agent	Cancer type
Benzo[a]-pyrene(Tobacco)	Lung
Alcohol	Mouth, Pharynx, Larynx, Esophagus
Dietary Fat	Breast
Asbestos	Respiratiry-tract
Estrogens	Endometrial, Ovarian, Breast
Tamoxifen	Endometrial
X-radiation and Gamma radiation	Leukemia, Thyroid, Lung, Colon and
Aflatoxin	skin
Nickel(Nickle refining)	Liver
Wood dust(wood working)	Lung and Nasal
2-napthylamine	Nasal
Mustard gas	Bladder,Respiratiry-tract and Lung

(Landrigan et al., 1999, Anderson, 2006, Ke et al., 2008)

2.3 Treatment for cancer

There are many methods used for treatment of cancer. The major method for treat the cancer such as (1) surgery, which a common method for treatment by removing the tumor tissue and normal tissue surrounding tumor tissue. (2) Chemotherapy, is the use of medicine to kill the cancer cells. (3) Radiotherapy or radiation treatment, this treatment kills the cancer cells by use X-rays from outside the body or inside the body via a liquid either injected or swallowed. (4). Hormonal therapy treats the cancer cells by lower the level of hormone in the body. (5). Monoclonal antibodies, this method is designed to a direct target in cancer cells. However above methods have some of disadvantages such as killing of the normal cell surround cancer cells, cost, internal organ can be damaged from surgery, increased risk of infections and affect to immune system in the body.

2.4 Natural plant a resources of compound for anti-cancer therapy

At present, researchers are interested in screening bioactive compounds from various plants for discovery of a novel drug. Plants are sources of drugs and secondary metabolites. Surprisingly, many bioactive compounds possess potential activity to anti-proliferation of cancer cells (Table 2.3). While the conventional chemotherapeutic agents have been shown to be implicated with unacceptable toxicity. Plant bioactive compounds, are new alternative for treatment of the cancer cell and developed to anti-cancer drug.

Table 2.3 List of compounds for anti-cancer from plants

Anti-cancer compound	Species and genus name	Type of cancer	Mode of action	Reference
Vinca alkaloids	*Catharanthus roseus* G. Don. (Apocynaceae)	Leukemia lymphomas, breast and lung cancer,	Mitotic block	(Okouneva et al., 2003, Simoens et al., 2008)
Podophyllotoxin	*Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich	Lymphomas, bronchial and testicular cancers	-	(Shoeb et al., 2005)
Taxanes	*Taxus brevifolia* Nutt. (Taxaceae)	Lung and prostate cancer	Mitotic block	(Hait et al., 2005)
Berbamine	*Berberis amurensis*	Leukemia	Induced apoptosis	(Xu et al., 2006, Xie et al., 2009)
Beta-lapachone	*Tabebuia avellanedae*	Breast, prostate , lung and pancreatic cancer	Inhibiting Topoisomerase I and II	(Almeida, 2009)

Table 2.3 List of compound for anti-cancer from plants (Cont.)

Anti-cancer compound	Species and genus name	Type of cancer	Mode of action	Reference
Colchicine	*Colchicum autumnale*	leukemic and solid tumor	Anti-mitotic	(Dubey et al., 2008)
Combretastatin A-4	*Combretum caffrum* Kuntze	Phase II clinical trials	Tubulin structure disruption	(Thomson et al., 2006, Ley et al., 2007)
Curcumin	*Curcuma longa*	Colorectal cancer, multiple myeloma and pancreatic cancer	Exact mechanism of action is still unknown	(Goel et al., 2008, Gaurisankar et al., 2010)
Daphnoretin	*Wikstroemia Indica*	Ehrlich ascites carcinomas	Suppression of protein and DNA synthesis	(Diogo et al., 2009, Lu et al., 2011)
Diadzein and Genistein	*Lupinus* species, *Vicia faba, Glycine max, Psoralea corylifolia*	Breast, stomach, bladder, lung and prostate cancer	Inhibits 3A 4-mediated metabolism and oxidative metabolism	(Kaufman et al., 1997, Dixon and Ferreira, 2002, Moon et al., 2006)
Emodin	Rhizome of rhubarb	Lung, liver, ovarian and blood cancer	Induced apoptosis	(Huang et al., 2009)
Flavopiridol	*Amoora rohituka* and *Dysoxylum binectariferum*	Colorectal, non-small cell lung cancer, renal cell carcinoma	Inhibits cell cycle progression at G1 or G2 phase	(Mans et al., 2000)

Table 2.3 List of compound for anti-cancer from plants (Cont.)

Anti-cancer compound	Species and genus name	Type of cancer	Mode of action	Reference
Indirubin	Chinese herb, Danggui Longhui Wan	Chronic myeloid leukemia	Inhibits cyclin dependent kinases	(Nam et al., 2005)
Ingenol 3-oangelate	*Euphorbia peplus* L.	Actinic keratosis and basal cell carcinoma	Causes necrosis of tumor	(Hampson et al., 2005)
4-Ipomeanol	*Ipomoeca batatas*	Lung cancer	Cytochrome P-450-mediated conversion into DNA-binding metabolites	(Ancuceanu and Istudor, 2004)
Salvicine	*Salvia prionitis* Hance	Malignant tumors	Inhibition of topoisomerase II	(Deng et al., 2011)
Schischkinnin	*Centaurea schischkinii*	Colon cancer lines *in vitro*	-	(Shoeb et al., 2005)
Montamine	*Centaurea Montana*	CaCo2 colon cancer cell line *in vitro*	-	(Shoeb et al., 2006)
Silvestrol	*Aglaia foveolata* Panell	Prostate, breast and lung cancers	Apoptosome/ Mitochondrial pathway	(Kim et al., 2007, Kinghorn et al., 2009)

2.5 Calcium signaling pathway

Calcium ions (Ca^{2+}) is a secondary messenger and an important signaling molecules. Once Ca^{2+} enter the cytoplasm they exert allosteric regulatory effects on many enzymes and proteins. Ca^{2+} signals play key roles in the regulation of diverse cellular processes, such as T-cell activation, secretion, muscle contraction and neurotransmitter release. If the calcium signaling was defected, it affected various cellular functions and led to muscular dystrophy, heart disease, alzheimer's disease, type II diabetes and various types of the cancer (Parkash and Asotra, 2010).

Small-molecule inhibitors of Ca^{2+} signaling pathway in human are of great medical importance, since Ca^{2+} signaling in mammalian cells plays pivotal roles in the regulation of diverse cellular processes, including T-cell activation, secretion, motility and apoptosis (Clapham, 1995). A key protein in calcium signaling pathway is calcineurin, a protein phosphatase involved in activation of the nuclear factor of activated T cell (NF-AT) which is a transcription factor required for the expression of cytokine gene in T cells (Im and Rao, 2004). The calcineurin inhibitors, FK506 and cyclosporine A, are widely used as potent immunosuppressants (Barten et al., 2003). In addition, several cell-cycle inhibitors have potential as anti-cancer agents (Crews and Shotwell, 2003). Many small-molecule inhibitors elicit their physiological effects through the common mechanisms from yeast to mammalian cells by acting on the evolutionarily conserved target molecules.

In *S. cerevisiae*, the Ca^{2+}-signals are implicated in the regulation of the G2/M cell-cycle progression (Mizunuma et al., 1998) (Fig. 2.2). Swe1 kinase specifically inhibits a G2 form of the Cdc28 cyclin-dependent protein kinase by phosphorylating it at Tyr-19 and delays the entry into mitosis. The cell-cycle regulation by Ca^{2+} is executed through the activation of the two parallel pathways, calcineurin and Mpk1 mitogen-activated protein (MAPK) kinase cascade, and these two pathways cooperatively activate Swe1. The effect of high concentration of $CaCl_2$ in the culture medium, which leads to the activation of the cellular Ca^{2+}-signaling pathway, on the cell-cycle regulation is particularly obvious in the genetic background of Δ*zds1* strain.

The activation of Ca^{2+}-signaling eventually leads to severe growth retardation which is accompanied by polarized bud growth and G2 cell-cycle arrest. These physiological effects are caused by the activation of Swe1, a negative regulatory kinase that phosphorylates Cdc28 of the G2 cell-cycle engine. (Mizunuma et al., 1998). A novel yeast-based 'positive screening' assay has been developed for convenient drug-screening procedure in yeast (Shitamukai et al., 2000). Screening of microbial metabolites for activity alleviating the deleterious physiological effects of external $CaCl_2$ identified the Hsp90 inhibitor, radicicol, as an inhibitor of Ca^{2+}-signal-dependent cell-cycle regulation in yeast (Chanklan et al., 2008). Radicicol alleviated analogous physiological effects due to the expression of a constitutively active form of calcineurin or overexpression of Swe1. Western blot analysis indicated that radicicol inhibited Ca^{2+}-induced accumulation of Swe1 and Clb2 (Chanklan et al., 2008).
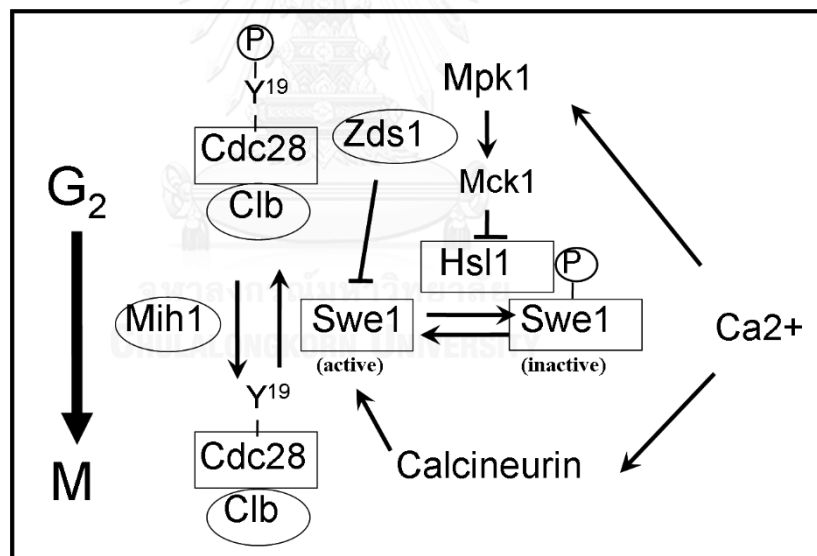
Figure 2.2 Model of Ca^{2+}-signaling pathways in *S. cerevisiae*

From: Mizunuma et al., 1998

2.6 Fingerroot *Bosenbergia pandurata*

Boesenbergia pandurata or the finger root plant, is a herb that belongs to the Zingiberaceae family. The fresh rhizomes are commonly used in Southeast and South Asia as a food ingredient and in Traditional Thai Medicine to maintain wellness, treat stomach discomfort, aphthous ulcer, anti-flatulence, diuresis, leukorrhea, oral diseases and dysentery.

Wangkangwan et al. (2009) utilized the $\triangle zds1$ yeast -based assay to search for an inhibitor of the Ca^{2+}-signaling pathway in yeast by screening of 141 crude medicinal plant extracts. The ethanol extract of fingerroot *Boesenbergia pandurata* was one of the strong positive samples from the screens (Wangkangwan et al., 2009). The yeast-based assay was used to guide fractionation and purification of the crude *B. pandurata* extract. A flavonoid 5-hydroxy-7-methoxy flavanone so-called pinostrobin, was identified. Pinostrobin was further studied and reported as an inhibitor of Ca^{2+}-signal-mediated cell-cycle regulation in the *S.cerevisiae* (Wangkangwan et al., 2009).

Suksawatamnuay (2009) extended the study of Wangkangwan et al. (2009) on the biological activity of pinostrobin in *S. cerevisiae*. It was found that pinostrobin treated yeast cells resulted in reduced the *SWE1* expression as well as Swe1 activity as compared to those in the untreated experiments, indicating that Swe1 kinase is the target protein of pinostrobin in the Ca^{2+}-signaling pathway of *S. cerevisiae*. In *S. cerevisiae,* Swe1 kinase is a negative regulator of Cdc28 kinase. It phosphorylates Cdc28 of the G2 cell-cycle engine. Previously, radicicol, a known inhibitor of Hsp90 and an anti-tumor agent, was demonstrated to be involved in inhibiting the Ca^{2+}-signaling pathway in the yeast-based assay (Chanklan et al., 2008). It was reported that radicicol acts at downstream of *SWE1* by reducing Swe1 level (Chanklan et al., 2008).

There are several reports on the activity of pinostrobin. Wu et al. (2002) reported that pinostrobin can inhibit Cyclooxygenase (COX) I and COX II in prostaglandin generation in inflammation process. Moreover, pinostrobin exhibited anti-oxidant activity (Kong et al., 2009), in *vivo* anti-ulcerogenic activity (Abdelwahab et al., 2011). Sukardiman et al. (2000) suggested one of the molecular mechanism of pinostrobin from *Temu kunci* (*Kempferia pandurata*) in human mammary carcinoma by inhibition of DNA topoisomerase I activity.

Trakoontivakorn et al. (2001) reported that pinostrobin has the antimutagenic activity from 3-amino-1-methyl-5*H*-pyrido [4,3-*b*]indole (Trp-P-2) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Furthermore pinostrobin exhibited anti-*Holicobacter pllori* activity and inhibited Herpes simplex virus-1, (Bhamarapravati et al., 2006, Wu et al., 2011). Pinostrobin from *Renealmia alpinia* inhibited the hemolytic activity and proteolytic activity of *Bothrops asper* venom (Gomez-Betancur et al., 2014). Pinostrobin from *Cajanus cajan (L.)* Mill sp. inhibit the TNF-α and IL-1β in RAW 264.7 cells (abelson murine leukemia virus-induced tumor) and J774a.1 cells (reticulum cell sarcoma) and inhibit voltage-channel of sodium in mammalian brain (Nicholson et al., 2010, Patel and Bhutani, 2014). It was found that pinostrobin from honey showed induction of mammalian phase 2 detoxification enzyme activity and antioxidation enzyme and quinone reductase (Fahey and Stephenson, 2002). Moreover pinostrobin from *Teloxys graveolens* (Chenopodiaceae) inhibited intestinal smooth muscle contractions in Guinea-pig ileum (Meckes et al., 1998).

There were several reports on anti-proliferative effect of pinostrobin on various human cancer cell lines. Smolarz et al. (2006) revealed that pinostrobin isolated from *Polygonum lapathifolium* induced apoptosis on Jurkat and HL60 cell lines. Ashidi et al. (2007) reported that pinostrobin from *Cajanus cajan* (L.) Mill sp. (Leguminosae) exhibited significant inhibition on a cell proliferation assay in human CCRF-CEM leukaemia cells and generated reactive oxygen species (ROS) in the ROS assay. Le Bail et al. (2000) found that pinostrobin possessed anti-aromatase activity and decreased the growth of MCF-7 cells induced by DHEAS and estradiol. However, the molecular mechanism of pinostrobin on anti-proliferation in some cancer cell lines has not yet been demonstrated.

2.7 Cell cycle and DNA repair

Cell cycle checkpoints are required to maintain genomic stability in cells. When cells loss checkpoint system especially at G1 and G2 checkpoint in cancer cells, promote genomic instability (Williams and Stoeber, 2012) and delays the cell entry into mitosis. These are associated with ataxia-telangiectasia mutated (ATM) protein kinase or ataxia- telangiectasia related (ATR). Cells monitor the integrity of their DNA at the

G1 to S phase and G2 to M phase (Kastan and Bartek, 2004). Thus, after the first checkpoint at G1, the cell cycle can be stopped again at the G2 checkpoint. P53 is a protein in the G1 checkpoint and induces the expression of p21, leading to cell cycle arrest for DNA repair.

The G2 checkpoint is an important cell cycle checkpoint. That checkpoint helps to maintain genomic stability when DNA was damaged. P53 assists in the regulation of the G2 checkpoint by inhibiting cyclin B and cyclin-dependent kinase 1 (Cdk1), or referred to Cdc2. ATM is a protein kinase which phosphorylates and activates the checkpoint kinase 2(Chk2), lead to suppression of the cell division cycle 25C phosphatase (Cdc25) by phosphorylation at Ser216. Down regulation of Cdc25 phosphatase activity affects inhibitory activity of Cdc2/cyclin B complex, maintaining the Cdk1 in an inactive form and preventing cell entry into mitosis. ATR is a protein kinase that phosphorylates and activates the checkpoint kinase 1 (Chk1). Chk1 can be activated by both ATM and ATR pathways, it can phosphorylate to activate the Wee1 activity and inactivate the Cdc25 phosphatase activity (Do et al., 2013).

Wee1 is a tyrosine kinase which plays role on regulating the transition between the G2 and M phases in human (Russell and Nurse, 1987). Wee1 negatively regulates the entry of the cell cycle into mitosis by phosphorylating the Tyr15 residue on Cdc2, thus inactivating the Cdc2/cyclin B complex and arresting the cycle. In contrast, the Cdc2 activity is balanced being activated by Cdc25 (Parker and Piwnica-Worms, 1992) (Fig. 2.3).

In several cancer cells, G1 checkpoint is defected. This might be a result from mutation of p53, thus cells with DNA damage can proceed from G1 to the S phase. However G2 checkpoint in cancer cells can check and repair the DNA damage before entry to M phase. Interference in the G2 arrest mechanism can lead to progression into mitosis with damaged DNA, resulting in mitotic disaster and cell death.
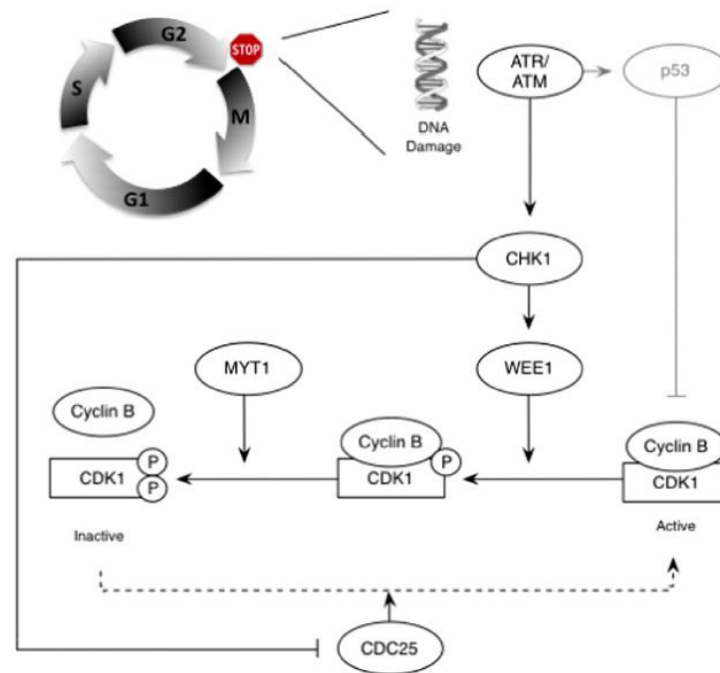
Figure 2.3 G2 checkpoint in mammalian cell. Cyclin B/CDK1 complex is active and lead to the cells entry to M phase. DNA damage Induces Myt1 and Wee1 to phosphorylate at amino acids Y15 and T14 in Cdk1. Then, cyclin B is ineffective to associate with Cdk1after that the G2 checkpoint is active, interrupt entry into M phase. Cdc25 can dephosphorylate Cdk1and activated cell cycle progression. P53 is a protein that can inhibit the cyclinB/Cdk1 complex upon DNA damage.
From: Vriend et al., 2003.

2.8 Wee1 in cancer

Wee1 is a tyrosine kinase, controlling the cell cycle in G2/M phase by phosphorylation of Cdc2 at Y15 and Y37 of H2B (Histone subunit) in nucleosome before cells enter into mitosis for the repair of the DNA damage (Fig 2.4). Overexpression of Wee1 resulted in decrease in the histone level and led to inefficient chromatin packing, making the DNA more accessible to the DNA damage repair machinery and stimulating radio resistance (Liang et al., 2012).
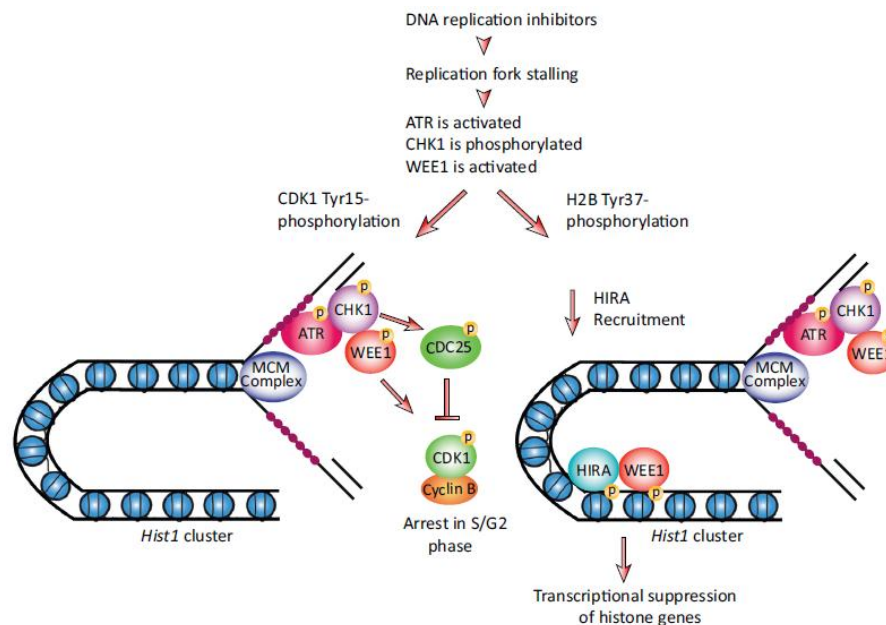
Figure 2.4 The phosphorylation of Wee1 on CDC2 and H2B arrests the cell cycle at G2 phase and down regulates histone gene transcription, respectively.

From: Mahajan and Mahajan, 2013.

Wee1 is up regulated in various cancers especially in p53-deficient cancers. Overexpression of Wee1 has previously been reported in various cancers. Wee1 expression increased in benign nevi, primary melanomas and metastatic melanomas, respectively (Magnussen et al., 2012) and high expression in the tested osteosarcoma (OS) tissue samples (PosthumaDeBoer et al., 2011). From data of microarray analysis, immunohistochemistry and quantitative PCR, revealed the high expression of Wee1 in glioblastoma (Mir et al., 2010). Wee1 was significantly up regulated in breast cancer (Wang et al., 2011). Furthermore the expression of Wee1 was correlated with cell survival in mantle cell lymphoma (Blenk et al., 2008). Iorns et al. (2009) reported that Wee1, is essential for viability of only in cancer cell lines that have an elevated level of expression of this kinase. Thus, inhibition of the Wee1 by Wee1 inhibitor is a new potential treatment strategy for anticancer therapy.

2.9 Known Wee1 inhibitors

PD-166285, is a potent Wee1 inhibitor that inhibits the phosphorylation at Y15 and T14 on Cdc2. However, PD-166285 was demonstrated to be a broad-spectrum tyrosine kinase inhibitor (Wang et al., 2011). Another known inhibitor, MK-1775 is a pyrazolo-pyrimidine derivative, as a small molecule inhibitor of the Wee1 tyrosine kinase with potential antineoplastic sensitizing activity. MK-1775 selectively targets and inhibits the activity of Wee1, a tyrosine kinase that phosphorylates Cdc2 to inactivate the Cdc2/cyclin B complex. Inhibition of Wee1 activity prevents phosphorylation of Cdc2 thus impair the G2 DNA damage checkpoint. There are several reports on MK-1775 activity such as Kreahling et al. (2012) supported that MK1775 showed the single agent antitumor activity against sarcoma cells by leading to unscheduled entry into mitosis and initiation of apoptotic cell death. Bridges et al. (2011) found that MK-1775 abrogated the radiation-induced G2 blocking in p53-defective cells and showed significantly enhanced antitumor efficacy of radiation in *vivo*. Kreahling et al. (2013) reported that MK-1775 treatment as a single agent at clinically relevant concentrations leads to unscheduled entry into mitosis and initiation of apoptotic cell death in all sarcomas tested. Guertin et al. (2013) revealed that MK-1775, as a single agent showed the effect to inhibit proliferation in A427 non-small lung cancer cell line.

PD-166285
MW = 512.43 g/mol

(A)

MK-1775
MW = 500.6 g/mol

(B)
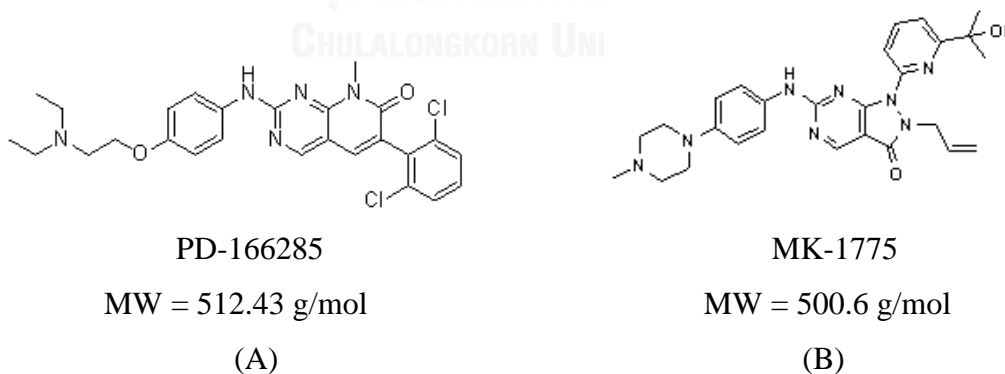
Figure 2.5 Structure of Wee1 inhibitor (A) PD-166285, (B) MK-1775
(Express, 2015, MedKoo Biosciences, 2015)

2.10 Combination of the Wee1 inhibitor with therapeutic agents

The phosphorylation of Wee1 was found to be associated with heat shock protein 90 (Hsp90). The Hsp90 is an essential molecular chaperone that is utilized by cancer cells to protect a number of overexpressed or mutated oncoproteins from misfolding and degradation (Trepel et al., 2010). Oncoproteins are proteins encoded by oncogenes which play important roles in cancer cell viability and tumor progression (Hede et al., 2014). For example, the overexpression of ErbB2 leads to increased breast cancer metastasis (Tan et al., 1997, Holbro et al., 2003). Activated Akt are involved cell growth and cell survival (Fresno Vara et al., 2004). Moreover the abnormality of Bcr-Abl is associated with the chronic myelogenous leukemia, CML (Primo et al., 2006). Recently, *in vitro* study showed that combination of Wee1 inhibitors and Hsp90 inhibitors is highly effective, Wee1 inhibitors seem to sensitize cancer cells to treatment with Hsp90 inhibitors (Mollapour et al., 2010). The combination of Wee1 inhibitor and Hsp90 inhibitors showed the increase in apoptotic cell and cell viability was found decreased in human prostate cancer cell line (Iwai et al., 2012). Furthermore Haarberg et al. (2013) reported that Wee1 inhibitor and Hsp90 inhibitor treatment in combination showed the significant level of apoptosis induction and growth inhibition.

There are a number of researches on combined treatment of MK-1775 with DNA-damaging agent showed synergistic effect on Wee1 inhibiton. Hirai et al. (2009) reported that MK-1775 can abrogates G2 DNA damage checkpoint, leading to apoptosis in combination with DNA-damaging chemotherapeutic agents such as gemcitabine, carboplatin, and cisplatin selectively in p53-deficient cells. MK-1775 treatment, solely lead to initiation of apoptotic cell death and unscheduled entry into M phase. Notably when MK-1775 was combined with gemcitabine, they significantly enhanced the cytotoxic effect in sarcoma cells lines (Kreahling et al., 2013). Moreover, MK-1775 showed enhanced effectiveness when combine with 5-fluorouracil, pemetrexed, doxorubicin, camptothecin and mytocin C, as observed by the increase of sub G1 population and caspase-3/7 in WiDr cells (Hirai et al., 2010). The combination of gemcitabine with MK-1775 showed promoted the mitotic entry of tumor cells and led to apoptotic cell death and delayed the tumor progression, compared to gemcitabine treatment (Rajeshkumar et al., 2011). Furthermore combination of MK-1775 and

panobinostat resulted in synergistic effect on antitumor activity in pancreatic cancer cell lines (Wang et al., 2015).

Aarts et al. (2012) found that combination treatment of MK-1775 and AZD7762 (Chk1 inhibitor) led to the synergistic effect forcing cell entry to M phase. Finally, MK-1775 in combination with MK-8776 (Chk1 inhibitor) enhanced inhibiting efficacy lead to apoptotic cell death in leukemic cells (Chaudhuri et al., 2014).

CHAPTER III
RESEARCH METHODOLOGY

3.1 Instruments used in this thesis

1. 37°C CO_2 incubator (Thermo Scientific, USA)

2. 37°C incubator (Thermo Scientific, USA)

3. Amersham Hyperfilm™ ECL (GE healthcare, Sweden)

4. Amersham™ Hybond™ 0.45 (GE healthcare, Sweden)

5. Autoclave MLS 3020 (Sanyo, Japan)

6. Balance AG285 PG2002-S and PB3002 (Mettler Toledo, Switzerland)

7. Beaker (Pyrex, USA)

8. Bench-top centrifuge 2600 (Denvelle, Germany)

9. Bottle for culture media (tissue culture) 100 ml (Corning Incorporation, USA)

10. Centrifuge tubes 15 and 50 ml (Corning Incorporation, USA)

11. Cover slips (Nissho Nipro, Japan)

12. Cryotube (NUNC™, Denmark)

13. Deep freezer -80°C ULT1786 (Forma Scientific, USA)

14. Deep freezer -20°C MDF-U332 (Sanyo Electric, Japan)

15. Disposable syringe (Nissho Nipro, Japan)

16. DNA Thermal Cycle T100™ (Bio-Rad, USA)

17. Filter paper (Whatman, England)

18. Gel Documentation and Quantity One version 4.4.1(Bio-Rad, USA)

19. Haemacytometer (Mettler Toledo, Switzerland)

20. Hot air oven UE (Memmert, Germany)

21. Hypercassatte™ (Amersham biosciences, UK)

22. Invert microscope (Olympus, USA)

23. Laminar flow Clean model V4 (LAB Service, Thailand)

24. Measuring cylinder (Pyrex, USA)

25. Microcentrifuge tube 15 and 50 ml (Corning Incorporation, USA)

26. Microcentrifuge tube 1.5 ml (Axygen Scientific, USA)

27. Micropipette P10, P20, P100, P200 and P1000 (Gilson, France)

28. Microplate reader Elx 800 (Bio-tek instrument, USA)

29. Microwave oven (Samsung, Korea)

30. MiniOpticon^{TM} Real-time PCR System (Bio-Rad, USA)

31. Optizen Nano Q (KAIA Bio-Ingenieria, Korea)

32. Parafilm (Parafilm®M, USA)

33. PCR tube 200 μl (Corning Incorporation, USA)

34. pH meter S-20K (Mettler-Toledo, Switzerland)

35. Pipette aid (Drummond, USA)

36. Polyvinylidine fluoride (PVDF) membrane 0.45 μm (GE healthcare, Sweden)

37. Power supply for electrophoresis (Bio-Rad, USA)

38. Refrigerator Tiara (Mitsubishi electric)

39. Seropipette 5 and 10 ml (Pyrex, USA)

40. SDS-polyacrylamide gel electrophoresis, Protein III System (Bio-Rad, USA)

41. Semi-dry electrophoretic transfer cell, Trans-Blot® SD (Bio-Rad, USA)

42. Sonicator RK100 (BANDELIN, Germany)

43. Stainless Alcohol burner

44. Syringe filter CA-CN 13 mm 0.45 μm (Restek, Thailand)

45. Thermo-block MylabTH Thermo-Block SLTDB-120 (Seoul in Bioscience, Korea)

46. Tissue culture Flask 25 and 75 cm^3 (Corning Incorporation, USA)

47. Tissue culture plate 6, 12, 24 and 96 well (NUNC^{TM}, Denmark)

48. Vortex mixer Genie II G-560E (Scientific Industries, USA)

49. Water bath (Memmert, USA)

3.2 Chemicals

1. Absolute ethanol (Lab Scan analytical science, USA)

2. Absolute methanol (Merck, Germany)

3. Acetic acid (Lab Scan analytical science, USA)

4. Acrylamide-solution (40%) Mix 37.5:1 (Sigma, USA)

5. Ammonium bicarbonate (Sigma, USA)

6. Ammonium persulfate (Bio Basic inc, Canada)

7. Anti-rabbit IgG, HRP-linked antibody; catalog#7074S (Cell signaling, USA)

8. ApopNexin^{TM} FITC (Merk, Germany)

9. BCA^{TM} protein assay (Thermo Scientific, USA)

10. β-mercapto-ethanol (Sigma, USA)

11. Bromophenol blue (Sigma, USA)

12. Clarity^{TM} Western ECL substrate (Bio-Rad, USA)

13. Chloroform (Lab Scan analytical science, USA)

14. Copper sulfate (Fisher Scienctific, UK)

15. Developer (Carestream Health Inc, USA)

16. DNA ladder 100 bp (Fermentas, Canada)

17. Diethylpyrocarbonate (DEPC) (Sigma, USA)

18. Dimethyl sulfoxide (DMSO) (Amesco, USA)

19. dNTPs mix (Fermentas, Canada)

20. Fetal Bovine Serum (FBS) (Hyclone, UK)

21. Ficoll-Hypaque (GE healthcare Bio-Sciences, UK)

22. Fixer (Kodak, USA)

23. Glycerol (Carlo ERBA, France)

24. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Hyclone, UK)

25. Hydrochloric acid (HCl) (LAB-SCAN, Ireland)

26. Isopropanol (Merk, Germany)

27. Ionomycin (Merk, Germany)

28. Magnesium chloride (MgCl$_2$) (Merk, Germany)

29. Magnesium sulfate (MgSO$_4$) (Merk, Germany)

30. MTT (3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide) (Bio Basic inc, Canada)

31. Nonidet P-40 (Bio Basic inc, Canada)

32. Penicillin (General Drugs House, Thailand)

33. Phorbol 12-myristate 13-acetate (Merk, Germany)

34. Phosphatase inhibitor cocktail 2 (Sigma, USA)

35. Potassium chloride (KCl) (Merk, Germany)

36. Potassium di-hydrogen phosphate (KH$_2$PO$_4$)

37. Propidium iodide (Sigma, USA)

38. Protease inhibitor (Sigma, USA)

39. Rabbit anti-Phospho-cdc2 (Tyr15); catalog#9111 (Cell signaling, USA)

40. Rabbit anti-Wee1; catalog#06-972 ((Millipore, Billerica, USA)

41. Rabbit anti-GAPDH (D16H11); catalog#5174 (Cell signaling, USA)

42. Random hexamer (Fermentas, Canada)

43. Reverse transcriptase (Fermentas, Canada)

44. Ribonuclease inhibitor (Fermentas, Canada)

45. RPMI 1640 (Hyclone, UK)

46. Streptomycin (AppliChem, USA)

47. SDS (sodium dodecyl sulfate) ($C_{12}H_{25}OSO_3$) (Sigma, USA)

48. Sodium di-hydrogen phosphate (Na_2HPO_4) (Merck, Germany)

49. Sodium hydroxide (NaOH) (Merck, Germany)

50. Sodium phosphate (NaH_2PO_4) (Merck, Germany)

51. Sodium pyruvate (Hyclone, UK)

52. SsoAdvanced^{TM} Universal SYBR® Green Supermix (Bio-Rad, USA)

53. Taq DNA polymerase (Fermentas, Canada)

54. TEMED (N, N, N, N-Tetramethyethylenediamide) (Bio Basic inc, Canada)

55. Tris buffer pH 6.8 (Preparation is described in appendix)

56. Tris buffer pH 8.8 (Preparation is described in appendix)

57. TriZol reagent (Invitrogen, USA)

58. Trypan blue 0.5% w/v (Hyclone, UK)

59. Tween 20 (Sigma, USA)

3.3 Primer sequences and conditions used in quantitative real-time PCR (qPCR)

Table 3.1 Primer sequences and conditions used in quantitative real-time PCR (qPCR)

Gene	Nucleotide sequence	Annealing temp.(°C)	Product size (bp)	Reference
WEE1	Fwd: 5'-ATTTCTCTGCGT GGGCAGAAG-3' Rev: 5'-CAAAAGGAGAT CCTTCAACTCTG C-3'	57	140	(Yuan et al., 2004)
GAPDH	Fwd: 5'-TGCACCACCAA CTGCTTAGC-3' Rev: 5'-GGCATGGACTG TGGTCATGAG-3'	55	87	(Yuan et al., 2004)

3.4 Antibody for Western blot analysis

Table 3.2 Antibody for Western blot analysis

Antigen	Ratio (Primary antibody:Bloking solution)	Ratio (Secondary antibody:Bloking solution)
Wee1	Rabbit anti-Wee1 1:2,000	Anti-rabbit IgG-HRP 1:4,000
P-Cdc2	Rabbit anti-P-Cdc2 1:2,000	Anti-rabbit IgG-HRP 1:4,000
GAPDH	Rabbit anti-GAPDH 1:4,000	Anti-rabbit IgG-HRP 1:4,000

3.5 Source of pinostrobin

A pure pinostrobin, was isolated and purified from crude extract of fingerroot *B. pandurata* (Wangkangwan et al., 2009). The stock solution of pinostrobin was prepared in dimethyl sulfoxide (DMSO).

3.6 Pinostrobin dissolving method

DMSO was added little by little into a microcentrifuge tube containing 2.7 mg of pinostrobin and vortexed until homogeneous was obtained. Then adjusting the volume to 100 µl by DMSO to final concentration of 100 mM of pinostrobin (Molecular weigh 270 g/mol).

3.7 Human cancer cell lines

Jurkat (Human acute T cell leukemia) ATCC number CRL-2603, KATO III (Human stomach carcinoma cell line) ATCC number HTB-103 and BT474 (Human breast carcinoma cell line) ATCC number HTB-20 were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand. Normal human blood cells were obtained from Thai Red Cross Society, Bangkok, Thailand.

3.8 Cell culture

Jurkat T-cells were cultivated in RPMI-1640 with L-Glutamine supplemented with 10% fetal bovine serum, 10^6 U/ml penicillin, 500 mg/ml streptomycin and mercaptoethanol. KATO III cell lines was cultivated in RPMI-1640 with L-Glutamine supplemented with 10% fetal bovine serum, 10^6 U/ml penicillin, 500 mg/ml streptomycin, sodium pyruvate and HEPES. BT474 cell lines was cultivated in RPMI-1640 with L-Glutamine supplemented with 10% fetal bovine serum and 10^6 U/ml penicillin, 500 mg/ml streptomycin. The cells were cultured at 37°C in 5% CO_2 containing atmosphere. The cells were examined under the microscope and the media was changed every 2-3 days.

3.9 Cell subculturing of Jurkat T-cell (suspension cells)

The complete medium was bring to 37°C in the water bath. After that mix the cell suspension in T-flask by sterile pipette. The cell suspension were removed and determine by cell density or the medium has an acidic pH (yellow from the phenol red). The new complete medium was added to T-flask and incubate at 37°C in 5% CO_2 containing atmosphere.

3.10 Cell subculturing of KATO III and BT474 (adherent cells)

The complete medium was bring to 37°C in the water bath. After that remove and discard the medium and gently wash cells with PBS (phosphate buffered saline). The PBS was removed and discard, after that the pre-warmed trypsin to cover the cell layer. Gently rock the T-flask to get complete coverage of the cell layer. The cells were incubated at 37°C in 5% CO_2 for approximately 3-5 min. Observe the cells under the microscope, when cells have detached add the complete medium (twice the volume used for the trypsin). Disperse the medium by pipetting over the cell layer surface several time. The cells were transferred to centrifuge tube and centrifuge at $1000 \times g$ for 5 min. The supernatant was discarded and resuspended cell pellet in complete medium. The cells suspension were transferred to complete medium by determine the total number of cells and percent viability and incubate at 37°C in 5% CO_2 containing atmosphere.

3.11 Cryopreservation procedure

Prepare the freezing medium (Appendix A) for cell culture. Collect cells by centrifuge for 5 min at $1000 \times g$ and resuspend cells in freezing medium. Then add 1 ml of the cell suspension to each of the vial and seal. Place the vial into 4°C or on ice and quickly transfer the vial to a liquid nitrogen or -80°C freezer.

3.12 Recovery of cryopreserved cells

Prepare a complete medium at 8-10 ml in T-flask. Remove the vial from liquid nitrogen or -80°C freezer and thaw by gentle agitation at 37°C. Transfer the cell suspension to sterile centrifuge tube containing 5 ml of RPMI-1640 medium free serum

and centrifuge $1000 \times$ *g* for 5 min. Discard the supernatant and resuspend cells in 1- 2 ml of complete medium. Pipette the cell pellet by gently mix to loosen the pellet. Transfer the cell suspension into the medium in T-flask and check the cell culture after 24 h.

3.13 Trypan blue dye exclusion test

Cells were centrifuged and the supernatant was discarded. Then, 1 ml complete media was added and 50 µl of the cell suspension was transferred to a new microcentrifuge tube. Fifty µl of trypan blue at concentration of 0.5% (w/v) was added prior to transferring of 10 µl of the cell suspension to haemacytometer. Finally, the unstained cells were counted under microscope and the cell viability was calculated using the formula as follow:

$$\text{Cell viability (cells/ml)} = (\text{unstained cells} / 4) \times 10^4 \times 2$$

3.14 MTT viability assay

MTT viability assay was carried out according to that described in Mosmann (1983). The Jurkat T-cell, KATO III and BT474 were cultivated in RPMI medium containing 10% fetal bovine serum (FBS) at 37 ℃ in a humidified atmosphere of 5% CO_2. Then, 50 µl of cells was seeded in 96-well plates at a concentration of 5×10^4 cells/well. After that, 50 µl of RPMI medium containing varying concentrations of pinostrobin (0.1, 1, 10, 100 µM) was added and incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 for 1 or 2 or 3 or 4 days. Subsequently, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 5 mg/ml was added to each well, and cells were incubated for an additional 4 h at 37 ℃ in a humidified atmosphere of 5% CO_2 . Finally, 100 µl of isopropanol was added to each well. The optical density of the dissolved material was measured by a microplate reader at 540 nm. The cell survival was expressed as percentage of viable cells of treated samples to control samples. The test was performed in triplicates.

$$\%\,\text{Viability} = [(\text{ODtest average} - \text{ODblank average})\,/$$
$$\text{ODcontrol cell average} - \text{ODblank average})] \times 100$$

ODtest average   = The average value of optical density of cell treated with pinostrobin or DMSO

ODcontrol cell average   = The average value of optical density of cell

ODblank average   = The average value optical density of medium

3.15 Preparation of human normal white blood cells

Human normal white blood cells were isolated from whole blood by density gradient centrifugation using Ficoll-Hypaque by following the manufacturer's instruction.

3.16 Hemolytic activity assay of pinostrobin on human red blood cell

The hemolytic activity of pinostrobin on human red blood cell was tested as described by Arpornsuwan et al. (2014). Briefly human red blood cell was washed three times with PBS and then 1% of cell suspension was diluted in PBS. Pinostrobin was diluted in PBS by 10-flod dilution from 100 - 0.01 μM. Human red blood cells were seeded in a microcentrifuge tube and then treated with various concentrations of pinostrobin and incubated at 37 °C in 5% CO_2 containing atmosphere for 24 h. The optical density of supernatant was measured at 540 nm. The PBS solution was used as a negative control and 1% Triton X-100 was used as a positive control. The % hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = [(\text{OD Pinostrobin treated} - \text{OD negative control})/$$
$$(\text{OD positive control} - \text{OD negative control})] \times 100$$

3.17 Cell cycle progression and apoptosis

For cell cycle analysis, 250 µl of Jurkat T-cells at a concentration of 1.25×10^5 cells/well were added onto 24-well plates and 250 µl of RPMI medium containing pinostrobin (at 2X IC_{50} value) or five hundred µl of KATO III or BT474 at a concentration of 1.25×10^5 cells/well were added onto 24-well plates for overnight, after that remove and replace with RPMI medium containing pinostrobin (at 2X IC_{50} value). Incubated for 1 or 2 or 3 or 4 days at 37°C in a humidified atmosphere of 5% CO_2. The cells were harvested by centrifuged at $4200 \times g$ for 5 min at room temperature. The cell pellets were resuspended in 500 µl of PBS and centrifuged at $4200 \times g$ for 5 min. The cells were fixed with 200 µl of 70% ice-cold ethanol and incubated at -20°C for 24 h or on ice for 4 h. After that, the fixed cells were washed with PBS containing 5% of fetal bovine serum and resuspended in a PBS containing RNase A (10 mg/mL), incubated at 37°C for 30 min and stained with propidium iodide (PI, 1 mg/mL). The cell suspensions were then be incubated at 37°C at least 30 min in the absence of light and analyzed by a flow cytometer.

For apoptotic cell analysis, 250 µl of Jurkat T-cell suspension at a concentration of 1.25×10^5 cells/well and 250 µl of RPMI medium containing pinostrobin (concentration at 2X IC_{50} value) were added into 24-well plates and incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 or five hundred µl of KATO III or BT474 at a concentration of 1.25×10^5 cells/well was added onto 24-well plates for overnight, after that supernatant was removed and replaced with RPMI medium containing pinostrobin (at 2X IC_{50} value) and incubated for 48 h in KATO III or 72 h in BT474 at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested by centrifuged at $4200 \times g$ for 5 min at room temperature. Cell pellets were then resuspended in 1 ml of ice-cold PBS and centrifuge at $4200 \times g$ for 5 min twice. After that the cell pellets were resuspended in 1X Binding Buffer and 200 µl of the cell suspension were taken and were added with 3 µl of the annexin conjugate ApopNexin™ FITC. Finally, 2 µl of 100x PI was be added to ApopNexin™ FITC labeled cells follow by mixing and incubating for 15 min at room temperature in the dark and analyzed by a flow cytometer.

3.18 Western blot analysis

3.18.1 Preparation of protein extract from Jurkat T-cell, KATO III and BT474

Five hundred µl of Jurkat T-cells, KATO III or BT474 were seeded at a concentration of 1×10^6 cells/well onto 12-well plates and 500 µl of RPMI medium containing pinostrobin (at 2X IC_{50} value), incubated with Ionomycin (Io) 1 µg/ml, Phobol 12-myristate 13-acetate (PMA) 25 µg/ml and harvested at 0, 30, 60 or 120 min, respectively at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested by centrifugation at $7500 \times g$ for 5 min at 4°C. The cell pellets were then washed with 1 ml of ice-cold PBS and centrifuged at $7500 \times g$ for 5 min. After that, the cells were resuspended in 40 µl of ice-cold LIPA buffer for 30 min and centrifuged at $12,000 \times g$ for 15 min. Finally, the supernatant were transferred into new microcentrifuge tube and kept at -80°C.

3.18.2 Protein determination by BCA assay

The standard protein (2 mg/ml) were added as gradient volume to each well followed by adding of sample protein (1 µl/well). After that BCA reagent was added and the plate was covered and incubated at 37°C for 30 min. The absorbance was measured at 540 nm by a microplate reader. Then, a standard curve was plotted using data of standard protein and concentration of protein samples were determined from the standard curve's equation.

3.18.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were mixed with sample buffer (loading buffer) and boil in boiling water for 5 minutes. The prepared samples and protein marker were loaded into wells of SDS-polyacrylamide followed the protocol described in Laemmli et al. (1976). The Electrophoresis was performed with 70 voltage for 150 min.

3.18.4 Western Blot Analysis

Proteins were transferred onto PVDF membrane. The membrane was blocked in a blocking buffer (5% of skim milk in PBST) for 30-60 min, after that membrane was incubated with primary antibody in the blocking buffer at 4°C overnight. The membrane was washed and incubated with a secondary antibody and the target proteins were detected by chemiluminescence. GAPDH expression level was used as a gel loading control.

3.19 Quantitative real-time PCR

3.19.1 RNA extraction

Five hundred µl of Jurkat T-cells, KATO III or BT474 were added at a concentration of 1×10^6 cells/well onto 12-well plates and 500 µl of RPMI medium containing with varying concentration of pinostrobin (at 2X IC_{50} value), and then incubated for 0, 15, 30, 60 and 120 min, respectively at 37°C in a humidified atmosphere of 5% CO_2. The cells were harvested by centrifuged at $4200\times g$ for 5 min and the supernatant was discarded. The cell pellet was resuspended in 1 ml of TriZol reagent, incubated at room temperature for 5 minutes. After that, 200 µl of chloroform was added, Tubes were vortexed and incubated at room temperature for 2-3 min, and then centrifuged at $12000\times g$ for 15 min at 4°C. Supernatant was transferred into the new microcentrifuge tube. Five hundred µl of isopropanol was added, follow by incubation at room temperature for 10 min. Then, tubes were subjected to centrifugation at $12000\times g$ for 10 min at 4°C. After discarding the supernatant, the RNA pellet was washed with 1 ml of 75% ethanol dissolved in DEPC water. After centrifugation at $7500\times g$ for 5 min, supernatant was discarded and the RNA pellet was dried. RNA will then be dissolved in DEPC water (16 µl) and then incubated at 60°C for 10 minutes. The concentration of RNA was measured by NanoDrop at 260 and 280 nm compared with DEPC water.

3.19.2 Removal of genomic DNA from RNA preparation

Total 1 µg of RNA was mixed with 1 µl of 10X reaction buffer with $MgCl_2$, 1 U of DNase I and adjusted the volume to 10 µl by DEPC water. The mixtures were incubated at 37°C for 30 min. The mixtures were added with 1 µl of 50 mM EDTA and incubated at 65°C for 10 min. RNA samples were stored at -80°C until use.

3.19.3 Generate complementary DNA (cDNA) by reverse transcriptase

To generate complementary DNA (cDNA), the RNA from 3.19.2 approximately 100 ng - 1 µg was added into PCR tube, then 2 µl of specific primer for *WEE1* (Rev: 5'-CAAAAGGAGATCCTTCAACTCTGC-3') (Yuan et al., 2004) and specific primer for *GAPDH* (Rev: 5'-GGCATGGACTGTGGTCATGAG-3') (Yuan et al., 2004) at concentration 10 µM were added to 12.5 µl by DEPC water. The tube was mixed and centrifuged about 2-3 second and transferred to DNA Thermal Cycle at 65°C for 5 min and 4°C for 5 min, respectively. Four µl of 5X Reverse Transcriptase buffer, 2 µl of

dNTP mix at concentration 10 mM and 0.5 µl of Ribonuclease inhibitor at concentration 40 unit/µl, were added and then mixed and centrifuged about 2-3 second. The tube was incubated at room temperature for 5 min, then 1 µl of Reverse transcriptase at concentration 200 unit/µl was added and incubated at room temperature for 10 minutes. After that, the reaction was continued at 25°C for 10 min and 42°C for 60 minutes on DNA Thermal Cycle, respectively, Finally, the reaction was stopped by heating to 70°C for 10 min and the cDNA samples were will be kept at -20°C.

3.19.4 Quantitative polymerase chain reaction (qPCR)

The cDNA from 3.19.3 was used as a template in the reaction and the specific primers for *WEE1* genes and specific primers for *GAPDH* gene were used for control gene. The qPCR reaction using by MiniOpticon^{TM} Real-time PCR System follow by component listed in table 3.3.

Table 3.3 Components and reagents for Quantitative polymerase chain reaction (qPCR)

Component	Concentration	Volume	Final concentration
SsoAdvanced^{TM} Universal SYBR® Green Supermix	2X	5 µl	1X
Forward primer	10 µM	0.5 µl	0.5 µM
Reverse primer	10 µM	0.5 µl	0.5 µM
cDNA	1 µg	1 µl	50 ng
Nuclease-free H$_2$O		3 µl	
Total volume		10 µl	

The conditions as follows by: 95°C for 3.00 min, followed by 40 cycles of 95°C for 30 sec, annealing temperature of each gene candidate for 30 sec, 57°C for *WEE1* and 55°C for *GAPDH*. The relative expression of mRNA level was calculated by $2^{(-\Delta\Delta Cq)}$.

3.20 Statistical analysis

The unpaired t-test (two-tailed) and one-way ANOVA was performed to determine statistical significance of treated cells and the untreated cells (GraphPad Prism5, USA).

CHAPTER IV
RESULTS

4.1 Pinostrobin exhibits chronic cytotoxic effect against Jurkat T-cell, KATO III and BT474 cell lines

In *S. cerevisiae*, Swe1 kinase specifically inhibits a G2 form of Cdc28 by phosphorylating it at Tyr19 (Booher et al., 1993) and delays the onset of mitosis. In human, *WEE1* is an ortholog gene of *S. cerevisiae SWE1*. Wee1 acts as a negative regulator of entry into mitosis (G2 to M transition) by protecting the nucleus from cytoplasmically activated cyclin B1-complexed CDK1 before the onset of mitosis by mediating phosphorylation of CDK1 on Tyr-15. Wee1 is a central regulator of G2/M cell cycle checkpoint. In case of DNA damage, Wee1 adds an inhibitory phosphorylation on the Tyr15 residue of CDK1, by so postponing progression to mitosis and yielding the cell time to either repair the damage or undergo apoptosis (K and MJ, 2009). Kinases, such as Wee1, represents potential therapeutic targets, however, their expression varies in different types of tumors. Overexpression of Wee1 has previously been reported in osteosarcoma, glioblastoma and breast cancer (Iorns et al., 2009, Mir et al., 2010, PosthumaDeBoer et al., 2011).

Smolarz et al. (2006) previously reported on the effect of pinostrobin from *Polygonum lapathifolium* L. ssp. *nodosum* (Pers.) Dans. on anti-leukemic activity against Jurkat and HL-60 cell lines. This has raised question whether pinostrobin that affected Swe1 activity in *S. cerevisiae*, will also affect Wee1 expression and activity in the cancer cell lines. The anti-proliferative activity of pinostrobin in Jurkat T-cell, KATO III and BT474 cell lines by MTT viability assay were determined. Jurkat T-cells, KATO III and BT474 were cultivated in RPMI 1640 containing either 0.1- 100 μM of pinostrobin or DMSO (control) and incubated at 37 ℃ in humidified conditions with 5% CO_2 and 95% air for 1 day, 2 days, 3 day and 4 days before the MTT assay. The results were shown in Fig. 4.1.

A) Jurkat T-cell
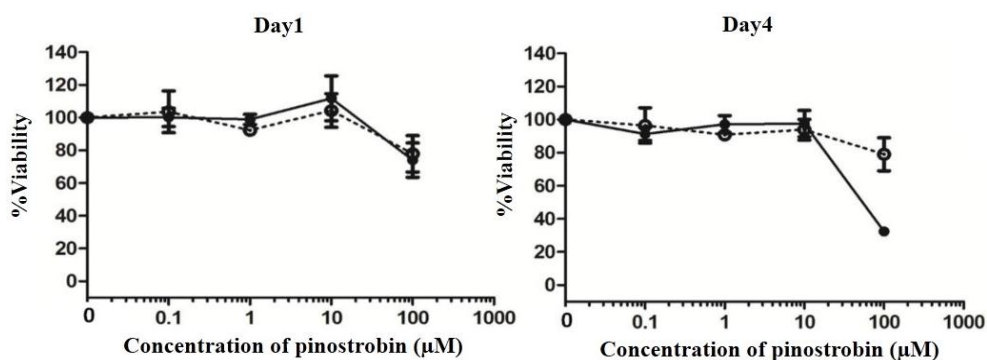
B) BT474
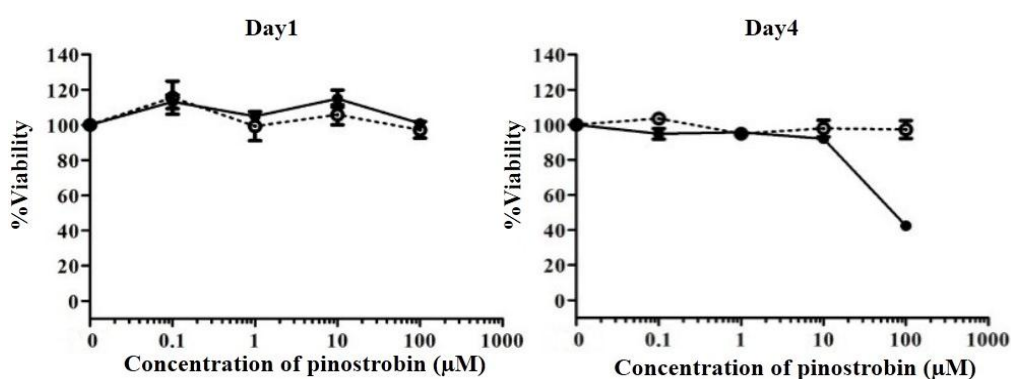
C) KATO III
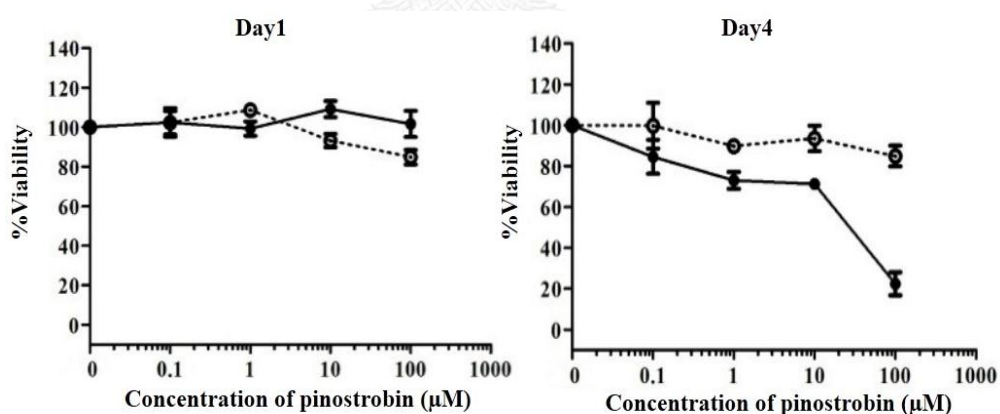
Figure 4.1 Cytotoxicity of pinostrobin on human cancer cell lines. The cell viability was measured by MTT assays and the viability against concentration result of Jurkat T-cell (A) or BT474 (B) or KATO III (C). Cells were treated with either varying concentrations of pinostrobin or DMSO and incubated at 37 °C in humidified conditions with 5%CO_2 for 1 day and 4 days, respectively. The data at each time point were mean ± SD from triplicates experiment. ( ): Control (DMSO), ( ): Pinostrobin treatment.

The treatment with or without pinostrobin (control) to Jurkat T-cells, BT474 and KATO III for 1 day showed similar pattern of cell viability (Fig. 4.1). This revealed that no acute cytotoxic effect was observed in the pinostrobin treatment. On days 4, however, cells treated with pinostrobin at concentration higher than 10 μM showed sharply decline in cell viability compared to that of control. Results demonstrated that pinostrobin contains potential chronic effect of cytotoxicity against Jurkat T-cells, BT474 and KATO III at IC$_{50}$ values of 51.2 ± 1.61 μM, 61.9 ± 1.15 μM and 24.7 ± 4.5 μM, respectively.

4.2 Effect of pinostrobin on human normal red blood cells

Next, I further investigated whether pinostrobin contain cytotoxic effect against human normal red blood cells or not. It was found that there was no different on % hemolysis of red blood cells between pinostrobin treatment and 0.5% DMSO treatment on red blood cells. Since pinostrobin was dissolved in 0.5% DMSO, we therefore concluded that pinostrobin caused no hemolytic activity to human red blood cells at least at concentration of 100 μM (Table 4.1).

Table 4.1 Pinostrobin showed no effect on human red blood cells

Concentration of pinostrobin (μM)	Hemolysis (%)	
	Pinostrobin*	DMSO
0.1	1.02 ± 1.2	1.04 ± 0.5
1	1.02 ± 0.3	1.04 ± 0.1
10	1.12 ± 1.0	2.14 ± 1.0
100	1.45 ± 0.9	2.27 ± 0.1

The data are expressed as the mean $\pm$ SD for three different preparations ($n = 3$).

*0.5% DMSO which was the same concentration used to dissolve pinostrobin.

4.3 The cytotoxic effect of pinostrobin on normal human white blood cells

From previous experiments, pinostrobin showed the cytotoxic effect on various human cancer cell lines and did not showed cytotoxic effect on hemolytic activity against human normal red blood cells. To see whether pinostrobin possesses the cytotoxic effect on normal human white blood cells or not. Normal human white blood cells (group O, A and B) were separated from whole blood and then treated with various concentration of pinostrobin (0.1-100 μM) for 1 and 4 days. The results showed that pinostrobin did not show toxicity against normal human white blood cells (group O, A and B) were observed at its IC_{50} dose (Fig. 4.2).

A) Group O
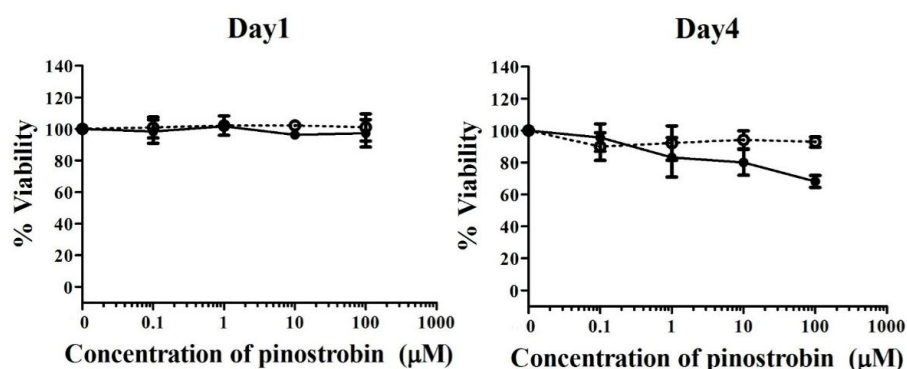
B) Group A
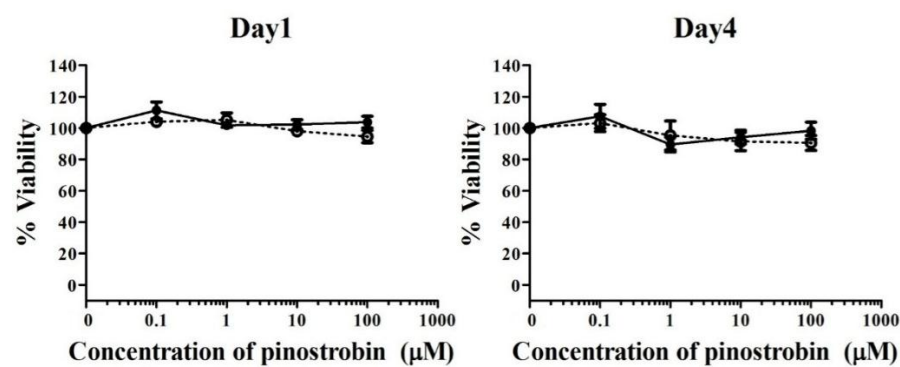
C) Group B
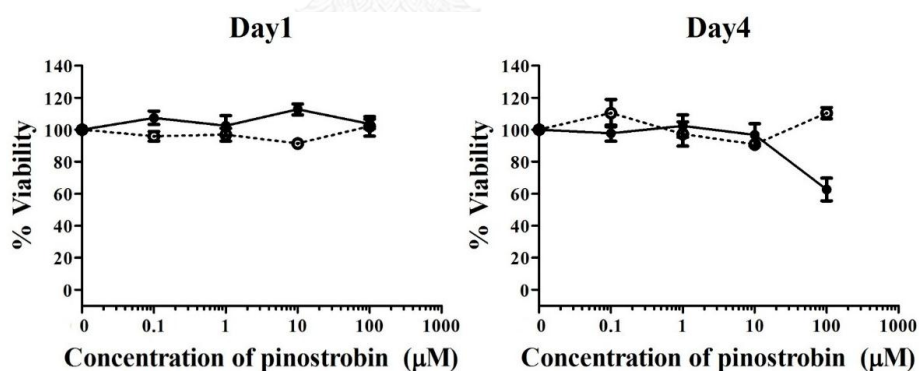
Figure 4.2 Cytotoxic effect of pinostrobin on normal human white blood cells. The cell viability was measured using MTT assay. The white blood cells isolated from whole blood of group O (A), group A (B) and group B (C) were treated with various concentration of pinostrobin compared with DMSO and incubated at 37 ℃ in humidified conditions with 5% CO_2 for 1 day and 4 days, respectively. The data at each time point were mean ± SD from triplicate experiments. (-Θ-): Control (DMSO), (-◆-): Pinostrobin treatment.

4.4 Down-regulation of Wee 1 kinase level and activity caused by pinostrobin

Suksawatamnuay (2009) demonstrated that Swe1 kinase is a molecular target of pinostrobin. Western blotting indicated that not only the level of Swe1 but also the activity of Swe1 kinase was decreased as observed from the decreasing level of P-Cdc28.

In general, proteins that are involved in various important biological processes are highly conserved along evolution from yeasts to humans (Barrientos, 2003). Among those, Swe1 in *S. cerevisiae* is an ortholog of Wee1 in human. Wee1 is a tyrosine kinase (Russell and Nurse, 1987) that plays role on regulating the transition between the G2 and M phases in human. Wee1 negatively regulates entry into mitosis by phosphorylating the Tyr15 residue of Cyclin Dependent Kinase 1(CDK1, also known as CDC2), thus inactivating the CDK1/cyclin B complex and arresting the cell cycle. I, therefore, evaluate the effect of pinostrobin on Wee1 level and Wee1 kinase activity in Jurkat T-cell, BT474 and KATO III. These cell lines were cultivated either in the absence (0.5% DMSO) or in the presence of 100 or 120 μM pinostrobin (2X IC$_{50}$) in Jurkat T-cell or BT474 and 100 μM pinostrobin (4X IC$_{50}$) 30 minutes prior to addition of Ionomycin (Io) and Phorbol 12-myristate 13-acetate (PMA) for high intracellular calcium concentration and activation of protein kinase, respectively. The treated cells were harvested at various time points: 0, 30, 60 and 120 minutes, respectively. Cell extracts were prepared and subjected to SDS-PAGE and Western blot analyses. I found that in the presence of 100 and 120 μM pinostrobin in Jurkat T-cell and BT474, the level of Wee1 was decreased after 30 min (Fig. 4.3A and 4.3B), whereas 100 μM pinostrobin in KATO III caused the level of Wee1 slightly decreased at 120 minutes (Fig. 4.3C).

Wee1 remained unchanged throughout 120 minutes in the control (untreated samples). The results suggested that pinostrobin down regulates the level of Wee 1 in Jurkat T-cell, BT474 and KATO III cell lines. Since Wee1 kinase regulates entry into mitosis by phosphorylating the Tyr15 residue of Cdc2 (Magnussen et al., 2012), to determine the activity of Wee1, anti-P-Tyr15-Cdc2 antibody was used in the Western blot analysis. We detected a band of 34 kDa of P-Cdc2 (Phosphorylated form) appeared relatively constant level in the untreated cells along with GAPDH (as loading control).

However, the level of P-Cdc2 in the pinostrobin treated Jurkat T-cells, BT474 and KATO III cell lines were decreased at 30 minutes after treatment. Notably, the decrease in P-Cdc2 was corresponded to the decrease in the level of Wee1 which also started to decline onwards 30 minutes. Taken together, results revealed that pinostrobin has an effect on down-regulating the Wee1 level which led to decrease in Wee 1 kinase activity (Fig. 4.3).
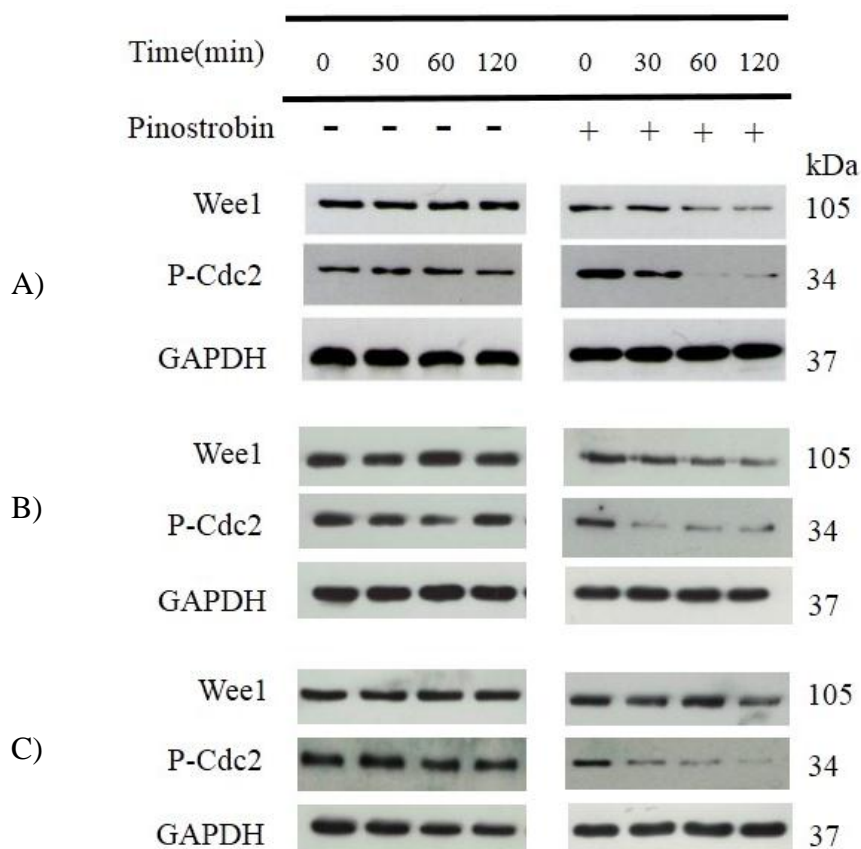
Figure 4.3 The effect of pinostrobin on level of Wee1 and P-Cdc2 in Jurkat T-cells (A), BT474 (B) and KATO III (C). Cells were incubated at 37°C in the atmosphere of 5%, harvested and extracted protein at 0, 15, 30, 60 and 120 min. Protein samples were subjected to SDS-PAGE, blotted and detected the level of Wee1 and P-Cdc2 by anti-Wee1 and anti-P-Cdc2. GAPDH was used as a loading control.

4.5 Down-regulation of Wee1 kinase level by pinostrobin or radicicol and the effect of combined treatment

It has been previously reported that radicicol, a macrocyclic antifungal antibiotic and an anti-tumor agent, is a drug targeting Swe1 in Ca^{2+}-signaling pathway in *S. cerevisiae* (Chanklan et al., 2008). Radicicol reacts specifically at the N-terminal domain of Heat Shock Protein 90 (Hsp90) (Schulte et al., 1998). In *S. cerevisiae*, Hsc82 is an ortholog of mammalian Hsp90. Radicicol inhibits Hsp90 homolog in yeast as well as in human (Goes and Martin, 2001, Ki et al., 2001). Swe1 is one of the substrates of Hsp82/Hsp90 (Donze and Picard, 1999). The physical interaction of Swe1 and Hsc82/Hsp90 has been demonstrated *in vitro* (Goes and Martin, 2001). Furthermore, Chanklan et al. (2008) reported that radicicol, acts at downstream of *SWE1* by reduction in Swe1 level and Suksawatamnuay (2009) demonstrated that pinostrobin reduced the level and activity of Swe1 kinase in *S. cerevisiae*. So, I expected that the level of Wee1 was decreased when treated Jurkat T-cell with pinostrobin and radicicol.

Jurkat T-cells were cultivated either in the absence (0.5% DMSO) or in the presence of 100 μM (2X IC_{50}) pinostrobin or 3 μM (1X IC_{50}) radicicol or the combine treatment for 30 min prior to addition of Ionomycin (Io) and Phorbol 12-myristate 13-acetate (PMA) for high intracellular calcium concentration induction and activation of protein kinase, respectively. The treated cells were harvested at various time points: 0, 30, 60 and 120 min, respectively. Cell extracts were prepared and subjected to SDS-PAGE and Western Blot analyse (Fig. 4.4).

Time(min)	0	30	60	120	0	30	60	120	0	30	60	120	0	30	60	120	
Radicicol	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	
Pinostrobin	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	kDa
Wee1																	105
P-Cdc2																	34
GAPDH																	37
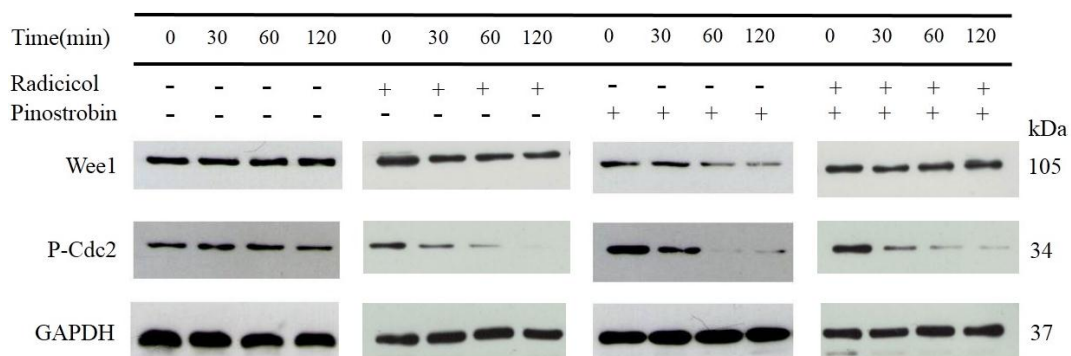
Figure 4.4 The effect of 100 µM pinostrobin or 3 µM radicicol or their combined treatment on the level of Wee1 kinase and P-Cdc2 in Jurkat T-cells and incubated at 37°C in the atmosphere of 5% CO_2. Cells were harvested at 0, 15, 30, 60 and 120 min. Protein samples were prepared and run on SDS-PAGE subjected to Western blotting and detected the Wee1 and P-Cdc2 by anti-Wee1 and anti-PCdc2, respectively. GAPDH was used as a loading control.

I found that Jurkat T-cells treated with 3 µM radicicol resulted in decreased Wee1 level, though in a lesser extent as compared to the cells treated with 100 µM pinostrobin. Accordingly, while activity of Wee1 as measured by the level of P-Cdc2 also was diminished although more than that of those treated with pinostrobin. The results of radicicol observed in Jurkat T-cells in this study were in agreement with those reported in yeast cells treated with radicicol (Chanklan et al., 2008). Since radicicol is a Hsp90 inhibitor, therefore, the inhibition of Hsp90 chaperone protein by radicicol may cause destabilization of Wee1 that led to decrease in level and activity of Wee1 (Fig. 4.4)

The effect of either pinostrobin or radicicol treatment on Jurkat T-cells caused decrease in level of Wee1 leading to diminished activity of Wee1. Further investigation on the combined of pinostrobin and radicicol on Wee1 level and activity was performed. Results revealed that combination of pinostrobin and radicicol showed the similar effect as those obtained from radicicol treatment alone. There was no decrease in level of Wee1 kinase as compared to that in the untreated cells (Fig. 4.4).

4.6 Effect of MK-1775 on activity of Wee1

A small molecule Wee1 inhibitor, MK-1775, was recently reported to possess selective inhibition activity on Wee1 tyrosine kinase (Kreahling et al., 2012). I wonder whether pinostrobin and MK-1775 might have similar biological activity on Wee1. Jurkat T-cells, BT474 and KATO III were treated with 2x IC_{50} dose of either 100, 120 and 4x IC_{50} 100 µM pinostrobin respectively or 500 nM MK-1775 (1X IC_{50}) and the cell suspensions were incubated at 37 °C in 5%CO_2 atmosphere. The samples of 500 µl were taken at different time points: 0, 30, 60 and 120 minutes, respectively, after the addition of Io and PMA. Crude protein extracts were prepared and subjected to SDS-PAGE, followed by Western blotting analysis using antibodies against Wee1, P-Cdc2 and GAPDH (as a loading control).

From Fig. 4.5, the results showed that unlike pinostrobin that decreased the level of Wee1 starting after 30 minutes of treatment, MK-1775 on the other hand did not affect the Wee1 levels throughout 120 minutes (Fig. 4.5). However, pinostrobin and MK-1775 inhibited the activity of Wee1 as determined by the decreasing in the amount of phosphorylated-Cdc2. MK-1775 showed more potent inhibition of Wee1 activity as the amount of P-Cdc2 decreased significantly from 30 min post-treatment in Jurkat T-cell, BT474 and KATO III. The results suggested that pinostrobin is an inhibitor of Wee1 but in different manner from MK-1775, a known Wee1 inhibitor.
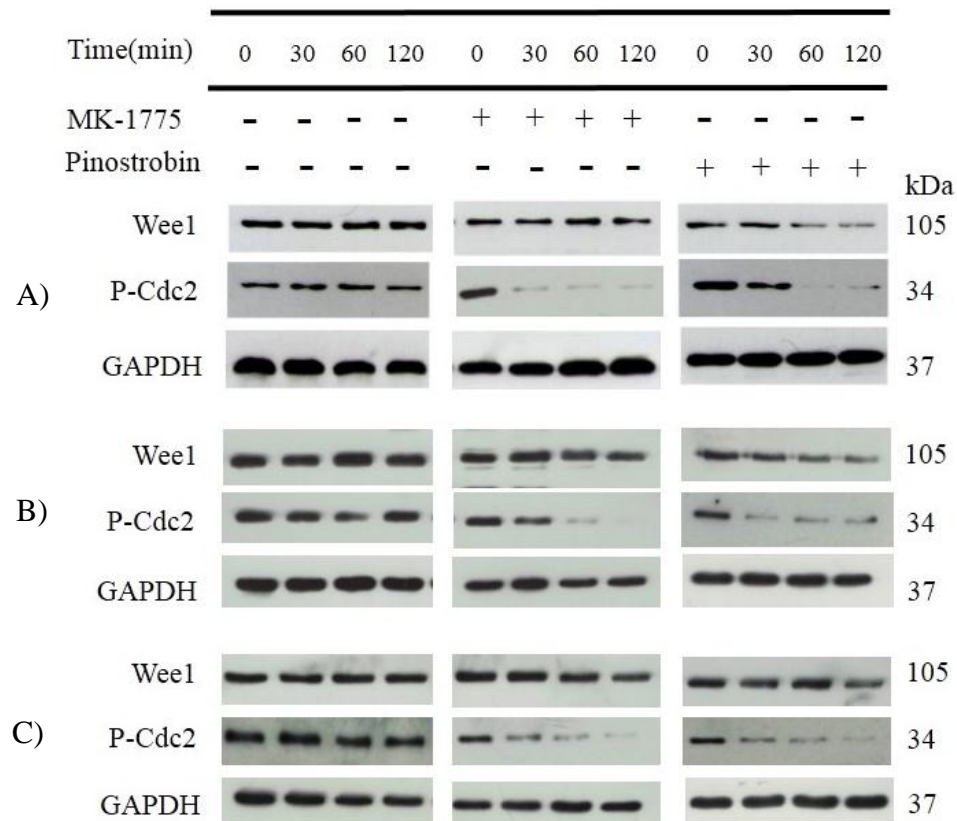
Figure 4.5 The effect of MK-1775 or pinostrobin on the levels of Wee1 kinase and P-Cdc2 in Jurkat T-cells (A), BT474 (B) and KATO III (C). Cells were treated with 500 nM MK-1775 or 100 μM, 120 μM or 100 μM pinostrobin, respectively and incubated at 37°C in the atmosphere of 5% CO_2. Cells were harvested at 0, 15, 30, 60 and 120 min. Protein samples were prepared and run by SDS-PAGE blotted and detected the Wee1 and P-Cdc2 by anti-Wee1 and anti-P-Cdc2. GAPDH was used as a loading control.

4.7 Effect of pinostrobin or MK-1775 on *WEE1* expression by quantitative real-time PCR

Both the level and the activity of Wee1 were down regulated in pinostrobin treated Jurkat-T cells, BT474 and KATO III (Fig. 4.3). To see whether pinostrobin down regulates *WEE1* gene expression in human cancer cell lines, quantitative real-time PCR was carried out. Jurkat T-cells, BT474 or KATO III of 500 µl were treated with 100 µM (2X IC_{50}), 120 µM (2X IC_{50}) or 100 µM (4X IC_{50}), respectively of pinostrobin or 500 nM (1X IC_{50}) MK-1775, and incubated at 37°C in a humidified atmosphere of 5% CO_2. The treated cells were harvested at 0, 15, 30, 60 and 120 min, followed by RNA extraction and cDNA synthesis for use as DNA template in quantitative real-time PCR.

The quantitative real-time PCR result revealed that pinostrobin, but not MK-1775 (Fig. 4.6A, B and C), down-regulated *WEE1* expression in Jurkat T cells after 30 min, and slightly decreased after 60 min in BT474 and KATO III.
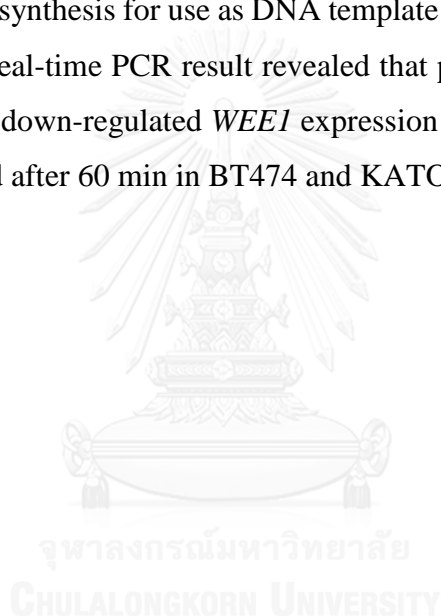
A)
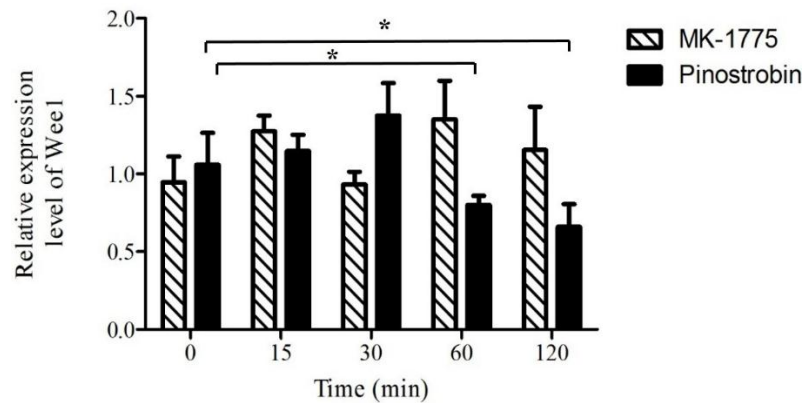
B)
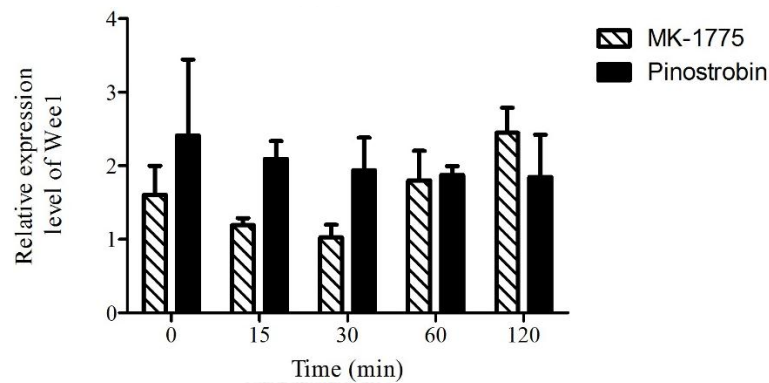
C)
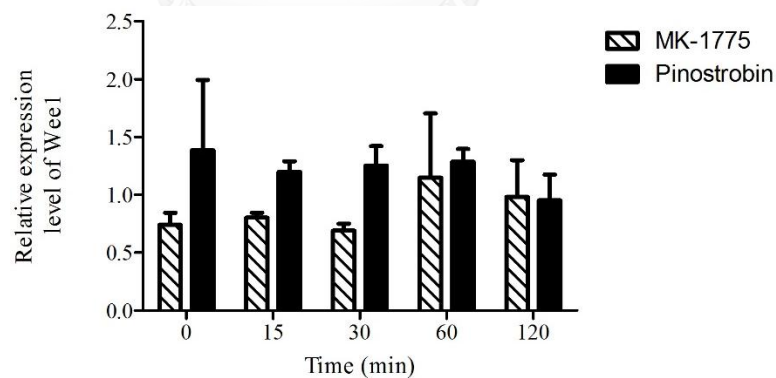
Figure 4.6 The relative expression level of WEE1 in Jurkat T-cells (A), BT474 (B) and KATO III (C). Cells were treated with 100 μM, 120 μM or 100 μM pinostrobin respectively or 500 nM MK-1775 and incubated at 37°C in the atmosphere of 5% CO_2. Cells were harvested and extracted for the RNA at 0, 15, 30, 60 and 120 min. Level of mRNA expression were determined by quantitative real-time PCR. Relative expression level of Wee1 was the fold of expression as compared from those of GAPDH expression level at each time point. * Significant differences at p-value <0.5.

4.8 Combination effect of pinostrobin with DNA-damaging agents on level and activity of Wee1 kinase in Jurkat T-cell, BT474 and KATO III

In previous studies, the combination of MK-1775 with DNA- damaging agents showed significant decrease in the activity of Wee1 kinase in human sarcomas and pancreatic cancer xenografts, respectively (Rajeshkumar et al., 2011, Kreahling et al., 2013). I, therefore, expected that pinostobin may cause synergistic effect when being combined with DNA-damaging agent. The effect of MK-1775 or pinostrobin when combined with DNA-damaging agents (gemcitabine, camptothecin or 5-fluorouracil) on the level and activity of Wee1 was determined by Western blotting analysis.

The Jurkat T-cells treated with 1X IC_{50} concentration of DNA-damaging agents (1 µM gemcitabine, 3 µM 5-fluorouracil or 10 nM camptothecin) showed no effect on the levels of Wee1 and P-Cdc2. In addition, the combination of treatment between MK-1775 or pinostrobin with either 1 µM gemcitabine, 3 µM 5-fluorouracil or 10 nM camptothecin showed rather similar results to those of MK-1775 singly or pinostrobin treatment (Fig. 4.7).

Time(min)	0 30 60 120	0 30 60 120	0 30 60 120	0 30 60 120	0 30 60 120	0 30 60 120	
MK-1775	- - - -	+ + + +	- - - -	- - - -	- - - -	- - - -	
Pinostrobin	- - - -	- - - -	+ + + +	- - - -	- - - -	- - - -	
Gemcitabine	- - - -	- - - -	- - - -	+ + + +	- - - -	- - - -	
5-Fluorouracil	- - - -	- - - -	- - - -	- - - -	+ + + +	- - - -	
Camptothecin	- - - -	- - - -	- - - -	- - - -	- - - -	+ + + +	kDa
Wee1							105
P-Cdc2							34
GAPDH							37
MK-1775	+ + + +	+ + + +	+ + + +	- - - -	- - - -	- - - -	
Pinostrobin	- - - -	- - - -	- - - -	+ + + +	+ + + +	+ + + +	
Gemcitabine	+ + + +	- - - -	- - - -	+ + + +	- - - -	- - - -	
5-Fluorouracil	- - - -	+ + + +	- - - -	- - - -	+ + + +	- - - -	
Camptothecin	- - - -	- - - -	+ + + +	- - - -	- - - -	+ + + +	kDa
Wee1							105
P-Cdc2							34
GAPDH							37

Figure 4.7 The combination effect between pinostrobin and DNA-damaging agents (gemcitabine, 5-fluorouracil or camptothecin) on the levels of Wee1 kinase and P-Cdc2 in Jurkat T-cells. Jurkat T-cells were treated with either 100 μM pinostrobin or 500 nM MK-1775 in combination with 1 μM gemcitabine, 3 μM 5-fluorouracil or 10 nM camptothecin. Treated cells were incubated at 37°C in the atmosphere of 5% CO_2. Cells were harvested at 0, 15, 30, 60 and 120 min, respectively. Protein samples were prepared and subjected to Western blotting analysis using anti-Wee1 and anti-P-Cdc2 and anti-GAPDH antibodies. GAPDH was used as a loading control.

In BT474, when treated with 500 nM (1X IC_{50}) of MK-1775 or 120 μM (2X IC_{50}) of pinostrobin, both treatments decreased the level of P-Cdc2 and that of Wee1. While the camptothecin treatment at concentration 2 μM (1X IC_{50}) showed no effect on either level of Wee1 and P-Cdc2. Furthermore, gemcitabine and 5-fluorouracil have the IC_{50} > 100 μM so I did not determine the combined treatment effect. The combined treatment of MK-1775 or pinostrobin with camptothecin showed the effect similar to camptothecin only (Fig. 4.8)

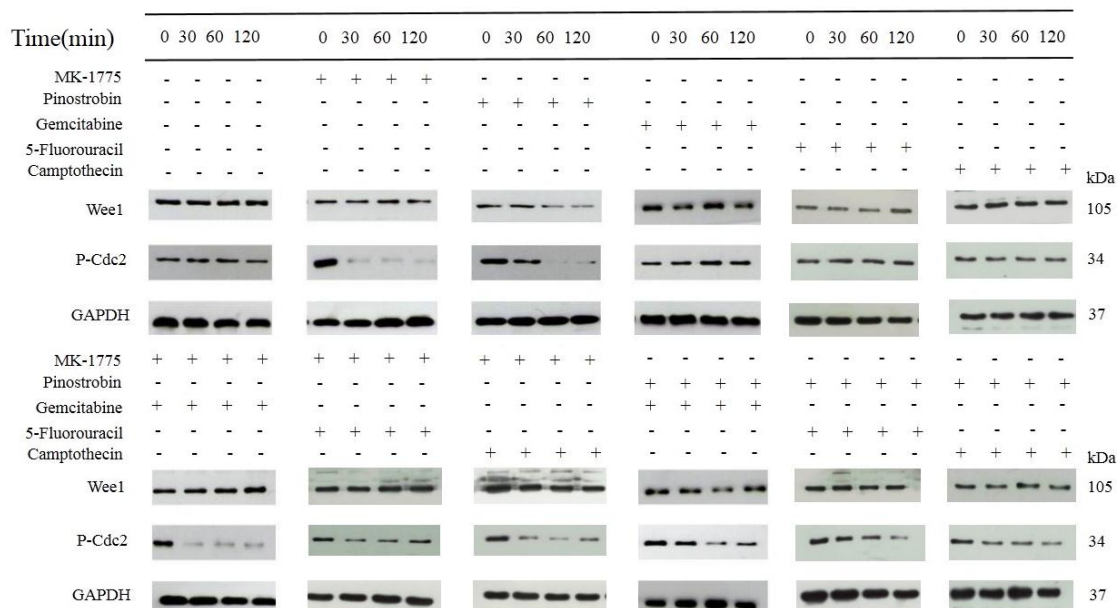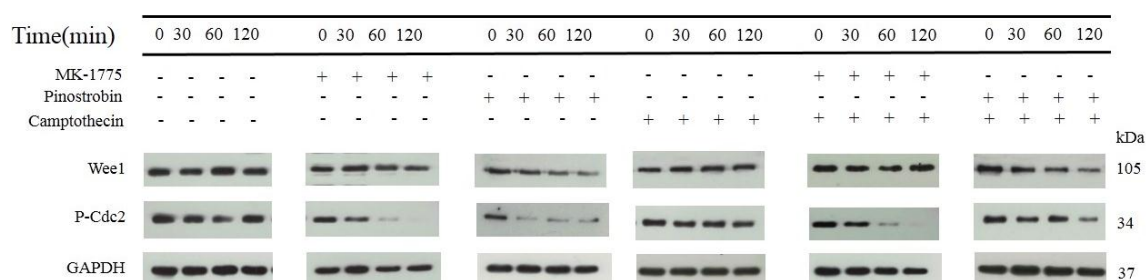**Figure 4.8** The combination effect between pinostrobin and DNA-damaging agent (Camptothecin) on the levels of Wee1 kinase and P-Cdc2 in BT474. The BT474 cells were treated with either 120 μM pinostrobin or 500 nM MK-1775 in combination with 2 nM camptothecin. The treated cells were incubated at 37°C in the atmosphere of 5% CO_2. Cells were harvested at 0, 15, 30, 60 and 120 min, respectively. Protein samples were prepared and subjected to Western blotting analysis using anti-Wee1 and anti-P-Cdc2 and anti-GAPDH antibodies. GAPDH was used as a loading control.

In KATO III, when treated with 500 nM (1X IC$_{50}$) of MK-1775 or 100 μM (4X IC$_{50}$) of pinostrobin, both treatments decreased the levels of P-Cdc2. While gemcitabine, 5-fluorouracil or camptothecin treatment at 10 nM (1X IC$_{50}$), 2 μM (1X IC$_{50}$) and 10 nM (1X IC$_{50}$), respectively showed no effect on levels of both Wee1 and P-Cdc2. The combined treatment of MK-1775 or pinostrobin with gemcitabine, 5-fluorouracil or camptothecin showed the effect similar to DNA-damaging agents only (Fig. 4.9)
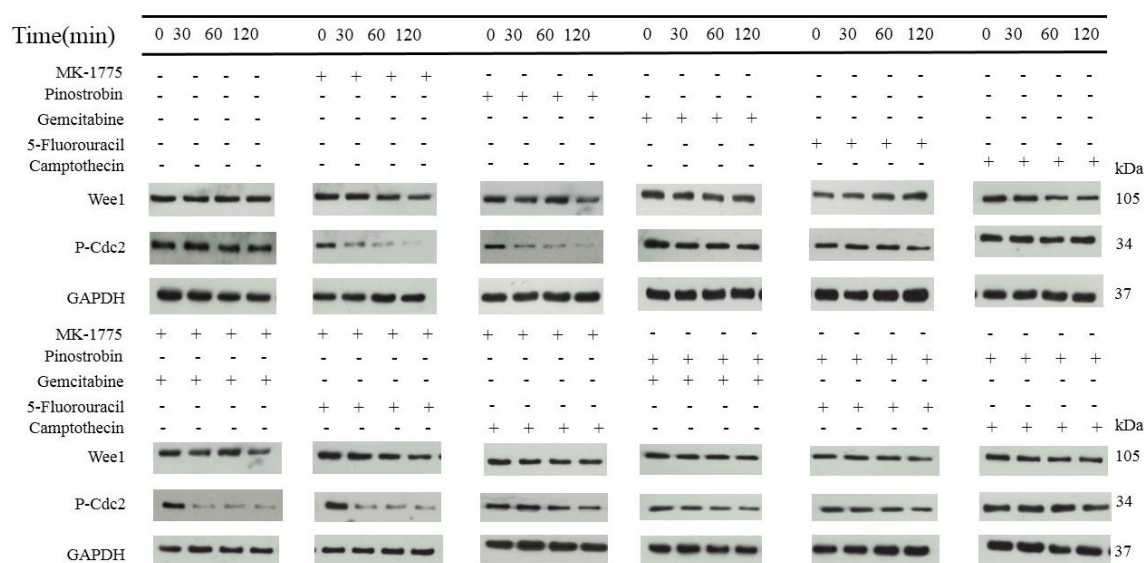
Figure 4.9 The combination effect between pinostrobin and DNA-damaging agents (gemcitabine, 5-fluorouracil or camptothecin) on levels of Wee1 kinase and P-Cdc2 in KATO III. KATO III were treated with either 100 μM pinostrobin or 500 nM MK-1775 in combination with 10 nM gemcitabine, 2 μM 5-fluorouracil or 10 nM camptothecin. The treated cells were incubated at 37°C in the atmosphere of 5% CO$_2$. Cells were harvested at 0, 15, 30, 60 and 120 min, respectively. Protein samples were prepared and subjected to Western blotting analysis using anti-Wee1 and anti-P-Cdc2 and anti-GAPDH antibodies. GAPDH was used as a loading control.

4.9 Effect of pinostrobin on cell cycle progression in Jurkat T-cell, BT474 and KATO III

Wee1 kinase is an important regulator of the G2 checkpoint and is overexpressed in various cancer cell lines. My data demonstrated that pinostrobin inhibited Wee1 kinase activity (Fig. 4.3). To address whether pinostrobin caused cell cycle perturbation, DNA content of Jurkat T-cells treated for 96 h with or without 100 µM ($2X$ IC_{50}) pinostrobin was analyzed. Results showed that pinostrobin treatment did not affect cell cycle progression but caused an increase in the sub G1 cell population from 2.4% in the control treatment (untreated cells) to 11.4% in the 100 µM pinostrobin treated Jurkat T-cells. The increase in sub G1 population could be observed from day 2 of treatment (Fig. 4.10).

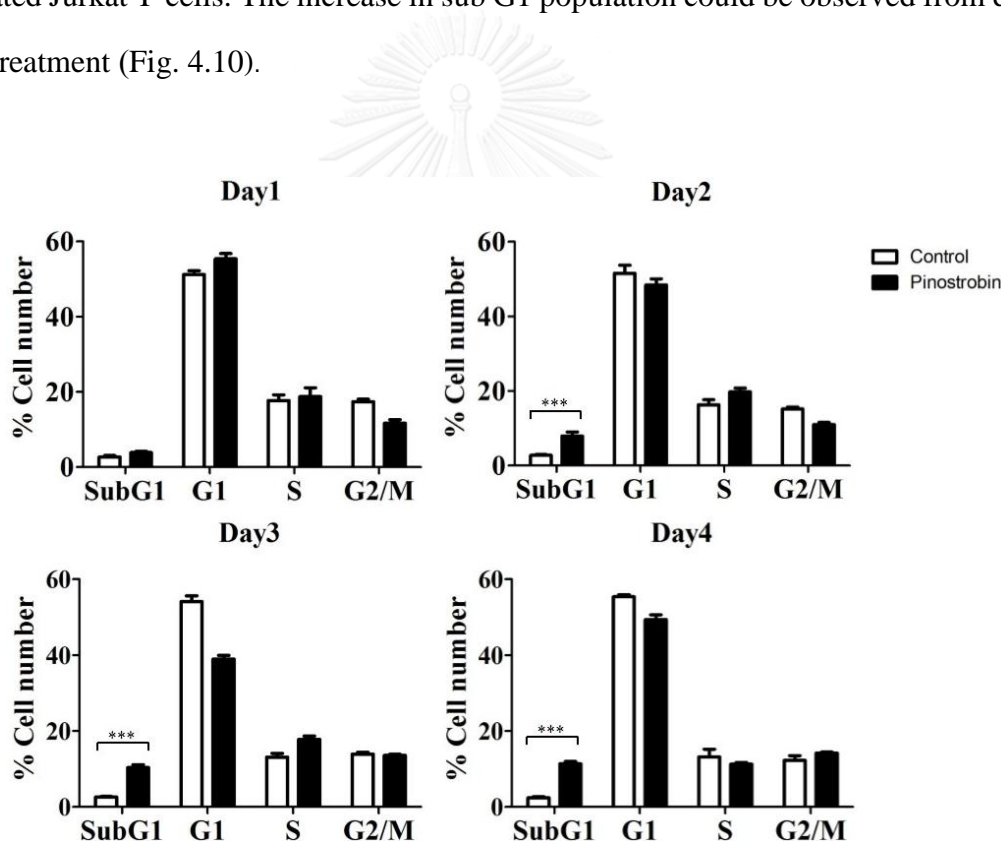**Figure 4.10** Effect of pinostrobin on cell cycle progression in Jurkat T-cells. Cells were incubated for 1 to 4 days in the absence (control, DMSO) or presence of 100 µM pinostrobin and incubated at 37°C in the atmosphere of 5% CO_2, after which the DNA content was analyzed by flow cytometry. The data showed values of mean ± SD from the triplicate experiments. *** Significant differences at p-value <0.001.

To see whether the known Wee 1 kinase inhibitor, MK-1775, caused cell cycle perturbation, DNA contents of Jurkat T-cell treated for 96 h with or without 500 nM MK-1775 were analyzed by flow cytometry. Results revealed that MK-1775 treatment also caused increase in the sub G1 population from 2.4% in the control treatment (untreated cells) to 73.6% in the Jurkat T-cells treated with 500 nM MK-1775 (Fig. 4.11). The increase in sub G1 population could be observed since day 1 post of treatment.

Figure 4.11 Effect of MK-1775 on cell cycle distribution in Jurkat T-cells. The cells were incubated for 1 to 4 days in the absence (control, DMSO) or presence of 500 nM MK-1775 and incubated at 37°C in the atmosphere of 5% CO_2, after which the DNA content was analyzed by flow cytometry. The data showed values of mean ± SD from the triplicate experiments. *** Significant differences at p-value <0.0001.

Pinostrobin showed similar effect as MK-1775 treatment on increase in sub G1 population of treated Jurkat T-cells without any change in profile of cell cycle progression.

BT474 cells were treated with 120 μM (2X IC$_{50}$) pinostrobin or without pinostrobin for 4 days. The result showed that pinostrobin treatment did not affect cell cycle progression but resulted in an increase in the sub G1 population from 3.1% in the control treatment (untreated cells) to 30.2% in the BT474 treated with 120 μM pinostrobin. The increase in sub G1 population could be observed since day 2 of treatment (Fig. 4.12).

Figure 4.12 Effect of pinostrobin on cell cycle progression in BT474. Cells were incubated for 1 to 4 days in the absence (DMSO, control) or presence of 120 μM pinostrobin and incubated at 37°C in the atmosphere of 5% CO_2, after which the DNA content was analyzed by flow cytometry. The data showed values of mean ± SD from the triplicate experiments. **, *** Significant differences at p-value <0.05 and <0.0001.

To see whether MK-1775 caused cell cycle perturbation, DNA contents of BT474 treated for 4 days with or without 500 nM (1X IC$_{50}$) MK-1775 were analyzed by flow cytometry. The results showed that MK-1775 treatment did not affect on cell cycle progression when compared to the control treatment (Fig. 4.13)
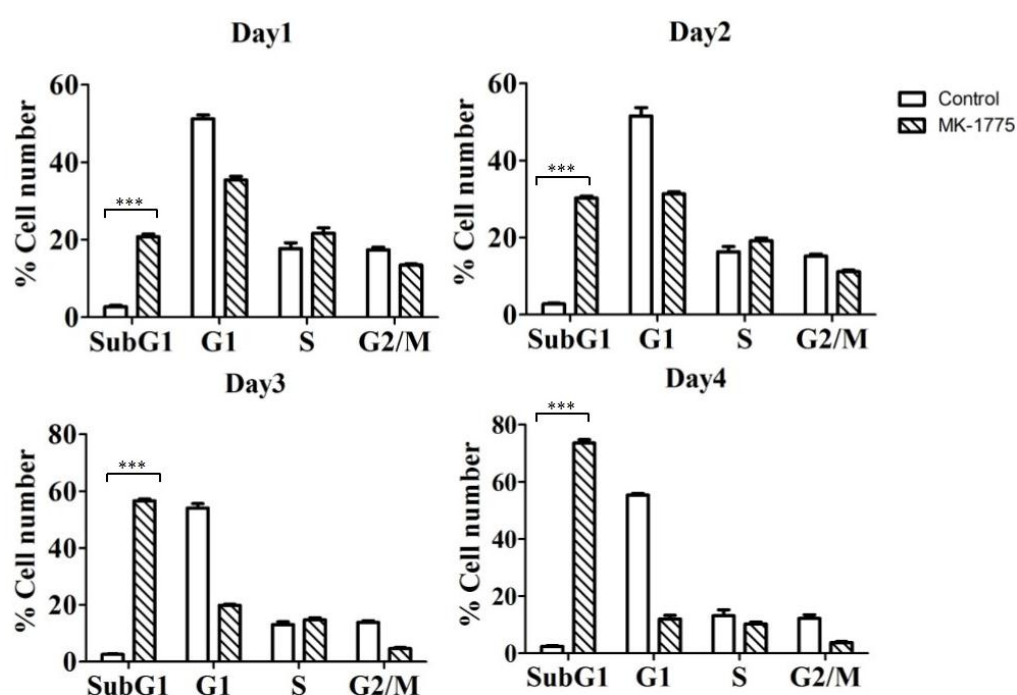

Figure 4.13 Effect of MK-1775 on cell cycle distribution in BT474. Cells were incubated for 1 to 4 days in the absence (control, DMSO) or presence of 500 nM MK-1775 and incubated at 37°C in the atmosphere of 5% CO_2, after which the DNA content was analyzed by flow cytometry. The data showed values of mean ± SD from the triplicate experiments.

Pinostrobin treatment caused significant increase in population of G2/M from 18.6% to 31.4% in the KATO III treated with 100 µM pinostrobin (Fig. 4.14).
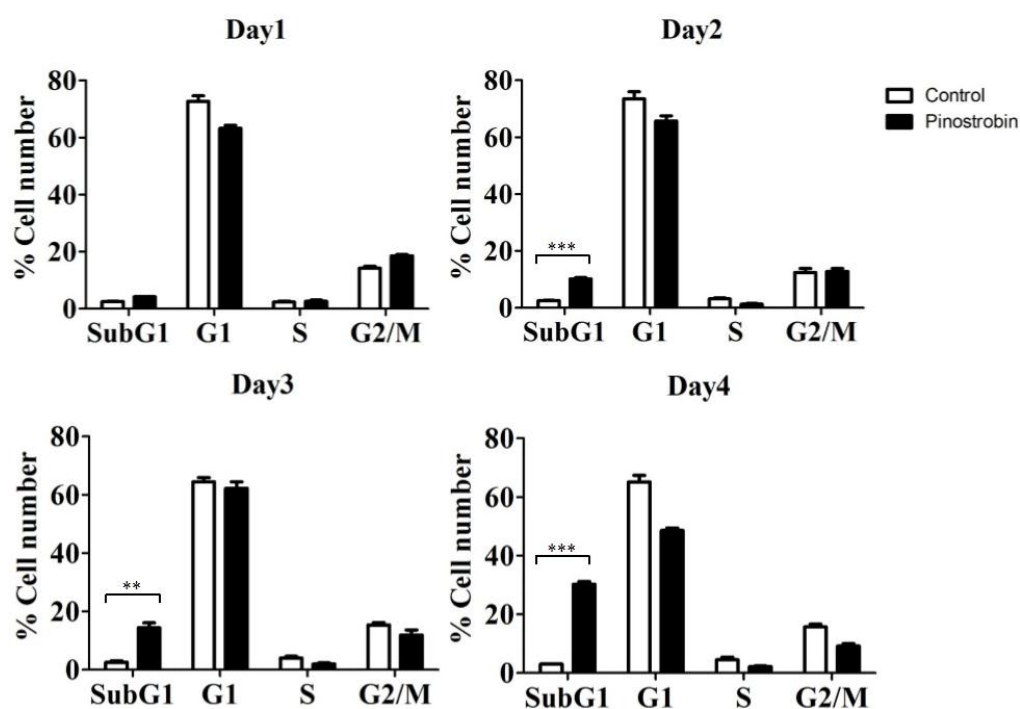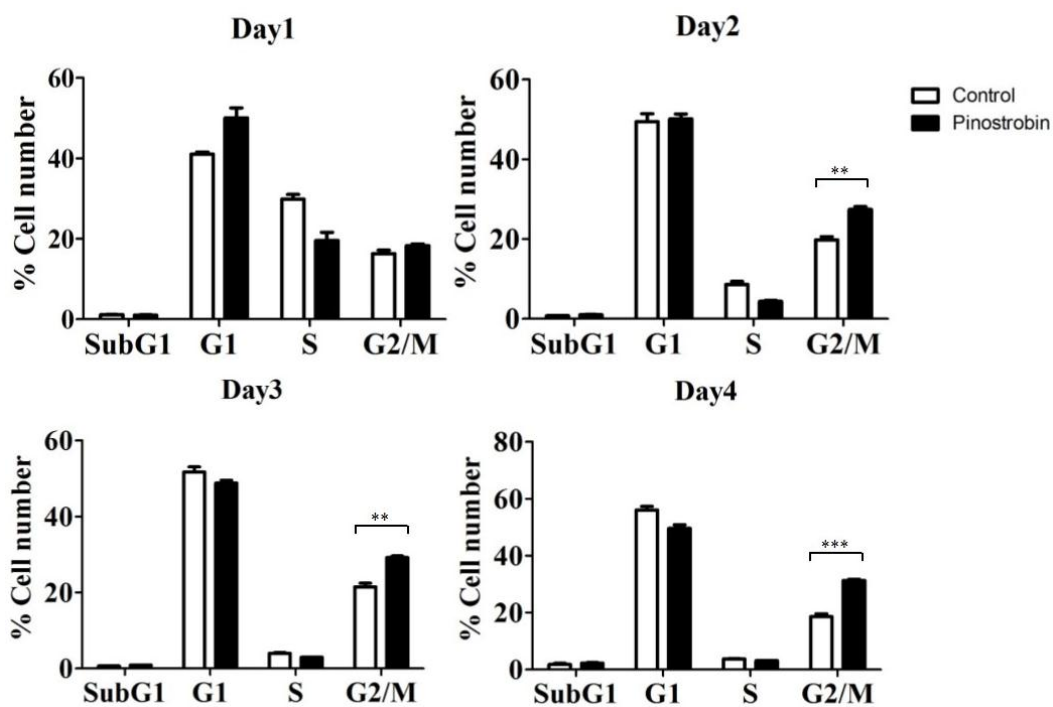

Figure 4.14 Effect of pinostrobin on cell cycle progression in KATO III. Cells were incubated for 1 to 4 days in the absence (control, DMSO) or presence of 100 µM pinostrobin and incubated at 37°C in the atmosphere of 5% CO_2, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. **,*** Significant differences at p-value <0.01 and <0.001 respectively.

To see whether MK-1775 caused cell cycle perturbation in KATO III, DNA contents of KATO III treated for 4 days with or without 500 nM (1X IC$_{50}$) MK-1775 were analyzed by flow cytometry. Surprisingly the results showed that, MK-1775 treatment caused S and G2/M arrest on days 2 and increased the G2/M arrest on days 4, when compared to the control treatment (Fig. 4.15).
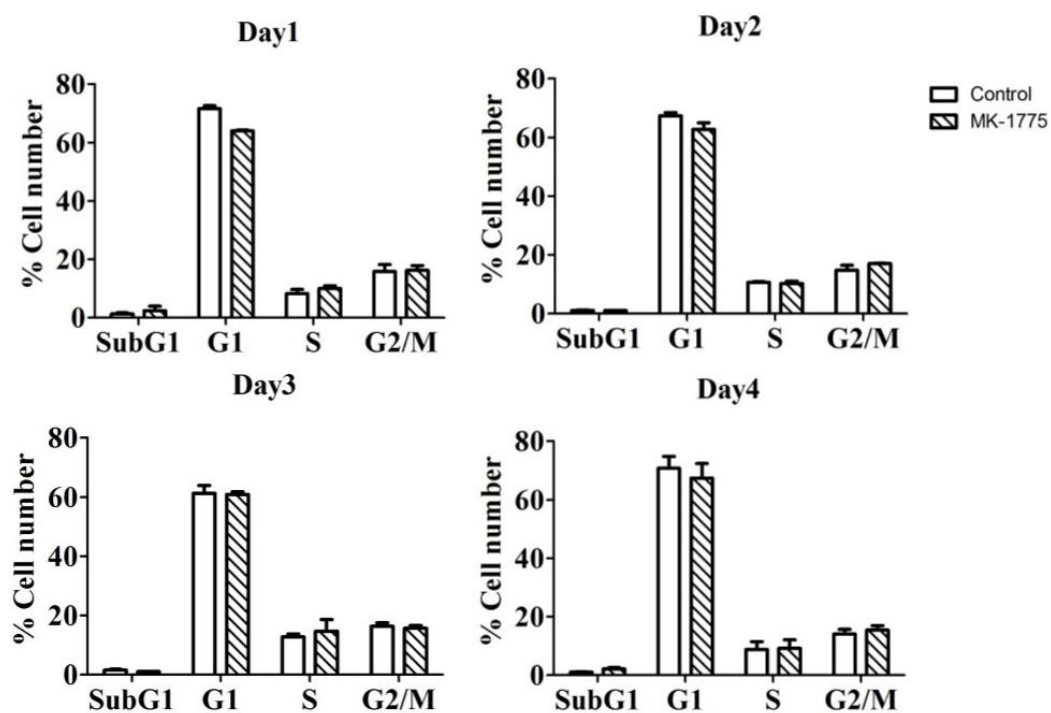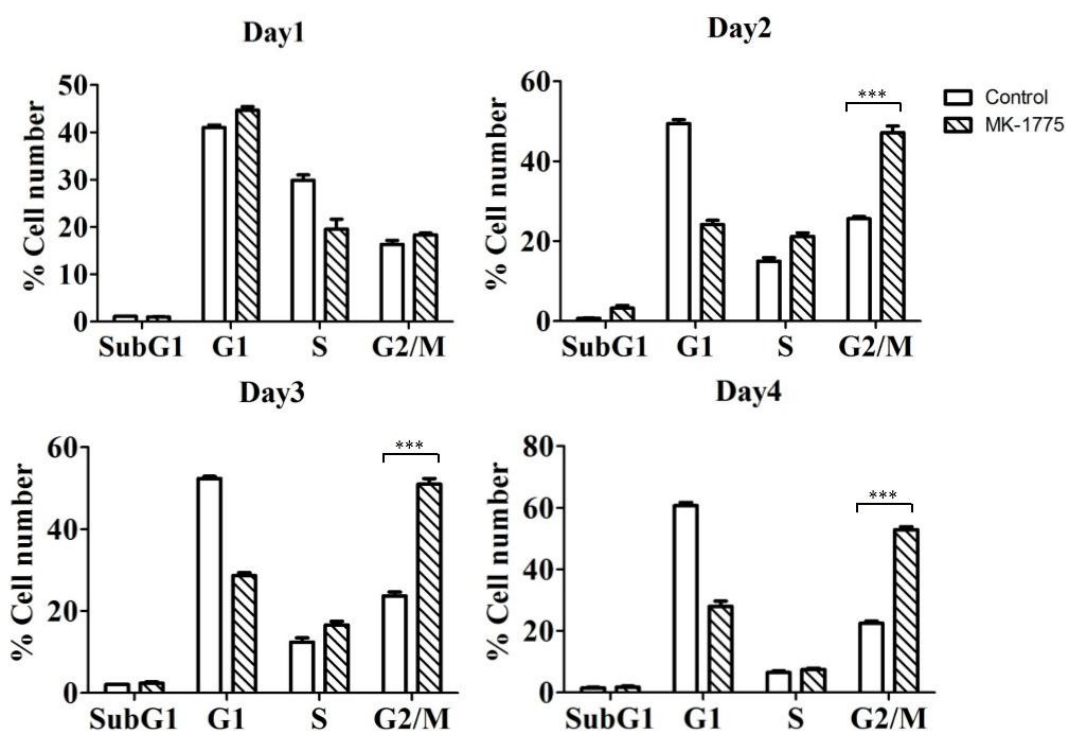

Figure 4.15 Effect of MK-1775 on cell cycle distribution in KATO III. Cells were incubated for 1 to 4 days in the absence (control, DMSO) or presence of 500 nM MK-1775 and incubated at 37°C in the atmosphere of 5% CO_2, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. **,*** Significant differences at p-value <0.01 and <0.001 respectively.

Pinostrobin showed the similar effect to MK-1775 treatment to KATO III on increase in G2/M population of treated cells and without increase in sub G1 in cell cycle progression profile.

4.10 Combination effect of pinostrobin with DNA-damaging agents on cell cycle progression in Jurkat T-cell, BT474 and KATO III

Hirai et al. (2010) reported that MK-1775 could enhance the antitumor efficacy of various DNA-damaging agents, including 5-fluorouracil in several colon cancer cell lines. Therefore, I examined the combination effect of pinostrobin with various DNA damaging drugs, including gemcitabine, 5-fluorouracil or camptothecin on cell cycle progression of Jurkat T-cells. Jurkat T-cells were treated with either 50 μM (1X IC$_{50}$) pinostrobin or 50 nM (0.1X IC$_{50}$) MK-1775 in combination with 0.1X IC$_{50}$ dose of each DNA- damaging agents (gemcitabine or 5-fluorouracil or camptothecin) for 4 days. DNA content of the treated cells was analyzed by flow cytometry. Results showed that treatment of either pinostrobin or MK-1775 or DNA-damaging agent alone did not affect cell cycle progression in Jurkat T-cells. However, the combined treatment between either pinostrobin or MK-1775 with DNA-damaging agent either, gemitabine or camptothecin showed significant increase in sub G1 population from 4.8% in the control treatment (DMSO) to 84.5%, 90.7%, 88.2% and 81.6% in the combined treatments: MK-1775+gemcitabine, MK-1775+camptothecin, pinostrobin+gemcitabine and pinostrobin+camptothecin, respectively. The increase in sub G1 population could be observed since day 1 of treatment (Fig. 4.16). However, the synergistic effect was not be observed in the combined treatment between either pinostrobin or MK-1775 with 5-fluorouracil.
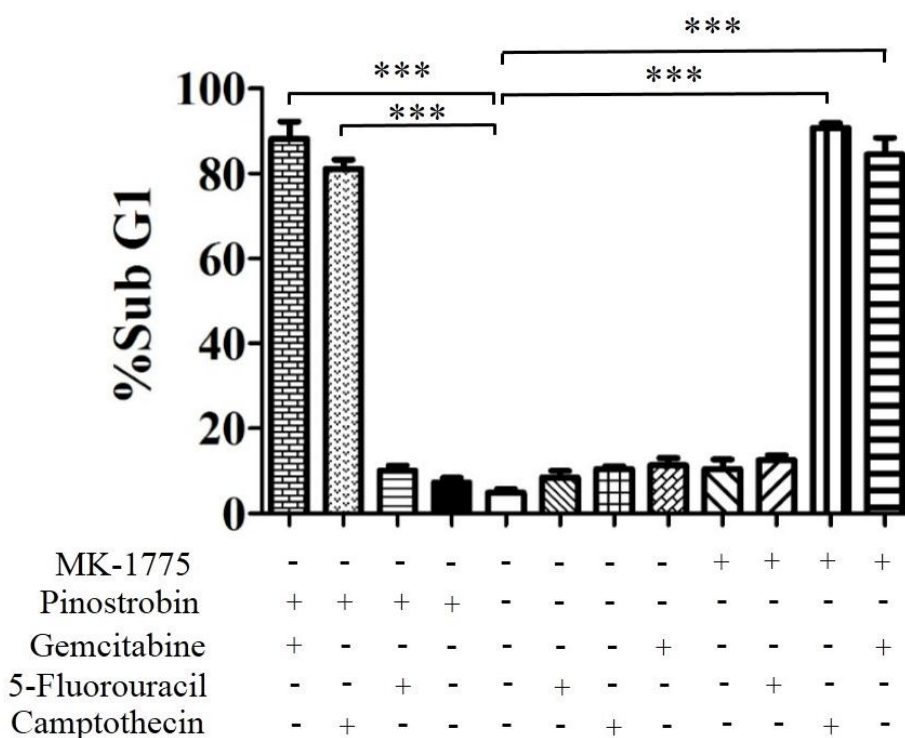
Figure 4.16 Combination effect of pinostrobin with DNA-damaging agent on population of sub G1 phase cell cycle in Jurkat T-cells. The Jurkat T-cells were treated with either 50 μM pinostrobin or 50 nM MK-1775 or the combination of either 50 μM pinostrobin or 50 nM MK-1775 with either 100 nM gemcitabine or 300 nM 5-fluorouracil or 1 nM camptothecin. The cells were incubated at 37°C in the atmosphere of 5% CO_2 for 4 days, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. *** Significant differences at p-value <0.0001.

In BT474, cells were treated with 30 μM (0.5X IC_{50}) of pinostrobin or 500 nM (1X IC_{50}) of MK-1775 and its combination with 0.2 μM (0.1X IC_{50}) camptothecin for 4 days. Either pinostrobin or MK-1775 and the combined treatment posed no effect on cell cycle progression but caused a highly increase in the sub G1 population in combined treatment when compare to control (DMSO) (Fig. 4.17) on 4 days.
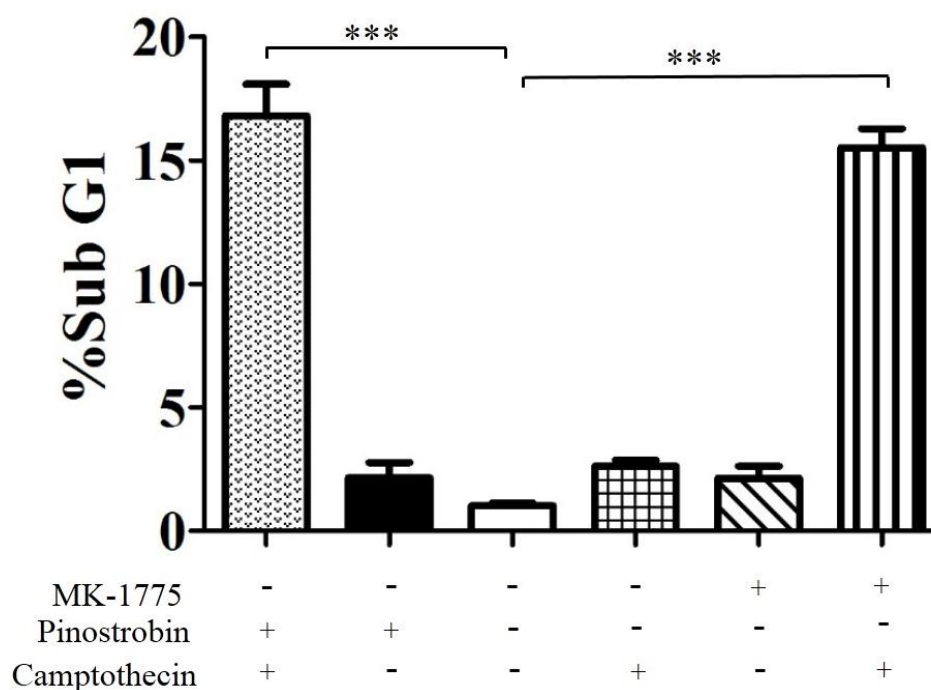
Figure 4.17 Combination effect of pinostrobin with camptothecin on population of sub G1 cell cycle in BT474. BT474 cells were treated with either 30 μM pinostrobin or 500 nM MK-1775 or the combination of either 30 μM pinostrobin or 500 nM MK-1775 with 0.2 μM camptothecin. The cells were incubated at 37°C in the atmosphere of 5% CO_2 for 4 days, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. *** Significant differences at p-value <0.0001.

In KATO III, cells were treated with 50 μM (2X IC$_{50}$) pinostrobin or 50 nM MK-1775 (0.1X IC$_{50}$) and in combination with DNA-damaging agents (1 nM (0.1X IC$_{50}$) gemcitabine, 1 nM (0.1X IC$_{50}$) camptothecin or 200 nM (0.1X IC$_{50}$) 5-fluorouracil) for 4 days. The individually treatment of pinostrobin, MK-1775 or DNA-damaging agents showed no effect upon cell cycle progression. However, the combination of treatment between pinostrobin or MK-1775 with DNA-damaging agents caused S phase arrest in range of 15.0% - 32.3% from 6.47% in the control (Fig 4.18), as measured at 4 days. In MK-1775 treatment combined with camptothecin or 5-fluorouracil caused G2/M arrest to 50.8% and 44.86%, respectively from 22.5% in control (DMSO), while pinostrobin combined with DNA-damaging agents did not show any different from pinostrobin singly treatment (Fig. 4.19) on 4 days.
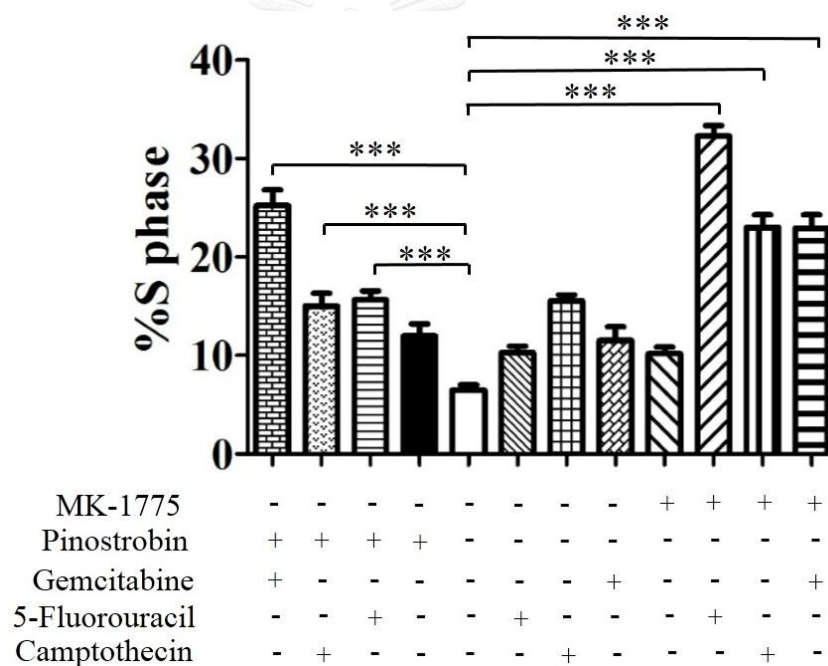
Figure 4.18 Combination effect of pinostrobin with DNA-damaging agents on population of S phase cell cycle distribution in KATO III. KATO III cells were treated with either 50 μM pinostrobin or 50 nM MK-1775 or the combination of either 50 μM pinostrobin or 50 nM MK-1775 with either 1 nM gemcitabine, 200 nM 5-fluorouracil or 1 nM camptothecin. Cells were incubated at 37°C in the atmosphere of 5% CO$_2$ for 4 days, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. *** Significant differences at p-value <0.0001.
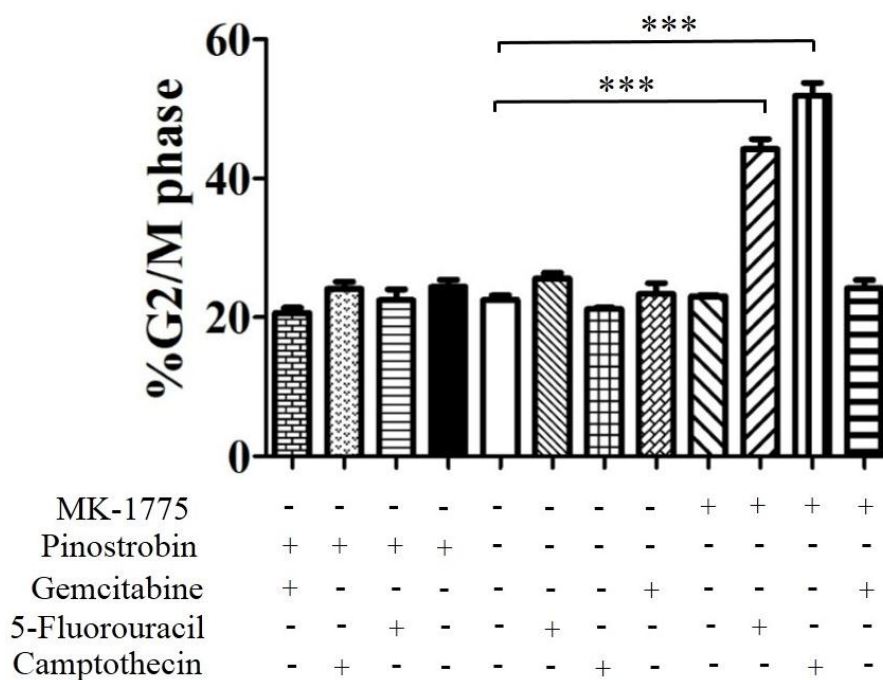
Figure 4.19 Combination effect of pinostrobin with DNA-damaging agents on population of G2/M phase cell cycle distribution in KATO III. KATO III cells were treated with either 50 μM pinostrobin or 50 nM MK-1775 or the combination of either 50 μM pinostrobin or 50 nM MK-1775 with either 1 nM gemcitabine, 200 nM 5-fluorouracil or 1 nM camptothecin. Cells were incubated at 37°C in the atmosphere of 5% CO_2 for 4 days, after which the DNA content was determined by flow cytometry. The data showed the values of mean ± SD from the triplicate experiments. *** Significant differences at p-value <0.0001.

4.11 Effect of pinostrobin on induction of apoptotic cell death in Jurkat T-cell, BT474 and KATO III

Apoptotic cell death was found correlated with sub G1 cell cycle population peak (Leers et al., 1999). Next, the effect of pinostrobin or MK-1775 on inducing apoptotic cell death in Jurkat T-cells, BT474 and KATO III were determined. The Jurkat T-cells were treated with either 100 μM (2X IC_{50}) pinostrobin or 500 nM (1X IC_{50}) MK-1775 for 48 h. Then the treated cells were stained with Annexin V-FITC and analyzed by flow cytometry. Quadrant regions were determined for apoptotic cell death. Quadrant regions were used to classified cells into four different populations: viable cells is in lower left quadrant (Annexin V -/PI -), early apoptotic cells is in lower right quadrant (Annexin V +/PI -), late apoptotic cells is in upper right quadrant (Annexin V +/PI +) and necrotic cells is in upper left quadrant (Annexin V -/PI +). The results from flow cytometry analysis showed that 100 μM pinostrobin or 500 nM MK-1775 induced apoptotic cell death (early and late apoptosis) from 4.01% (control) to 19.01% and 33.22% respectively after 48 h (Fig. 4.20).

Figure 4.20 Effect of pinostrobin on induction of apoptotic cells in Jurkat T-cells. Jurkat T-cells were incubated for 48 h at 37°C in the atmosphere of 5% CO_2 in the absence (control), presence of 100 μM pinostrobin or 500 nM MK-1775. Original dot plots of the PI versus the Annexin V-FITC fluorescence intensities. Annexin V-FITC -/PI - region of the lower left quadrant are counted as viable cells. Annexin V-FITC +/PI - region of the lower right quadrant are counted as early apoptotic cells. Annexin V-FITC +/PI + region of the upper right quadrant are counted as late apoptosis cells. Annexin V-FITC -/PI + region of the upper left quadrant are counted as necrotic cells: A) Control, B) MK-1775 and C) Pinostrobin. D) Bar diagrams showed the percentages of apoptotic cells in different treatments. The data shown are representative of three independent experiments with the similar results. * p-value < 0.001 as compared with the control group.

In BT474, cells were treated with 120 µM (2X IC_{50}) pinostrobin or 500 nM (1X IC_{50}) MK-1775, respectively for 72 h. Flow cytometry analysis showed that 120 µM of pinostrobin induced apoptotic cell (early and late apoptosis) to 16.32%, while MK-1775 treatment did not induce apoptotic cell (7.77%), when compared with control (DMSO) 4.58% (Fig. 4.21).
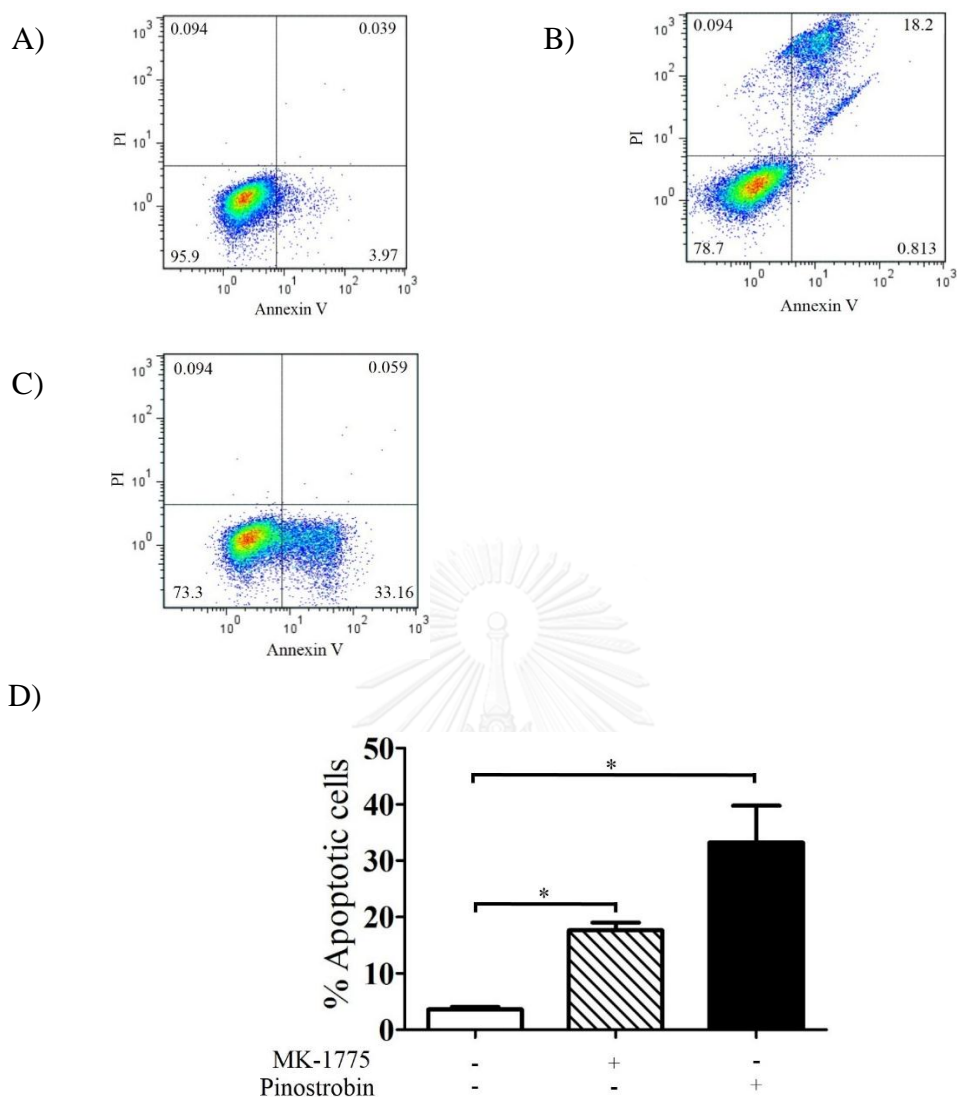
Figure 4.21 Effect of pinostrobin on induction of apoptotic cells in BT474. BT474 cells were incubated for 72 h at 37°C in the atmosphere of 5% CO_2 in the absence (control) or presence of 120 µM pinostrobin or 500 nM MK-1775. Original dot plots of the PI versus the Annexin V-FITC fluorescence intensities. Annexin V-FITC -/PI - region of the lower left quadrant are counted as viable cells. Annexin V-FITC +/PI - region of the lower right quadrant are counted as early apoptotic cells. Annexin V-FITC +/PI + region of the upper right quadrant are counted as late apoptosis cells. Annexin V-FITC -/PI + region of the upper left quadrant are counted as necrotic cells: A) Control, B) MK-1775 and C) Pinostrobin. D) Bar diagrams showed the percentages of apoptotic cells in different treatments. The data shown are representative of three independent experiments with the similar results. * p-value < 0.001 as compared with the control group.

In KATO III, cells were treated with 50 μM (2X IC_{50}) pinostrobin or 500 nM (1X IC_{50}) MK-1775, respectively for 48 h. The results from flow cytometry analysis showed that 50 μM of pinostrobin induced apoptotic cell (early and late apoptosis) to 22.38%. While MK-1775 treatment did not induced apoptotic cell (10.08%), when compared to control (DMSO) at 7.50% (Fig. 4.22).
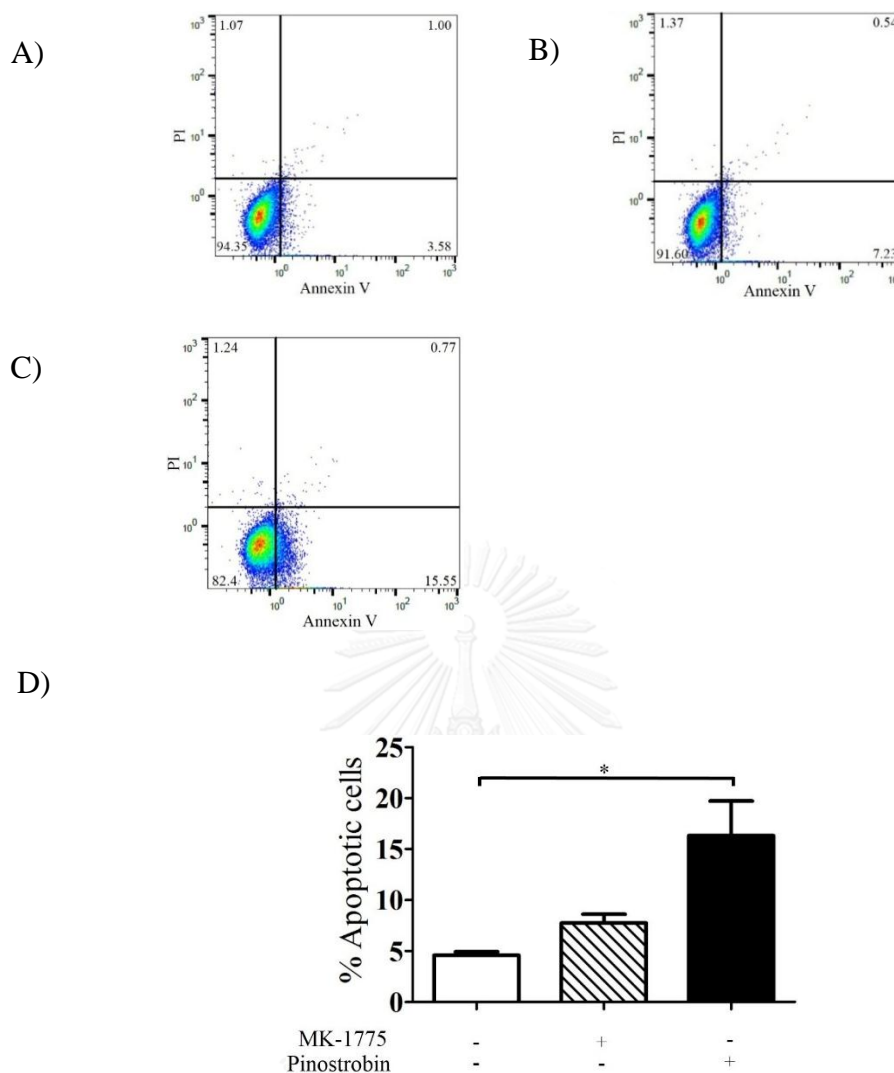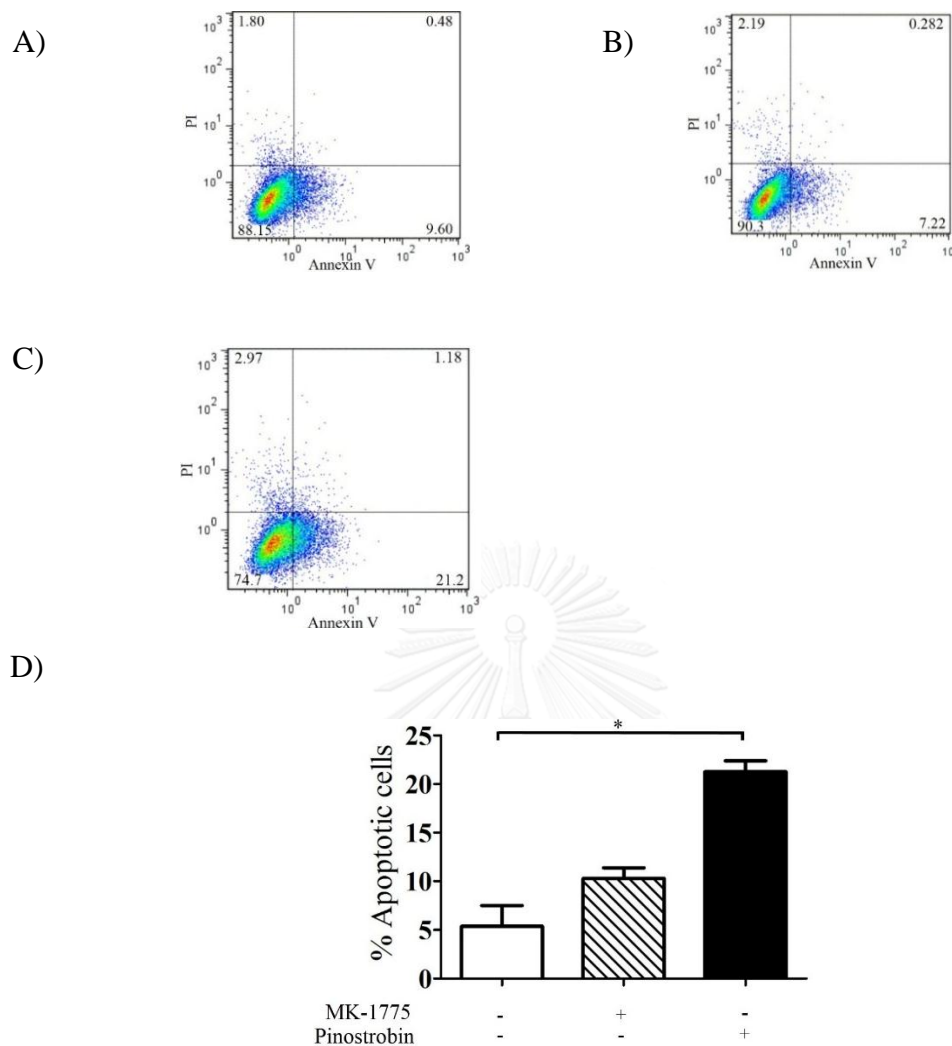
Figure 4.22 Effect of pinostrobin on induction of apoptotic cells in KATO III. The KATO III were incubated for 48 h at 37°C in the atmosphere of 5% CO_2 in the absence (control) or presence of 50 μM pinostrobin or 500 nM MK-1775. Original dot plots of the PI versus the Annexin V-FITC fluorescence intensities. Annexin V-FITC -/PI - region of the lower left quadrant are counted as viable cells. Annexin V-FITC +/PI - region of the lower right quadrant are counted as early apoptotic cells. Annexin V-FITC +/PI + region of the upper right quadrant are counted as late apoptosis cells. Annexin V-FITC -/PI + region of the upper left quadrant are counted as necrotic cells: A) Control, B) MK-1775 and C) Pinostrobin. D) Bar diagrams show the percentages of apoptotic cells in different treatments. The data shown are representative of three independent experiments with the similar results. * p-value < 0.01 as compared with the control group.

4.12 Combination effect of pinostrobin and DNA-damaging agents on induction of apoptotic cell death in Jurkat T-cell, BT474 and KATO III

Next, I examined the effect of pinostrobin or MK-1775 and its combination with a DNA damaging agent (either gemcitabine or 5-fluorouracil or camptothecin). Jurkat T cells were subjected to combined treatment between 50 µM (1X IC$_{50}$) pinostrobin or or 50 nM (0.5X IC$_{50}$) MK-1775 with either 0.1 µM (0.1 X IC$_{50}$) gemcitabine, 0.3 µM (0.1 X IC$_{50}$) 5-fluorouracil or 1 nM (0.1 X IC$_{50}$) camptothecin. The treated cells were incubated at 37°C under 5% CO$_2$ atmosphere for 12 h followed by cell harvesting and staining with Annexin V-FITC. Apoptotic cell death was analyzed by flow cytometry. The combined treatment of MK-1775+gemcitabine, MK-1775+camptothecin, pinostrobin+gemcitabine and pinostrobin+camptothecin revealed an increase in % apoptotic cells at 15.9%, 12.4%, 16.7% and 10.4%, respectively compared to 4.73% of the control DMSO treatment (Fig. 4.23).
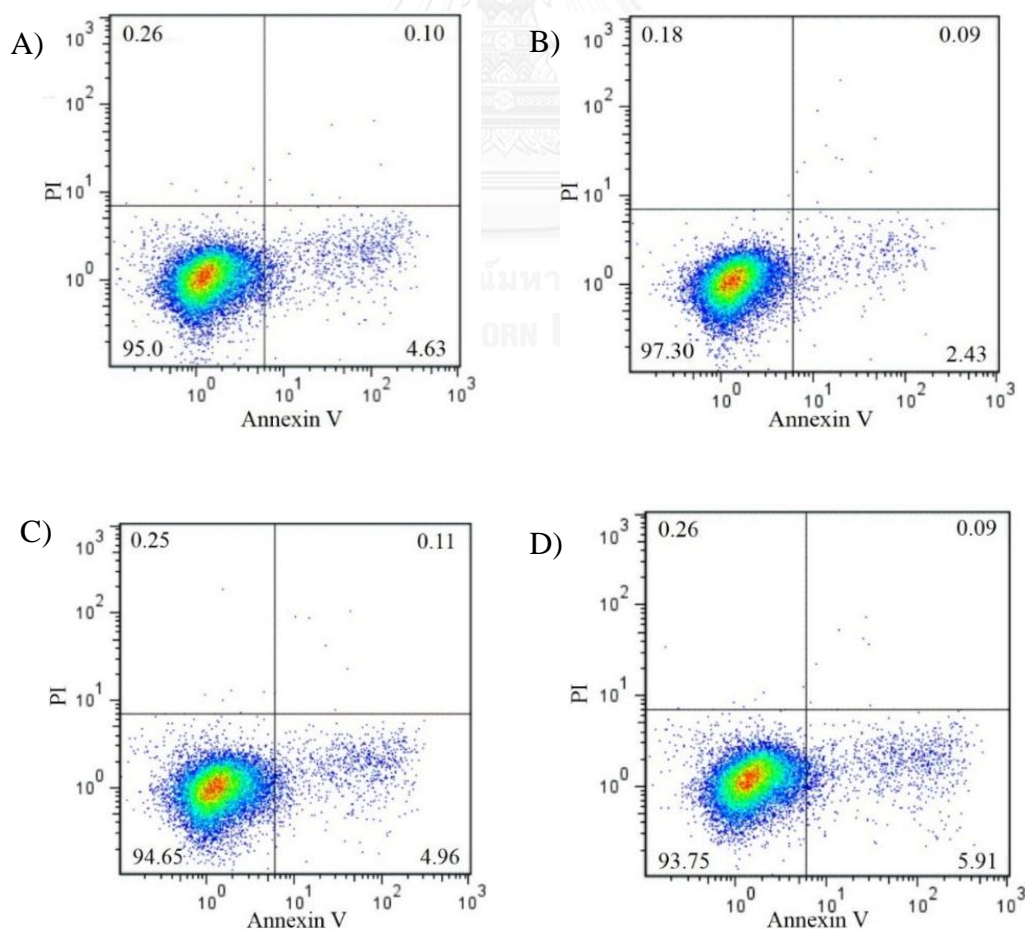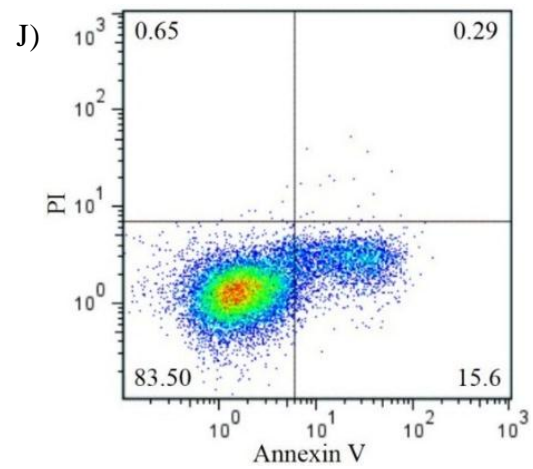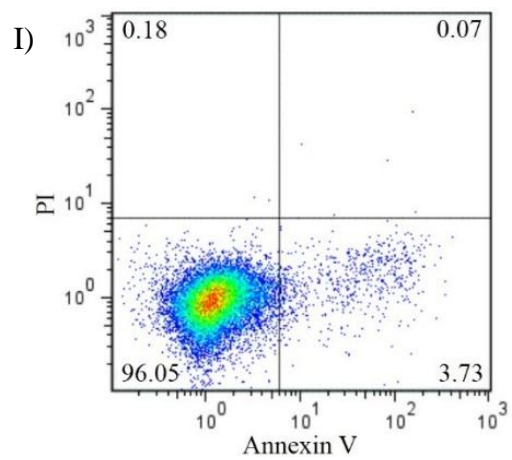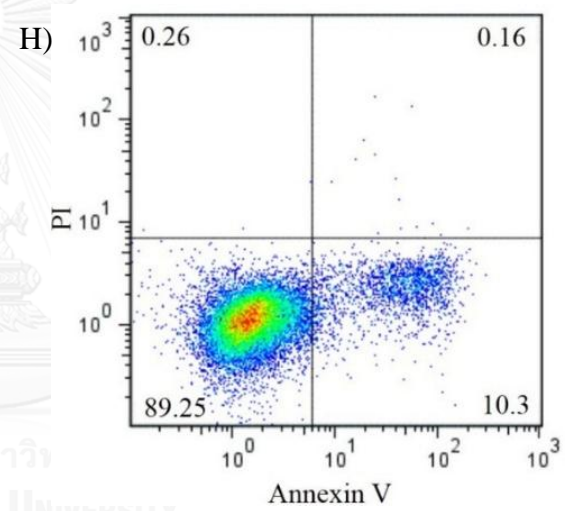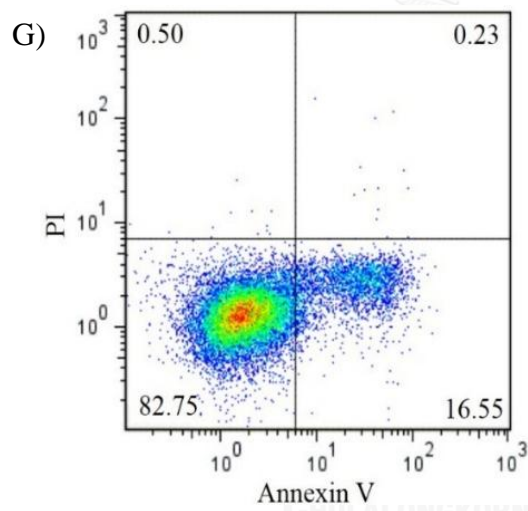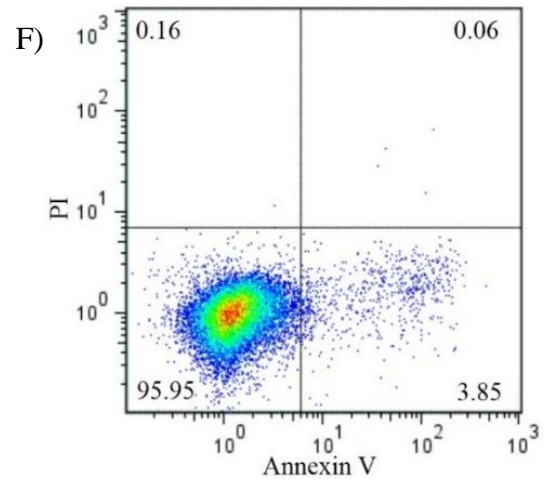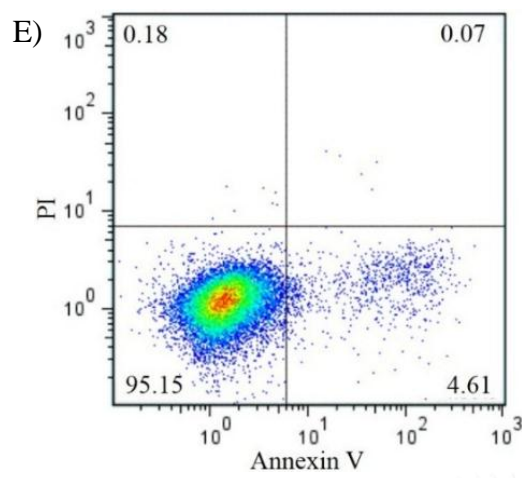
Figure 4.23 Combination effect of pinostrobin with DNA-damaging agents on number of apoptotic cell death in Jurkat T-cells. Jurkat T-cells were incubated at 37°C in the atmosphere of 5% CO_2 for 12 h in the absence (control) or presence of 50 μM pinostrobin or 50 nM MK-1775 and its combination with either 100 nM gemcitabine, 1 nM camptothecin or 300 nM 5-fluorouracil, after which the apoptotic cells were determined by flow cytometry. Original dot plots of PI versus Annexin V-FITC fluorescence intensities: A) Control, B) Pinostrobin, C) MK-1775, D) Gemcitabine, E) Camptothecin, F) 5-fluorouracil, G) Pinostrobin+Gemcitabine, H) Pinostrobin+Camptothecin, I) Pinostrobin+5-fluorouracil, J) MK-1775+Gemcitabine, K) MK-1775+Camptothecin, L) MK-1775+5-Fluorouracil. M) Bar diagrams showed percentages of apoptotic cells. The data shown are representative of three independent experiments with the similar results. *** p-value < 0.0001 as compared with the control group.

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In BT474, Cells were treated with 30 μM (0.5X IC_{50}) pinostrobin or 500 nM (1X IC_{50}) MK-1775 and combination with 0.2 μM (0.1X IC_{50}) camptothecin, respectively for 72 h. The combination treatment between pinostrobin or MK-1775 with camptothecin showed an enhanced cyctotoxic effect on induction of apoptotic cells, when compared to individual treatment with pinostrobin, MK-1775 or camptothecin and control DMSO treatment (Fig. 4.24).

G)
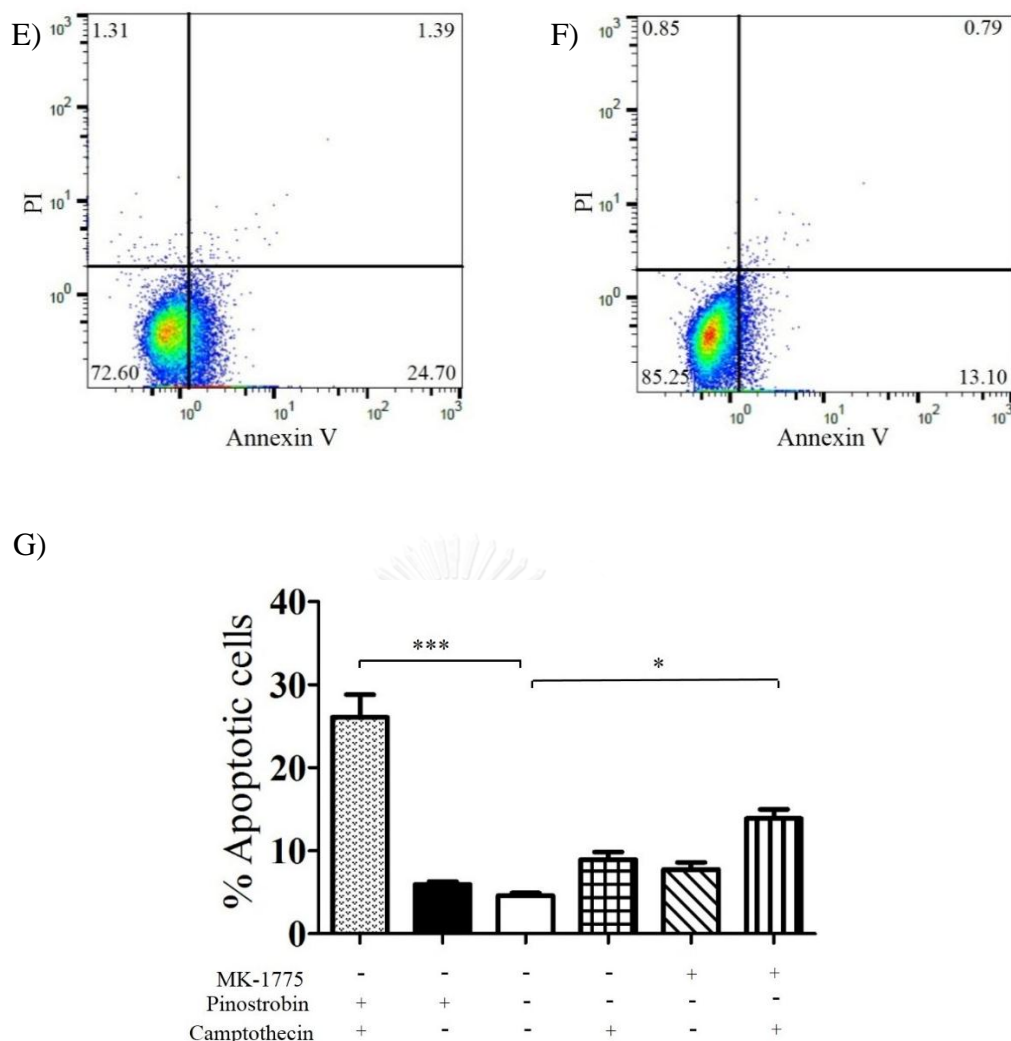
Figure 4.24 Combination effect of pinostrobin and camptothecin on number of apoptotic cell death in BT474. BT474 cells were incubated at 37°C in the atmosphere of 5% CO_2 for 72 h in the absence (control) or presence of 30 μM pinostrobin or 500 nM MK-1775 and its combination with 0.2 μM camptothecin after which the apoptotic cells were determined by flow cytometry. Original dot plots of PI versus Annexin V-FITC fluorescence intensities: A) Control, B) Pinostrobin, C) MK-1775, D) Camptothecin, E) Pinostrobin+Camptothecin, F) MK-1775+Camptothecin. G) Bar diagrams showed the percentages of apoptotic cells. The data shown are representative of three independent experiments with the similar results. *, *** $p < 0.001$ as compared with the control group.

In KATO III, Cells were treated with 25 μM (1X IC$_{50}$) pinostrobin or 50 nM(0.1X IC$_{50}$) MK-1775 with either 1 nM (0.1X IC$_{50}$) gemcitabine, 1 nM (0.1X IC$_{50}$) camptothecin or 200 nM (0.1X IC$_{50}$) 5-fluorouracil, respectively for 48 h. Both individual and combination treatment did not showed the cytotoxic effect to induce apoptotic cells death, when compared to control DMSO treatment (Fig. 4.25).
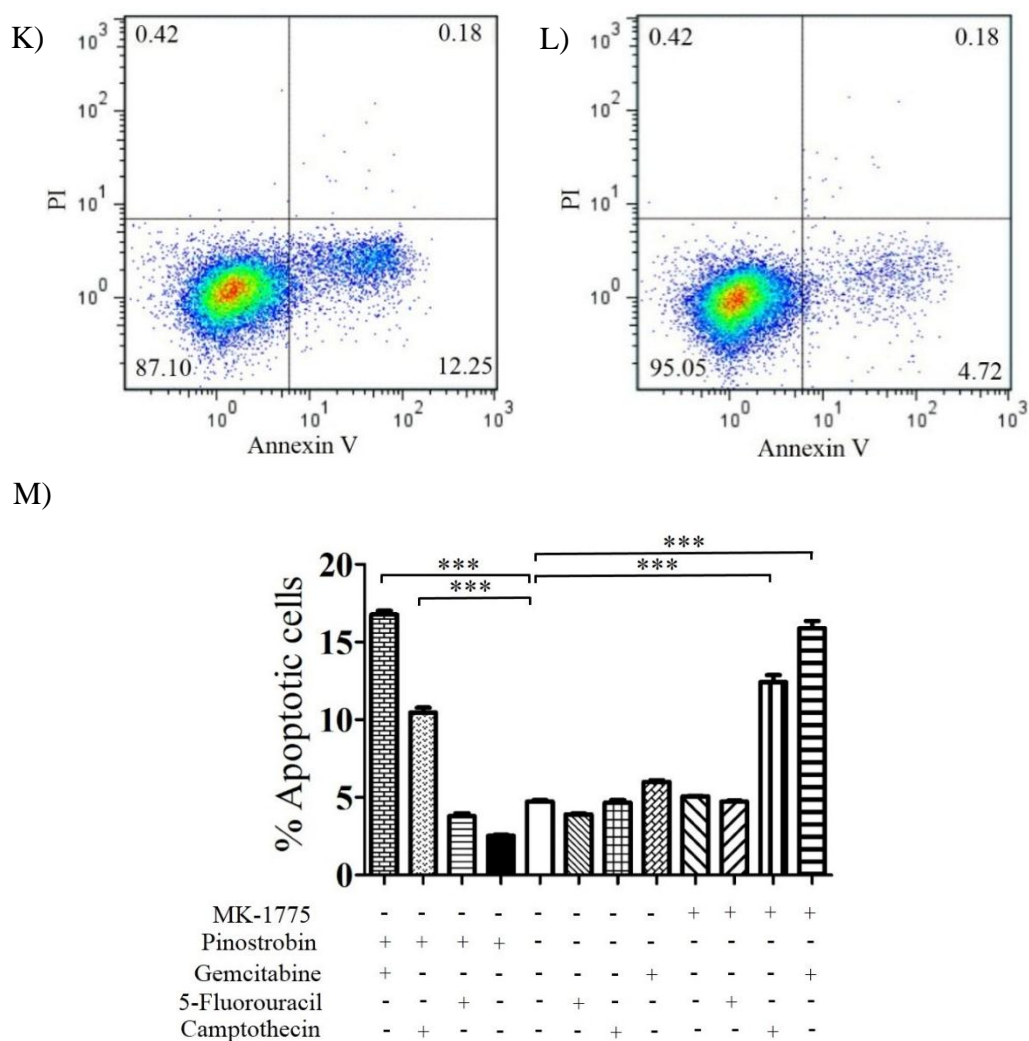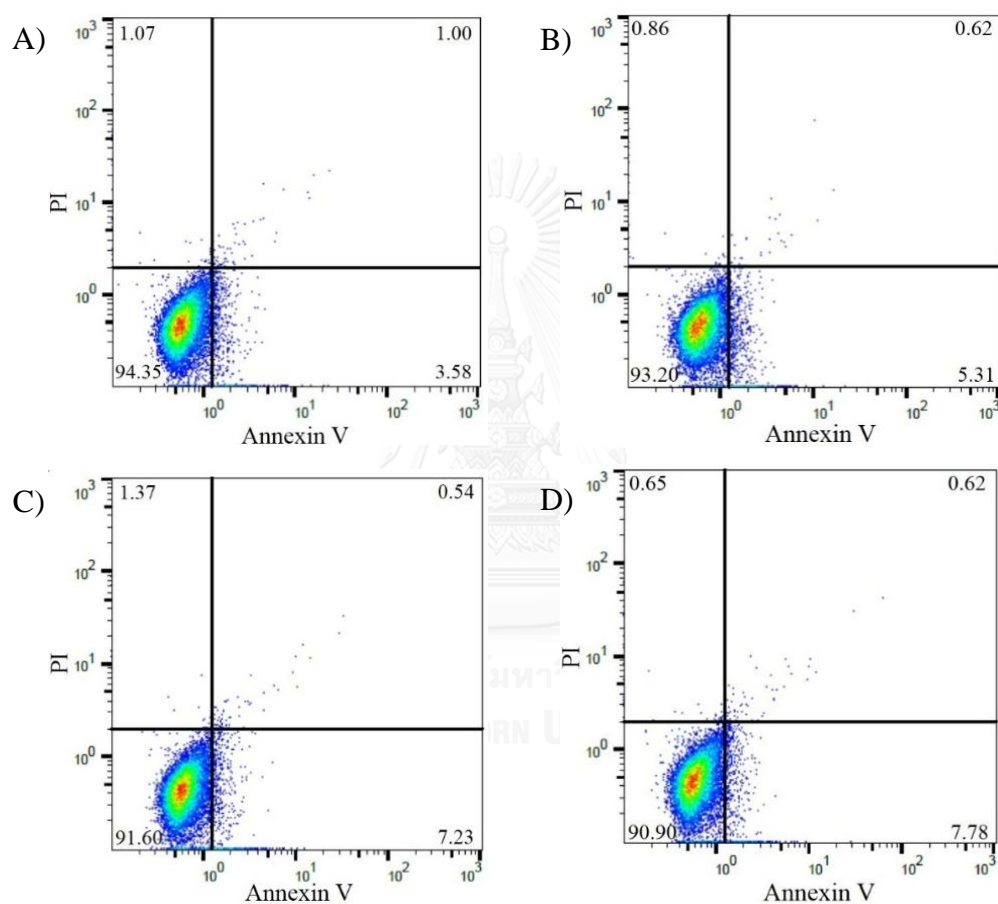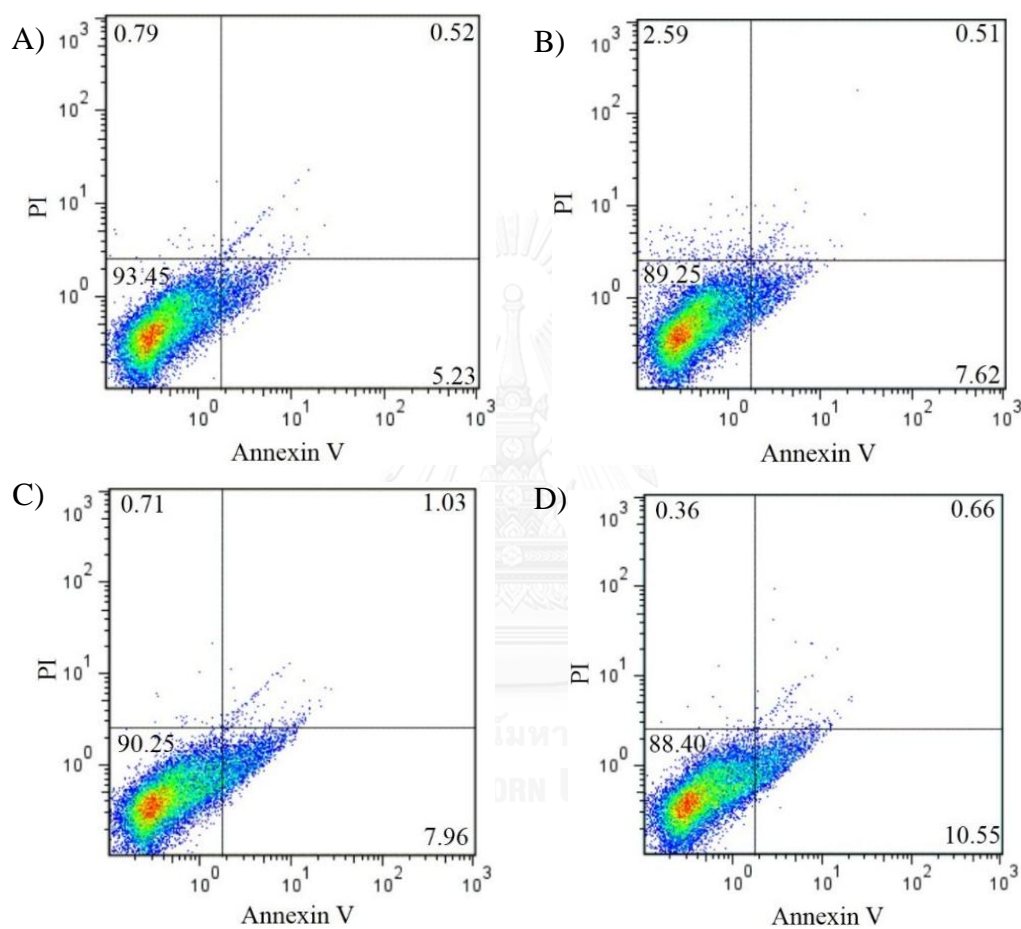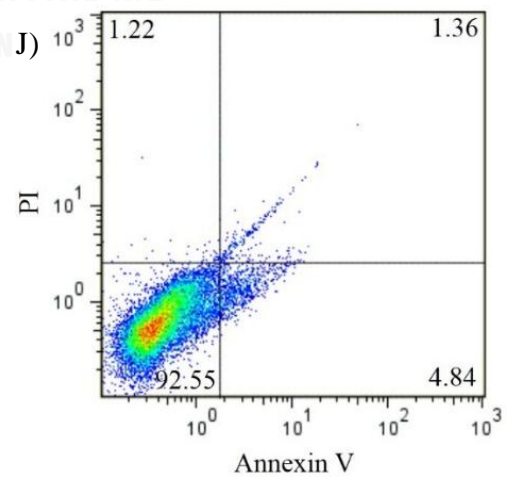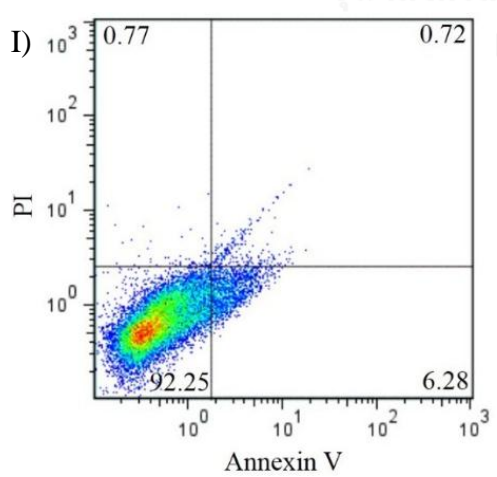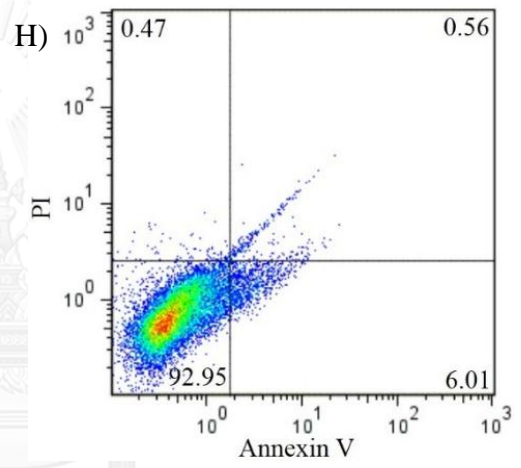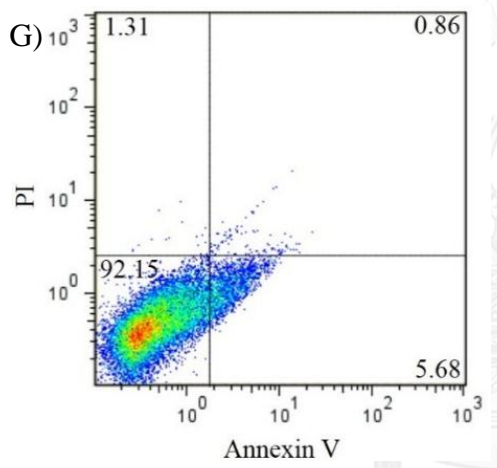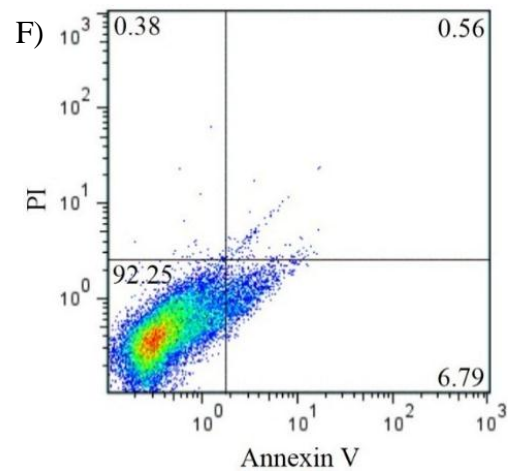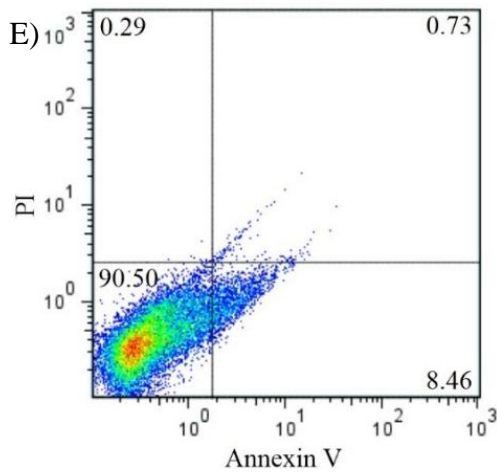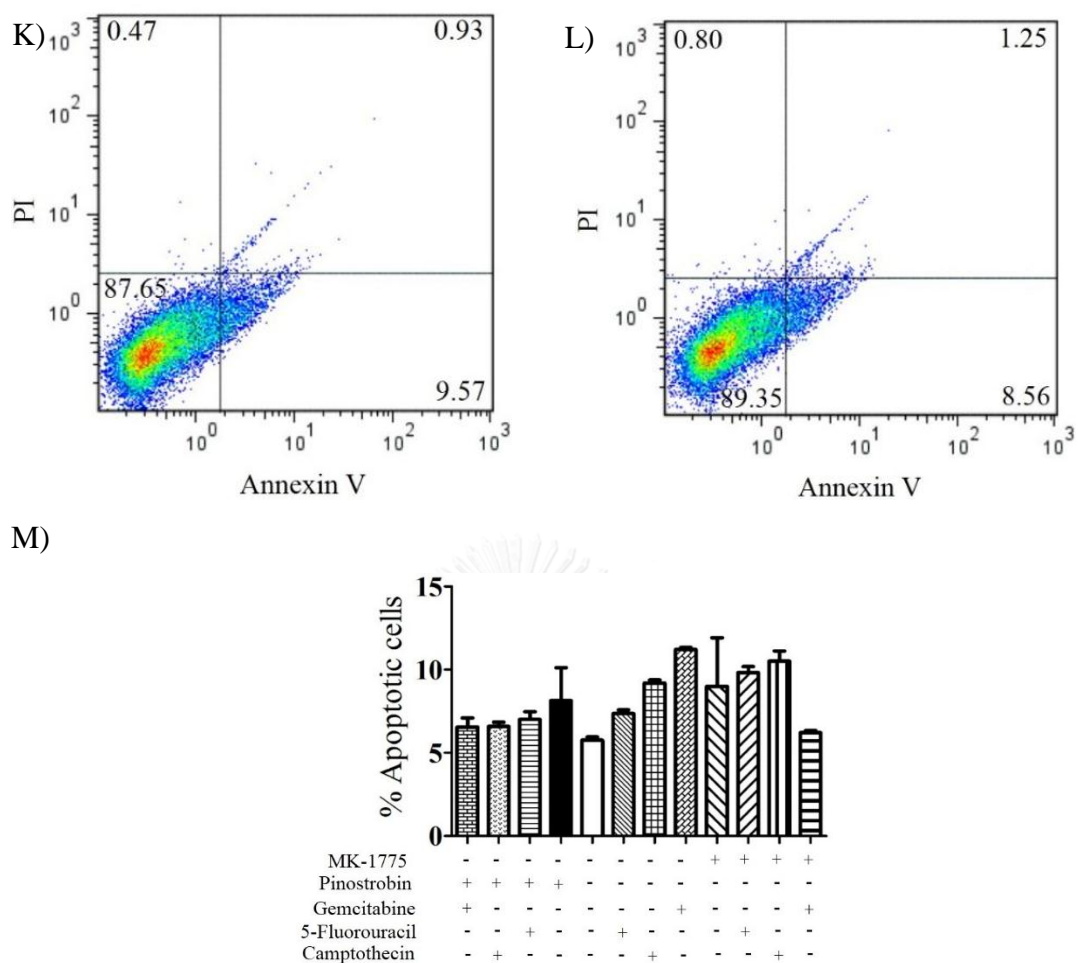
Figure 4.25 Combination effect of pinostrobin with DNA-damaging agents on number of apoptotic cell death in KATO III. KATO III cells were incubated at 37°C in the atmosphere of 5% CO_2 for 12 h in the absence (control) or presence of 25 µM pinostrobin or 50 nM MK-1775 and its combination with either 1 nM Gemcitabine or 1 nM Camptothecin or 200 nM 5-fluorouracil, after which the apoptotic cells were determined by flow cytometry. Original dot plots of PI versus Annexin V-FITC fluorescence intensities, A) Control, B) Pinostrobin, C) MK-1775, D) Gemcitabine, E) Camptothecin, F) 5-fluorouracil, G) Pinostrobin+Gemcitabine, H) Pinostrobin+Camptothecin, I) Pinostrobin+5-fluorouracil, J) MK-1775+Gemcitabine, K) MK-1775+Camptothecin, L) MK-1775+5-Fluorouracil. M) Bar diagrams showed the percentages of apoptotic cells. The data shown are representative of three independent experiments with the similar results.

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Discussion

Previously, pinostrobin has been reported to exert an anti-proliferative effect on various human cancer cell lines. Jurkat T-cells and HL60 cell lines (Smolarz et al., 2006), CCRF-CEM leukaemia cells (Ashidi et al., 2007), MCF-7 cells (Le Bail et al., 2000). Also, Anti-oxidant activity (Kong et al., 2009), in *vivo* anti-ulcerogenic activity (Abdelwahab et al., 2011) were observed. In this study, I found that pinostrobin could inhibit cell proliferation in Jurkat T-cells, BT474 and KATO III in time- and dose-dependent manner. Results from the MTT viability assay showed that pinostrobin did not acute cytotoxic effects against Jurkat T-cell, BT474 and KATO III, when treated cell with pinostrobin for 1 day. However, pinostrobin caused chronic cytotoxic effects in Jurkat T-cells, BT474 and KATO III on 4 days with IC_{50} values of 51.2, 61.6 and 24.7 µM, respectively (Fig. 4.1). Moreover pinostrobin did not showed harmful cytotoxic effect to the normal human red blood cells (Table 4.1) and normal white blood cells at IC_{50} dose (Fig. 4.2)

Human cancer cell lines with p53-deficient override their G1/S check point and depends on the G2/M checkpoint for the DNA damage repair (Mueller and Haas-Kogan, 2015). Wee1 kinase, plays a key role in G2/M checkpoint, that the complex negatively regulates entry of the cells into mitosis by phosphorylating the Tyr15 residue of Cdc2, therefore inactivating the Cdc2/cyclin B complex and arresting the cell cycle for the DNA damage repair (Magnussen et al., 2012). Previously reported that Jurkat T-cell, BT474 and KATO III were p53-deficient cell line (Bartek et al., 1990, Cheng and Haas, 1990, Sugimoto et al., 1992, O'Connor et al., 1997). Here I found that pinostrobin down-regulated Wee1 kinase level in those listed human cell lines and hence led to down regulation of Wee1 kinase activity (P-Cdc2). However MK-1775 (known Wee1 inhibitor) treatment only down-regulating only Wee1 kinase activity (Fig. 4.5A, B and C).

The results suggested that pinostrobin might act as a Wee1 inhibitor in various human cancer cell lines with different mechanism, when compared to MK-1775. In this work the results were confirmed by quantitative real-time PCR, that pinostrobin behaved differently from MK-1775 that it down regulated *WEE1* expression while MK-1775 did not pose the same effect in Jurkat T-cell, BT474 and KATO III. Among these cell lines, down regulated *WEE1* expression was the most obvious in Jurkat T-cell at 60 min after treatment. On the other hand MK-1775 treatment showed inconstant expression of *WEE1*. To the best of my knowledge, pinostrobin is the first natural product that possess a Wee1 kinase inhibition.

In this research, I found that pinostrobin behaved differently from MK-1775 on Wee1 kinase inhibition. Pinostrobin caused increased populations of subG1 but did not affect cell cycle progression in Jurkat T-cells and BT474 (Fig 4.10 and 4.12). The result suggested that inhibition of Wee1 kinase by pinostrobin prevents the phosphorylation of Cdc2 and block DNA damage repair, lead to an abrogate premature mitotic entry and finally causing cell death (Russell and Nurse, 1987, De Witt Hamer et al., 2011). I further demonstrated that pinostrobin caused G2/M cell cycle arrest in KATO III (Fig. 4.14). Nomura et al. (2007) reported that gelnadamycin (Hsp90 inhibitor) caused G2/M arrest in U87MG glioblastoma by decreasing the expression of Cdc2, cyclin B1 and phosphorylated Cdc2. Ashidi et al. (2007) revealed that flavanone (including pinostrobin) from *Cajanus cajan* leaves caused G1 arrest in CCRF-CEM leukaemia cells. Haddad et al. (2006) showed that the flavonoids (including pinostrobin) on cell cycle of many prostate cancer cell lines in G2/M phase arrest. Here, I found that Jurkat T-cell and BT474 showed increased in sub G1 population when were treated with pinostrobin but no effect on sub G1 was found in KATO III (Fig. 4.14). Wee1 kinase is found overexpressed in various cancer types, including leukemia, brain tumors, colon cancer, breast cancer and osteosarcoma (Mir et al., 2010, PosthumaDeBoer et al., 2011, Wang et al., 2011, Porter et al., 2012, Harris et al., 2014). Therefore, the use of the Wee1 inhibitor to inhibit the Wee1 kinase may be a new cancer therapeutic strategy on the overexpression of Wee1 kinase in various human cancer cell lines (Harris et al., 2014). MK-1775 cause increase in sub G1 population only in Jurkat T-cells but did not affected on both BT474 and KATO III, That be suggested that pinostrobin and MK-1775 possess different mode of action on cell cycle progression. Cell cycle analysis

showed that pinostrobin caused increased sub G1 or G2/M population in Jurkat T-cells and BT474 or KATO III, respectively (Fig. 4.10, 4.12 and 4.14). The sub G1 cell cycle population peak was found correlated with apoptotic cell death (Leers et al., 1999). This effect may be crucial for pinostrobin to induce apoptosis in Jurkat T-cell lines, BT474 and KATO III. Pinostrobin was formed to induce apoptotic cell death in these human cancer cell lines (Fig. 4.20, 4.21 and 4.22). This finding was supported by the report of Smolarz et al. (2006) which detected apoptotic cells by pinostrobin treated Jurkat or HL60 cell lines. Also, pinostrobin from *Kaempferia pandurata* Roxb. was reported to induce apoptosis in T47D human breast cancer cell line by decreasing the expression of Bcl-2 and increasing the expression of Bax, caspase-3 and p53 (Sukardiman et al., 2014). However, the molecular mechanisms of pinostrobin-mediated down regulation of Wee1 kinase and Wee1 activity to induced apoptotic cells death remain ambiguous.

Furthermore it was, shown that pinostrobin possess synergistic effects with some DNA-damaging agents (e.g. gemcitabine, camptothecin or 5-fluorouracil) on anti-proliferation by tremendously increase in sub G1 population of the treated Jurkat T-cells and BT474 or increase G2/M arrest in KATO III (Fig. 4.16, 4.17, 4.18 and 4.19) and induced apoptotic cell death (Fig. 4.23 and 4.24). These findings were supported by several reports. For example, MK-1775 enhanced the anti-proliferation efficacy of various DNA-damaging agents such as camptothecin, 5-fluorouracil, doxorubicin, mytocin C and pemetrexed in p53-deficient human colon cancer cell lines (Hirai et al., 2010) and enhanced gemcitabine in p53-deficient pancreatic cancer xenografts (Rajeshkumar et al., 2011).

From these data, it is still elusive as there are several mechanisms and pathways which might correspond to pinostrobin's mode of action on cell cycle progression. It was reported that okadaic acid posed effects on apoptosis, cell cycle progression and morphological change in various human cancer cell lines (Valdiglesias et al., 2011). Sukardiman et al. (2000) suggested that one of the molecular target of pinostrobin from *Temu kunci* (*Kempferia pandurata*) in human mammary carcinoma was DNA topoisomerase I. Here I found that different cell lines responded differently to pinostrobin treatment eg. Jurkat T-cell and KATO III.

To the best of our knowledge, up to the present, there was no any report on natural bioactive compound with inhibitory activity against Wee1 kinase. Here, it was demonstrated that pinostrobin, a flavonone from fingerroot *B. pandurata*, is a potential Wee1 kinase inhibitor. It will be interesting to further derivatize pinostrobin to increase or modify its activity for was as a potential anti-cancer drug.

5.2 Conclusion
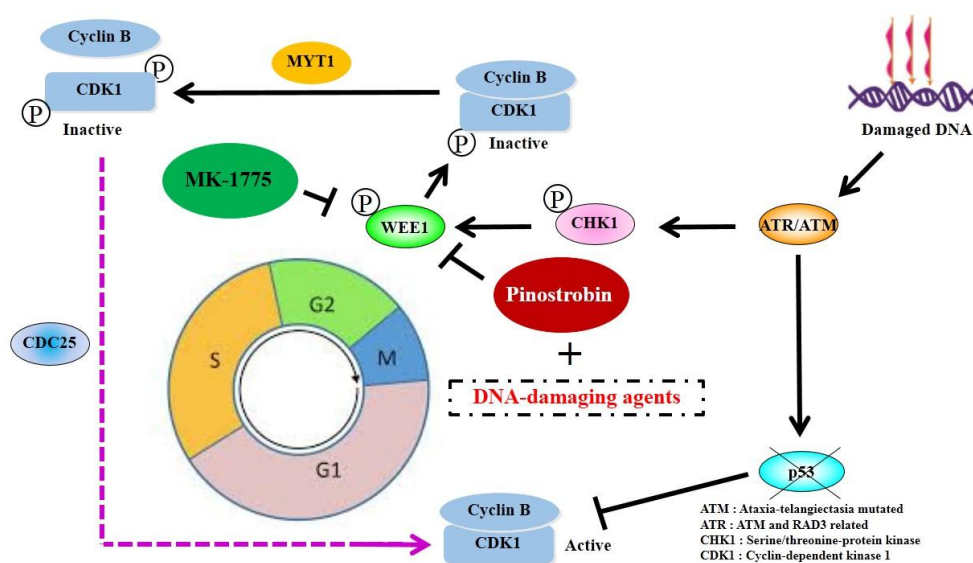
Figure 5.1 Proposed model on mechanism of pinostrobin in human cancer cell lines (Jurkat T-cells, BT474 and KATO III)

In this study, pinostrobin from *B. pandurata* was found to be a potential Wee1 kinase inhibitor. Its anti-proliferative activity on several cancer cell lines especially those with p53, a G1 phase cell cycle checkpoint, deficiency including human acute T cell leukemia (Jurkat), human breast carcinoma cell line (BT474) and human stomach carcinoma cell line (KATO III) was revealed. No cytotoxicity against human normal white and red blood cells was observed at its IC_{50} dose. Its mechanism of action was proposed here as pinostrobin inhibits activity of Wee1 kinase, a G2 cell cycle checkpoint, thus accelerating the abnormal cells to divide (Fig. 5.1).

As a result enhanced the treated cells died by apoptosis. Combination treatment of pinostrobin with ten-fold dosage reduction of some DNA-damaging agents: camptothecin or gemcitabine revealed synergistic effects on percentage of apoptotic cell death. Thus, much lowering the side effect of the DNA-damaging agents. The results suggested that potential use of pinostrobin especially in combination with some DNA-damaging agents for anti-cancer therapy in some kinds of cancer.

REFERENCES

Aarts, M., R. Sharpe, I. Garcia-Murillas, H. Gevensleben, M. S. Hurd, S. D. Shumway, C. Toniatti, A. Ashworth and N. C. Turner (2012). "Forced mitotic entry of S-phase cells as a therapeutic strategy induced by inhibition of WEE1." Cancer Discov 2(6): 524-539.

Abdelwahab, S. I., S. Mohan, M. A. Abdulla, M. A. Sukari, A. B. Abdul, M. M. Taha, S. Syam, S. Ahmad and K. H. Lee (2011). "The methanolic extract of *Boesenbergia rotunda* (L.) Mansf. and its major compound pinostrobin induces anti-ulcerogenic property in vivo: possible involvement of indirect antioxidant action." J Ethnopharmacol 137(2): 963-970.

Almeida, E. R. d. (2009). "Preclinical and Clinical Studies of Lapachol and Beta-Lapachone." The Open Natural Products Journal 2: 42-47.

Ancuceanu, R. V. and V. Istudor (2004). "Pharmacologically active natural compounds for lung cancer." Altern Med Rev 9(4): 402-419.

Anderson, L. M. (2006). "Environmental genotoxicants/carcinogens and childhood cancer: bridgeable gaps in scientific knowledge." Mutat Res 608(2): 136-156.

Arpornsuwan, T., W. Sriwai, J. Jaresitthikunchai, N. Phaonakrop, H. Sritanaudomchai and S. Roytrakul (2014). "Anticancer Activities of Antimicrobial BmKn2 Peptides Against Oral and Colon Cancer Cells." Int J Pept Res Ther 20(4): 501-509.

Ashidi, J., P. Houghton, P. Hylands, S. Sieber and T. Efferth (2007). "Molecular mechanism of action of the flavanone pinostrobin from *Cajanus cajan* leaves in cancer cells." Planta Med 73: 103.

Barrientos, A. (2003). "Yeast models of human mitochondrial diseases." IUBMB Life 55(2): 83-95.

Bartek, J., J. Bartkova, B. Vojtesek, Z. Staskova, A. Rejthar, J. Kovarik and D. P. Lane (1990). "Patterns of expression of the p53 tumour suppressor in human breast tissues and tumours in situ and in vitro." Int J Cancer 46(5): 839-844.

Barten, M. J., S. Dhein, H. Chang, H. B. Bittner, A. Tarnok, A. Rahmel, F. W. Mohr and J. F. Gummert (2003). "Assessment of immunosuppressive drug interactions: inhibition of lymphocyte function in peripheral human blood." J Immunol Methods 283(1-2): 99-114.

Bhamarapravati, S., S. Juthapruth, W. Mahachai and G. Mahady4 (2006). "Antibacterial activity of *Boesenbergia rotunda* (L.) Mansf. and *Myristica fragrans* Houtt. against *Helicobacter pylori*." Songklanakarin J Sci Technol 28: 157-163.

Blenk, S., J. C. Engelmann, S. Pinkert, M. Weniger, J. Schultz, A. Rosenwald, H. K. Muller-Hermelink, T. Muller and T. Dandekar (2008). "Explorative data analysis of MCL reveals gene expression networks implicated in survival and prognosis supported by explorative CGH analysis." BMC Cancer 8: 106.

Booher, R. N., R. J. Deshaies and M. W. Kirschner (1993). "Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins." EMBO J 12(9): 3417-3426.

Bridges, K. A., H. Hirai, C. A. Buser, C. Brooks, H. Liu, T. A. Buchholz, J. M. Molkentine, K. A. Mason and R. E. Meyn (2011). "MK-1775, a novel Wee1 kinase inhibitor, radiosensitizes p53-defective human tumor cells." Clin Cancer Res 17(17): 5638-5648.

Chanklan, R., E. Aihara, S. Koga, H. Takahashi, M. Mizunuma and T. Miyakawa (2008). "Inhibition of Ca2+-signal-dependent growth regulation by radicicol in budding yeast." Biosci Biotechnol Biochem 72(1): 132-138.

Chaturvedi, A. K., C. A. Gaydos, P. Agreda, J. P. Holden, N. Chatterjee, J. J. Goedert, N. E. Caporaso and E. A. Engels (2010). "*Chlamydia pneumoniae* infection and risk for lung cancer." Cancer Epidemiol Biomarkers Prev 19(6): 1498-1505.

Chaudhuri, L., N. D. Vincelette, B. D. Koh, R. M. Naylor, K. S. Flatten, K. L. Peterson, A. McNally, I. Gojo, J. E. Karp, R. A. Mesa, L. O. Sproat, J. M. Bogenberger, S. H. Kaufmann and R. Tibes (2014). "CHK1 and WEE1 inhibition combine synergistically to enhance therapeutic efficacy in acute myeloid leukemia ex vivo." Haematologica 99(4): 688-696.

Cheng, J. and M. Haas (1990). "Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines." Mol Cell Biol 10(10): 5502-5509.

Clapham, D. E. (1995). "Calcium signaling." Cell 80(2): 259-268.

Correa, P. and M. B. Piazuelo (2011). "*Helicobacter pylori* Infection and Gastric Adenocarcinoma." US Gastroenterol Hepatol Rev 7(1): 59-64.

Crews, C. M. and J. B. Shotwell (2003). "Small-molecule inhibitors of the cell cycle: an overview." Prog Cell Cycle Res 5: 125-133.

Das, D., R. B. Shah and M. J. Imperiale (2004). "Detection and expression of human BK virus sequences in neoplastic prostate tissues." Oncogene 23(42): 7031-7046.

De Witt Hamer, P. C., S. E. Mir, D. Noske, C. J. Van Noorden and T. Wurdinger (2011). "WEE1 kinase targeting combined with DNA-damaging cancer therapy catalyzes mitotic catastrophe." Clin Cancer Res 17(13): 4200-4207.

Deng, F., J. J. Lu, H. Y. Liu, L. P. Lin, J. Ding and J. S. Zhang (2011). "Synthesis and antitumor activity of novel salvicine analogues." Chin Chem Lett 22(1): 25-28.

Diogo, C. V., L. Felix, S. Vilela, A. Burgeiro, I. A. Barbosa, M. J. Carvalho, P. J. Oliveira and F. P. Peixoto (2009). "Mitochondrial toxicity of the phyotochemicals daphnetoxin and daphnoretin--relevance for possible anti-cancer application." Toxicol In Vitro 23(5): 772-779.

Dixon, R. A. and D. Ferreira (2002). "Genistein." Phytochemistry 60(3): 205-211.

Do, K., J. H. Doroshow and S. Kummar (2013). "Wee1 kinase as a target for cancer therapy." Cell Cycle 12(19): 3159-3164.

Donze, O. and D. Picard (1999). "Hsp90 binds and regulates Gcn2, the ligand-inducible kinase of the alpha subunit of eukaryotic translation initiation factor 2 [corrected]." Mol Cell Biol 19(12): 8422-8432.

Dubey, K. K., A. R. Ray and B. K. Behera (2008). "Production of demethylated colchicine through microbial transformation and scale-up process development." Process Biochem 43(3): 251-257.

Etoh, T., H. Inoue, Y. Yoshikawa, G. F. Barnard, S. Kitano and M. Mori (2000). "Increased expression of collagenase-3 (MMP-13) and MT1-MMP in oesophageal cancer is related to cancer aggressiveness." Gut 47(1): 50-56.

Express, M. (2015). "MK-1775 Chemical Structure [Online]. A vailiable from: http://www.medchemexpress.com/mk-1775.html [2015, 13 October].".

Fahey, J. W. and K. K. Stephenson (2002). "Pinostrobin from honey and Thai ginger (*Boesenbergia pandurata*): a potent flavonoid inducer of mammalian phase 2 chemoprotective and antioxidant enzymes." J Agric Food Chem 50(25): 7472-7476.

Fresno Vara, J. A., E. Casado, J. de Castro, P. Cejas, C. Belda-Iniesta and M. Gonzalez-Baron (2004). "PI3K/Akt signalling pathway and cancer." Cancer Treat Rev 30(2): 93-204.

Gaurisankar, S., DasTanya, B. Shuvomoy and C. Juni (2010). "Curcumin: From Exotic Spice to Modern Anticancer Drug." Al Ameen J Med Science 3(21-37).

Goel, A., A. B. Kunnumakkara and B. B. Aggarwal (2008). "Curcumin as "Curecumin": from kitchen to clinic." Biochem Pharmacol 75(4): 787-809.

Goes, F. S. and J. Martin (2001). "Hsp90 chaperone complexes are required for the activity and stability of yeast protein kinases Mik1, Wee1 and Swe1." Eur J Biochem 268(8): 2281-2289.

Gomez-Betancur, I., D. Benjumea, A. Patino, N. Jimenez and E. Osorio (2014). "Inhibition of the toxic effects of Bothrops asper venom by pinostrobin, a flavanone isolated from *Renealmia alpinia* (Rottb.) MAAS." J Ethnopharmacol 155(3): 1609-1615.

Guertin, A. D., J. Li, Y. Liu, M. S. Hurd, A. G. Schuller, B. Long, H. A. Hirsch, I. Feldman, Y. Benita, C. Toniatti, L. Zawel, S. E. Fawell, D. G. Gilliland and S. D. Shumway (2013). "Preclinical evaluation of the WEE1 inhibitor MK-1775 as single-agent anticancer therapy." Mol Cancer Ther 12(8): 1442-1452.

Haarberg, H. E., K. H. Paraiso, E. Wood, V. W. Rebecca, V. K. Sondak, J. M. Koomen and K. S. Smalley (2013). "Inhibition of Wee1, AKT, and CDK4 underlies the efficacy of the HSP90 inhibitor XL888 in an in vivo model of NRAS-mutant melanoma." Mol Cancer Ther 12(6): 901-912.

Haddad, A. Q., V. Venkateswaran, L. Viswanathan, S. J. Teahan, N. E. Fleshner and L. H. Klotz (2006). "Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines." Prostate Cancer Prostatic Dis 9(1): 68-76.

Hait, W. N., E. Rubin and S. Goodin (2005). "Tubulin-targeting agents." Cancer Chemother Biol Response Modif 22: 35-59.

Hampson, P., K. Wang and J. M. Lord (2005). "Treatment of actinic keratoses, acute myeloid leukemia therapy, Treatment of basal cell carcinoma, Protein kinase C activator." Drugs Fut 30: 1003.

Harris, P. S., S. Venkataraman, I. Alimova, D. K. Birks, I. Balakrishnan, B. Cristiano, A. M. Donson, A. M. Dubuc, M. D. Taylor, N. K. Foreman, P. Reigan and R. Vibhakar (2014). "Integrated genomic analysis identifies the mitotic checkpoint kinase WEE1 as a novel therapeutic target in medulloblastoma." Mol Cancer 13: 72.

He, W., Z. Wang, Q. Wang, Q. Fan, C. Shou, J. Wang, K. E. Giercksky, J. M. Nesland and Z. Suo (2009). "Expression of HIWI in human esophageal squamous cell carcinoma is significantly associated with poorer prognosis." BMC Cancer 9: 426.

Hede, S. M., V. Savov, H. Weishaupt, O. Sangfelt and F. J. Swartling (2014). "Oncoprotein stabilization in brain tumors." Oncogene 33(39): 4709-4721.

Hirai, H., T. Arai, M. Okada, T. Nishibata, M. Kobayashi, N. Sakai, K. Imagaki, J. Ohtani, T. Sakai, T. Yoshizumi, S. Mizuarai, Y. Iwasawa and H. Kotani (2010). "MK-1775, a small molecule Wee1 inhibitor, enhances anti-tumor efficacy of various DNA-damaging agents, including 5-fluorouracil." Cancer Biol Ther 9(7): 514-522.

Hirai, H., Y. Iwasawa, M. Okada, T. Arai, T. Nishibata, M. Kobayashi, T. Kimura, N. Kaneko, J. Ohtani, K. Yamanaka, H. Itadani, I. Takahashi-Suzuki, K. Fukasawa, H. Oki, T. Nambu, J. Jiang, T. Sakai, H. Arakawa, T. Sakamoto, T. Sagara, T. Yoshizumi, S. Mizuarai and H. Kotani (2009). "Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents." Mol Cancer Ther 8(11): 2992-3000.

Holbro, T., R. R. Beerli, F. Maurer, M. Koziczak, C. F. Barbas, 3rd and N. E. Hynes (2003). "The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation." Proc Natl Acad Sci U S A 100(15): 8933-8938.

Huang, Z., G. Chen and P. Shi (2009). "Effects of emodin on the gene expression profiling of human breast carcinoma cells." Cancer Detect Prev 32(4): 286-291.

Im, S. H. and A. Rao (2004). "Activation and deactivation of gene expression by Ca^{2+}/calcineurin-NFAT-mediated signaling." Mol Cells 18(1): 1-9.

Iorns, E., C. J. Lord, A. Grigoriadis, S. McDonald, K. Fenwick, A. Mackay, C. A. Mein, R. Natrajan, K. Savage, N. Tamber, J. S. Reis-Filho, N. C. Turner and A. Ashworth (2009). "Integrated functional, gene expression and genomic analysis for the identification of cancer targets." PLoS One 4(4): e5120.

Iwai, A., D. Bourboulia, M. Mollapour, S. Jensen-Taubman, S. Lee, A. C. Donnelly, S. Yoshida, N. Miyajima, S. Tsutsumi, A. K. Smith, D. Sun, X. Wu, B. S. Blagg, J. B. Trepel, W. G. Stetler-Stevenson and L. Neckers (2012). "Combined inhibition of Wee1 and Hsp90 activates intrinsic apoptosis in cancer cells." Cell Cycle 11(19): 3649-3655.

K, K. and O. C. MJ (2009). "The G(2) DNA damage checkpoint: could this ancient regulator be the Achilles heel of cancer?" Cancer Biol Ther 15: 1439-1439.

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

Kaufman, P. B., J. A. Duke, H. Brielmann, J. Boik and J. E. Hoyt (1997). "A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: implications for human nutrition and health." J Altern Complement Med 3(1): 7-12.

Ke, Q., T. P. Ellen and M. Costa (2008). "Nickel compounds induce histone ubiquitination by inhibiting histone deubiquitinating enzyme activity." Toxicol Appl Pharmacol 228(2): 190-199.

Ki, S. W., K. Kasahara, H. J. Kwon, K. Ishigami, T. Kitahara, T. Beppu, M. Yoshida and S. Horinouchi (2001). "Radicicol binding to Swo1/Hsp90 and inhibition of growth of specific temperature-sensitive cell cycle mutants of fission yeast." Biosci Biotechnol Biochem 65(11): 2528-2534.

Kim, S., B. Y. Hwang, B. N. Su, H. Chai, Q. Mi, A. D. Kinghorn, R. Wild and S. M. Swanson (2007). "Silvestrol, a potential anticancer rocaglate derivative from *Aglaia foveolata*, induces apoptosis in LNCaP cells through the mitochondrial/apoptosome pathway without activation of executioner caspase-3 or -7." Anticancer Res 27(4B): 2175-2183.

Kinghorn, A. D., E. J. Carcache de Blanco, H. B. Chai, J. Orjala, N. R. Farnsworth, D. D. Soejarto, N. H. Oberlies, M. C. Wani, D. J. Kroll, C. J. Pearce, S. M. Swanson, R. A. Kramer, W. C. Rose, C. R. Fairchild, G. D. Vite, S. Emanuel, D. Jarjoura and F. O. Cope (2009). "Discovery of anticancer agents of diverse natural origin." Pure Appl Chem 81(6): 1051-1063.

Kong, Y., Y.-J. Fu, Y.-G. Zu, W. Liu, W. Wang, X. Hua and M. Yang (2009). "Ethanol modified supercritical fluid extraction and antioxidant activity of cajaninstilbene acid and pinostrobin from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves." Food Chem 117(1): 152-159.

Kostic, A. D., E. Chun, L. Robertson, J. N. Glickman, C. A. Gallini, M. Michaud, T. E. Clancy, D. C. Chung, P. Lochhead, G. L. Hold, E. M. El-Omar, D. Brenner, C. S. Fuchs, M. Meyerson and W. S. Garrett (2013). "Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment." Cell Host Microbe 14(2): 207-215.

Kreahling, J. M., P. Foroutan, D. Reed, G. Martinez, T. Razabdouski, M. M. Bui, M. Raghavan, D. Letson, R. J. Gillies and S. Altiok (2013). "Wee1 inhibition by MK-1775 leads to tumor inhibition and enhances efficacy of gemcitabine in human sarcomas." PLoS One 8(3): e57523.

Kreahling, J. M., J. Y. Gemmer, D. Reed, D. Letson, M. Bui and S. Altiok (2012). "MK1775, a selective Wee1 inhibitor, shows single-agent antitumor activity against sarcoma cells." Mol Cancer Ther 11(1): 174-182.

Laemmli, U. K., L. A. Amos and A. Klug (1976). "Correlation between structural transformation and cleavage of the major head protein of T4 bacteriophage." Cell 7(2): 191-203.

Landrigan, P. J., L. Claudio, S. B. Markowitz, G. S. Berkowitz, B. L. Brenner, H. Romero, J. G. Wetmur, T. D. Matte, A. C. Gore, J. H. Godbold and M. S. Wolff (1999). "Pesticides and inner-city children: exposures, risks, and prevention." Environ Health Perspect 107 Suppl 3: 431-437.

Le Bail, J. C., L. Aubourg and G. Habrioux (2000). "Effects of pinostrobin on estrogen metabolism and estrogen receptor transactivation." Cancer Lett 156(1): 37-44.

Leers, M. P., W. Kolgen, V. Bjorklund, T. Bergman, G. Tribbick, B. Persson, P. Bjorklund, F. C. Ramaekers, B. Bjorklund, M. Nap, H. Jornvall and B. Schutte (1999). "Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis." J Pathol 187(5): 567-572.

Ley, C. D., M. R. Horsman and P. E. Kristjansen (2007). "Early effects of combretastatin-A4 disodium phosphate on tumor perfusion and interstitial fluid pressure." Neoplasia 9(2): 108-112.

Liang, D., S. L. Burkhart, R. K. Singh, M. H. Kabbaj and A. Gunjan (2012). "Histone dosage regulates DNA damage sensitivity in a checkpoint-independent manner by the homologous recombination pathway." Nucleic Acids Res 40(19): 9604-9620.

Littman, A. J., L. A. Jackson and T. L. Vaughan (2005). "Chlamydia pneumoniae and lung cancer: epidemiologic evidence." Cancer Epidemiol Biomarkers Prev 14(4): 773-778.

Liu, X., Y. Sun, J. Guo, H. Ma, J. Li, B. Dong, G. Jin, J. Zhang, J. Wu, L. Meng and C. Shou (2006). "Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells." Int J Cancer 118(8): 1922-1929.

Lu, C.-L., Y.-M. Li, G.-Q. Fu, L. Yang, J.-G. Jiang, L. Zhu, F.-L. Lin, J. Chen and Q.-S. Lin (2011). "Extraction optimisation of daphnoretin from root bark of *Wikstroemia indica* (L.) C.A. and its anti-tumour activity tests." Food Chem 124(4): 1500-1506.

Maginnis, M. S. and W. J. Atwood (2009). "JC virus: an oncogenic virus in animals and humans?" Semin Cancer Biol 19(4): 261-269.

Magnussen, G. I., R. Holm, E. Emilsen, A. K. Rosnes, A. Slipicevic and V. A. Florenes (2012). "High expression of Wee1 is associated with poor disease-free survival in malignant melanoma: potential for targeted therapy." PLoS One 7(6): e38254.

Mahajan, K. and N. P. Mahajan (2013). "WEE1 tyrosine kinase, a novel epigenetic modifier." Trends Genet 29(7): 394-402.

Mans, D. R. A., A. B. D. Rocha and G. Schwartsmann (2000). "Anti-Cancer Drug Discovery and Development in Brazil:Targeted Plant Collection as a Rational Strategy to Acquire Candidate Anti-Cancer Compounds." The Oncologist 5: 185-198.

Meckes, M., D. Paz, J. Acosta and R. Mata (1998). "The effects of chrysin and pinostrobin, two flavonoids isolated from Teloxys graveolens leaves, on isolated guinea-pig ileum." Phytomedicine 5(6): 459-463.

MedKoo Biosciences, I. (2015). "PD-166285 Chemical Structure [Online]. A vailiable from: http://www.medkoo.com/Anticancer-preclinical/PD0166285.htm [2015, 13 October].".

Mendonsa, A. M., M. N. VanSaun, A. Ustione, D. W. Piston, B. M. Fingleton and D. L. Gorden (2015). "Host and tumor derived MMP13 regulate extravasation and establishment of colorectal metastases in the liver." Mol Cancer 14: 49.

Mir, S. E., P. C. De Witt Hamer, P. M. Krawczyk, L. Balaj, A. Claes, J. M. Niers, A. A. Van Tilborg, A. H. Zwinderman, D. Geerts, G. J. Kaspers, W. Peter Vandertop, J. Cloos, B. A. Tannous, P. Wesseling, J. A. Aten, D. P. Noske, C. J. Van Noorden and T. Wurdinger (2010). "In silico analysis of kinase expression identifies WEE1 as a gatekeeper against mitotic catastrophe in glioblastoma." Cancer Cell 18(3): 244-257.

Mizunuma, M., D. Hirata, K. Miyahara, E. Tsuchiya and T. Miyakawa (1998). "Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast." Nature 392(6673): 303-306.

Mollapour, M., S. Tsutsumi, A. C. Donnelly, K. Beebe, M. J. Tokita, M. J. Lee, S. Lee, G. Morra, D. Bourboulia, B. T. Scroggins, G. Colombo, B. S. Blagg, B. Panaretou, W. G. Stetler-Stevenson, J. B. Trepel, P. W. Piper, C. Prodromou, L. H. Pearl and L. Neckers (2010). "Swe1Wee1-dependent tyrosine phosphorylation of Hsp90 regulates distinct facets of chaperone function." Mol Cell 37(3): 333-343.

Moon, Y. J., X. Wang and M. E. Morris (2006). "Dietary flavonoids: effects on xenobiotic and carcinogen metabolism." Toxicol In Vitro 20(2): 187-210.

Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." J Immunol Methods 65(1-2): 55-63.

Mueller, S. and D. A. Haas-Kogan (2015). "WEE1 Kinase As a Target for Cancer Therapy." J Clin Oncol 33(30): 3485-3487.

Nam, S., R. Buettner, J. Turkson, D. Kim, J. Q. Cheng, S. Muehlbeyer, F. Hippe, S. Vatter, K. H. Merz, G. Eisenbrand and R. Jove (2005). "Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells." Proc Natl Acad Sci U S A 102(17): 5998-6003.

Nicholson, R. A., L. S. David, R. L. Pan and X. M. Liu (2010). "Pinostrobin from *Cajanus cajan* (L.) Millsp. inhibits sodium channel-activated depolarization of mouse brain synaptoneurosomes." Fitoterapia 81(7): 826-829.

Nomura, N., M. Nomura, E. W. Newcomb and D. Zagzag (2007). "Geldanamycin induces G2 arrest in U87MG glioblastoma cells through downregulation of Cdc2 and cyclin B1." Biochem Pharmacol 73(10): 1528-1536.

O'Connor, P. M., J. Jackman, I. Bae, T. G. Myers, S. Fan, M. Mutoh, D. A. Scudiero, A. Monks, E. A. Sausville, J. N. Weinstein, S. Friend, A. J. Fornace, Jr. and K. W. Kohn (1997). "Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents." Cancer Res 57(19): 4285-4300.

Okouneva, T., B. T. Hill, L. Wilson and M. A. Jordan (2003). "The effects of vinflunine, vinorelbine, and vinblastine on centromere dynamics." Mol Cancer Ther 2(5): 427-436.

Parkash, J. and K. Asotra (2010). "Calcium wave signaling in cancer cells." Life Sci 87(19-22): 587-595.

Parker, L. L. and H. Piwnica-Worms (1992). "Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase." Science 257(5078): 1955-1957.

Patel, N. K. and K. K. Bhutani (2014). "Pinostrobin and Cajanus lactone isolated from *Cajanus cajan* (L.) leaves inhibits TNF-alpha and IL-1beta production: in vitro and in vivo experimentation." Phytomedicine 21(7): 946-953.

Porter, C. C., J. Kim, S. Fosmire, C. M. Gearheart, A. van Linden, D. Baturin, V. Zaberezhnyy, P. R. Patel, D. Gao, A. C. Tan and J. DeGregori (2012). "Integrated genomic analyses identify WEE1 as a critical mediator of cell fate and a novel therapeutic target in acute myeloid leukemia." Leukemia 26(6): 1266-1276.

PosthumaDeBoer, J., T. Wurdinger, H. C. Graat, V. W. van Beusechem, M. N. Helder, B. J. van Royen and G. J. Kaspers (2011). "WEE1 inhibition sensitizes osteosarcoma to radiotherapy." BMC Cancer 11: 156.

Press, M. F., M. C. Pike, V. R. Chazin, G. Hung, J. A. Udove, M. Markowicz, J. Danyluk, W. Godolphin, M. Sliwkowski, R. Akita and et al. (1993). "Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease." Cancer Res 53(20): 4960-4970.

Primo, D., J. Flores, S. Quijano, M. L. Sanchez, M. E. Sarasquete, J. del Pino-Montes, P. I. Gaarder, M. Gonzalez and A. Orfao (2006). "Impact of BCR/ABL gene expression on the proliferative rate of different subpopulations of haematopoietic cells in chronic myeloid leukaemia." Br J Haematol 135(1): 43-51.

Qiao, D., A. M. Zeeman, W. Deng, L. H. Looijenga and H. Lin (2002). "Molecular characterization of hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas." Oncogene 21(25): 3988-3999.

Rajeshkumar, N. V., E. De Oliveira, N. Ottenhof, J. Watters, D. Brooks, T. Demuth, S. D. Shumway, S. Mizuarai, H. Hirai, A. Maitra and M. Hidalgo (2011). "MK-1775, a potent Wee1 inhibitor, synergizes with gemcitabine to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts." Clin Cancer Res 17(9): 2799-2806.

Rubinstein, M. R., X. Wang, W. Liu, Y. Hao, G. Cai and Y. W. Han (2013). "Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin." Cell Host Microbe 14(2): 195-206.

Russell, P. and P. Nurse (1987). "Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog." Cell 49(4): 559-567.

Said, A. H., J. P. Raufman and G. Xie (2014). "The role of matrix metalloproteinases in colorectal cancer." Cancers (Basel) 6(1): 366-375.

Schulte, T. W., S. Akinaga, S. Soga, W. Sullivan, B. Stensgard, D. Toft and L. M. Neckers (1998). "Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin." Cell Stress Chaperones 3(2): 100-108.

Shitamukai, A., M. Mizunuma, D. Hirata, H. Takahashi and T. Miyakawa (2000). "A positive screening for drugs that specifically inhibit the Ca^{2+}-signaling activity on the basis of the growth promoting effect on a yeast mutant with a peculiar phenotype." Biosci Biotechnol Biochem 64(9): 1942-1946.

Shoeb, M., S. Celik, M. Jaspars, Y. Kumarasamy, S. M. MacManus, L. Nahar, P. K. Thoo-Lin and S. D. Sarker (2005). "Isolation, structure elucidation and bioactivity of schischkiniin, a unique indole alkaloid from the seeds of *Centaurea schischkinii*." Tetrahedron 61(38): 9001-9006.

Shoeb, M., S. M. MacManus, M. Jaspars, J. Trevidu, L. Nahar, P. Kong-Thoo-Lin and S. D. Sarker (2006). "Montamine, a unique dimeric indole alkaloid, from the seeds of *Centaurea montana* (Asteraceae), and its in vitro cytotoxic activity against the CaCo2 colon cancer cells." Tetrahedron 62(48): 11172-11177.

Shou, J., S. Massarweh, C. K. Osborne, A. E. Wakeling, S. Ali, H. Weiss and R. Schiff (2004). "Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer." J Natl Cancer Inst 96(12): 926-935.

Simoens, C., F. Lardon, B. Pauwels, C. M. De Pooter, H. A. Lambrechts, G. G. Pattyn, F. Breillout and J. B. Vermorken (2008). "Comparative study of the radiosensitizing and cell cycle effects of vinflunine and vinorelbine, in vitro." BMC Cancer 8: 65.

Smolarz, H. D., E. Mendyk, A. Bogucka-Kocka and J. Kocki (2006). "Pinostrobin--an anti-leukemic flavonoid from *Polygonum lapathifolium* L. ssp. nodosum (Pers.) Dans." Z Naturforsch C 61(1-2): 64-68.

Sugimoto, K., H. Toyoshima, R. Sakai, K. Miyagawa, K. Hagiwara, F. Ishikawa, F. Takaku, Y. Yazaki and H. Hirai (1992). "Frequent mutations in the p53 gene in human myeloid leukemia cell lines." Blood 79(9): 2378-2383.

Sukardiman, D. A, T. M and D. MO (2000). "Cytotoxic mechanism of flavonoid from *Temu Kunci* (*Kaempferia pandurata*) in cell culture of human mammary carcinoma." Clin Hemorheol Microcirc 23: 185-190.

Sukardiman, A. Widyawaruyanti, R. Widyowati, Sismindari and N. C. Zaini (2014). "Pinostrobin isolated from *Kaempferia pandurata* Roxb induced apoptosis in T47D human beast cancer cell line." <u>J Planta Husada</u> 2: 20-26.

Suksawatamnuay (2009). "Biological activity of pinostrobin from *Boesenbergia pandurata*, A calcium signaling pathway inhibitor in *Saccharomyces cerevisiae* and activity against proliferation of human cancer cell lines." <u>Bachelor's thesis, Graduate School, Department of Microbiology, Faculty of Science, Chulalongkorn University.</u>

Tan, M., J. Yao and D. Yu (1997). "Overexpression of the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities." <u>Cancer Res</u> 57(6): 1199-1205.

Thomson, P., M. A. Naylor, S. A. Everett, M. R. Stratford, G. Lewis, S. Hill, K. B. Patel, P. Wardman and P. D. Davis (2006). "Synthesis and biological properties of bioreductively targeted nitrothienyl prodrugs of combretastatin A-4." <u>Mol Cancer Ther</u> 5(11): 2886-2894.

Trakoontivakorn, G., K. Nakahara, H. Shinmoto, M. Takenaka, M. Onishi-Kameyama, H. Ono, M. Yoshida, T. Nagata and T. Tsushida (2001). "Structural analysis of a novel antimutagenic compound, 4-Hydroxypanduratin A, and the antimutagenic activity of flavonoids in a Thai spice, fingerroot (*Boesenbergia pandurata* Schult.) against mutagenic heterocyclic amines." <u>J Agric Food Chem</u> 49(6): 3046-3050.

Trepel, J., M. Mollapour, G. Giaccone and L. Neckers (2010). "Targeting the dynamic HSP90 complex in cancer." <u>Nat Rev Cancer</u> 10(8): 537-549.

Valdiglesias, V., B. Laffon, E. Pasaro and J. Mendez (2011). "Okadaic acid induces morphological changes, apoptosis and cell cycle alterations in different human cell types." <u>J Environ Monit</u> 13(6): 1831-1840.

Vriend, L. E., P. C. De Witt Hamer, C. J. Van Noorden and T. Wurdinger (2013). "WEE1 inhibition and genomic instability in cancer." <u>Biochim Biophys Acta</u> 1836(2): 227-235.

Wang, G., X. Niu, W. Zhang, J. T. Caldwell, H. Edwards, W. Chen, J. W. Taub, L. Zhao and Y. Ge (2015). "Synergistic antitumor interactions between MK-1775 and panobinostat in preclinical models of pancreatic cancer." <u>Cancer Lett</u> 356(2 Pt B): 656-668.

Wang, H., M. Huang, D. Y. Zhang and F. Zhang (2011). "Global profiling of signaling networks: study of breast cancer stem cells and potential regulation." Oncologist 16(7): 966-979.

Wangkangwan, W., S. Boonkerd, W. Chavasiri, K. Sukapirom, K. Pattanapanyasat, N. Kongkathip, T. Miyakawa and C. Yompakdee (2009). "Pinostrobin from *Boesenbergia pandurata* is an inhibitor of Ca^{2+}-signal-mediated cell-cycle regulation in the yeast *Saccharomyces cerevisiae*." Biosci Biotechnol Biochem 73(7): 1679-1682.

Williams, G. H. and K. Stoeber (2012). "The cell cycle and cancer." J Pathol 226(2): 352-364.

Wu, D., M. G. Nair and D. L. DeWitt (2002). "Novel Compounds from *Piper methysticum* Forst (Kava Kava) Roots and Their Effect on Cyclooxygenase Enzyme." J Agric Food Chem 50(4): 701-705.

Wu, N., Y. Kong, Y. Zu, Y. Fu, Z. Liu, R. Meng, X. Liu and T. Efferth (2011). "Activity investigation of pinostrobin towards herpes simplex virus-1 as determined by atomic force microscopy." Phytomedicine 18(2-3): 110-118.

Xie, J., T. Ma, Y. Gu, X. Zhang, X. Qiu, L. Zhang, R. Xu and Y. Yu (2009). "Berbamine derivatives: a novel class of compounds for anti-leukemia activity." Eur J Med Chem 44(8): 3293-3298.

Xu, R., Q. Dong, Y. Yu, X. Zhao, X. Gan, D. Wu, Q. Lu, X. Xu and X. F. Yu (2006). "Berbamine: a novel inhibitor of bcr/abl fusion gene with potent anti-leukemia activity." Leuk Res 30(1): 17-23.

Yamada, T., T. Oshima, K. Yoshihara, S. Tamura, A. Kanazawa, D. Inagaki, N. Yamamoto, T. Sato, S. Fujii, K. Numata, C. Kunisaki, M. Shiozawa, S. Morinaga, M. Akaike, Y. Rino, K. Tanaka, M. Masuda and T. Imada (2010). "Overexpression of MMP-13 gene in colorectal cancer with liver metastasis." Anticancer Res 30(7): 2693-2699.

Yuan, H., M. Kamata, Y. M. Xie and I. S. Chen (2004). "Increased levels of Wee-1 kinase in G(2) are necessary for Vpr- and gamma irradiation-induced G(2) arrest." J Virol 78(15): 8183-8190.

APPENDIX

APPENDIX A

Media preparation

RPMI-1640 medium (1X) + 2.05 mM L-Glutamine (Complete media)

Jurkat

RPMI-1640 medium	90	ml
Fetal bovine serum	10	ml
Penicillin G (10^6 U/ml)	0.01	ml
Streptomycin (500 mg/ml)	0.05	ml
2-Mercaptoethanol	0.091	ml

KATO III

RPMI-1640 medium	90	ml
Fetal bovine serum	10	ml
Penicillin G (10^6 U/ml)	0.01	ml
Streptomycin (500 mg/ml)	0.05	ml
Sodium pyruvate	1	ml
HEPES	1	ml

BT474

RPMI-1640 medium	90	ml
Fetal bovine serum	10	ml
Penicillin G (10^6 U/ml)	0.01	ml
Streptomycin (500 mg/ml)	0.05	ml

RPMI freezing media

RPMI complete media	9	ml
DMSO	1	ml

Fetal bovine serum activation

Fetal bovine serum (FBS) must be activated at 56°C for 30 min in water bath before use.

APPENDIX B

The chemical preparation

10% SDS

Dissolve sodium dodecyl sulfate 10 g in distilled water up to 100 ml and autoclaved at 121°C, 15 psi for 20 min.

10% Ammonium persulfate (APS)

Dissolve the 0.5 g of ammonium persulfate in sterile ddH_2O 5 ml. Aliquot and kept at 4°C.

50X TAE buffer

Trisma base	242 g
Acetic acid	57.1 ml

Dissolve Trisma base and acetic acid in 0.5 M pH 8.0 100 ml and adjust to 1000 ml by ddH_2O. Autoclaved at 121°C, 15 psi for 15 min.

RNase A solution (10mg/ml)

Weigh 10 mg of powdered RNase A in 1 ml of 10 mM sodium acetate pH 7.2. Heat to 100°C for 15 min, allow to cool and then adjust pH with 1 M Tris-HCl pH 7.2. Aliquot and store at -20°C.

Ethanol 70%

Ethanol 99%	700	ml
ddH_2O	300	ml

5% Stacking gel

ddH_2O	3.01	ml
40% Acrylamide	0.625	ml
1 M Tris pH 6.8	1.260	ml
10% SDS	0.05	ml
10% APS	0.05	ml
TEMED	0.005	ml

12% Separating gel

ddH$_2$O	8.59	ml
40% Acrylamide	6	ml
1 M Tris pH 8.8	5	ml
10% SDS	0.2	ml
10% APS	0.2	ml
TEMED	0.01	ml

10X Phosphate buffer saline (PBS, Ca2+, Mg2+ free) pH 7.4

NaCl	80	g
KCl	2	g
Na$_2$HPO$_4$	14.4	g
KH$_2$PO$_4$	2.4	g

Dissolve in ddH$_2$O up to 1 l and adjusted pH up to 7.4 with 1N HCl or 1 N NaOH and autoclaved at 121°C, 15 psi for 15 min.

MTT solution (5mg/ml)

MTT	50	mg
ddH$_2$O	10	ml

Dissolve MTT in ddH$_2$O and filter by syringe filter pore size 0.45 μM. Aliquot 1 ml in microcentrifuge tube and store at 4°C.

0.04 N HCl in isopropanaol

Add HCl 0.331 ml in 80 ml isopropanaol and adjust volume to 100 ml by isopropanol.

RIPA solution

1 M Tris-HCl pH 7.4	0.05	ml
0.5 M NaCl	0.3	ml
20% NP-40	0.05	ml
10% Sodium deoxycholate	0.05	ml
20% SDS	0.005	ml
Phosphatase inhibitor	0.01	ml
Protease inhibitor (100X)	0.01	ml
ddH$_2$O	525	ml

BCA^{TM} protein assay solution

BCA Reagent A	50	part
BCA Reagent B	1	part

1 M Tris pH 6.8 solution

Dissolve trisma base 12.11 g in ddH$_2$O 80 ml. Adjusted pH up to 6.8 with 1N HCl or 1 N NaOH and adjust volume to 100 ml. Autoclaved at 121°C, 15 psi for 15 min.

1 M Tris pH 8.8 solution

Dissolve trisma base 90.85 g in ddH$_2$O 400 ml. Adjusted pH up to 8.8 with 1N HCl or 1 N NaOH and adjust volume to 500 ml. Autoclaved at 121°C, 15 psi for 15 min.

5X Running buffer

Trisma base	15.1	g
Glycine	94	g
SDS	5	g

Dissolve trisma base, glycine and SDS in ddH$_2$O 800 ml and adjust to 1000 ml. Autoclaved at 121°C, 15 psi for 15 min.

Transfer buffer

Trisma base	15.1	g
Glycine	2.9	g
SDS	0.37	g

Dissolve trisma base, glycine and SDS in ddH$_2$O 800 ml and autoclaved at 121°C, 15 psi for 15 min. Add absolute methanol 200 ml up to 1000 ml.

2X loading buffer

10% SDS	4	ml
Glycerol 87%	2.29	ml
1.0 M Tris pH 6.8	1	ml
ddH$_2$O	2.71	ml
Bromphenol blue	0.001	g

Staining buffer

β-mercapto-ethanol	0.1	ml
2X loading buffer	0.9	ml

Phosphate buffer saline tween 20 (PBST)

1X Phosphate buffer saline	1000	ml
Tween 20	0.5	ml

Developer and Fixer

Developer	1 part : H$_2$O 4 part	
Fixer	1 part : H$_2$O 4 part	

Blocking solution (5% skim milk in PBST)

PBST	100	ml
Skim milk	5	g

ECL Western blot reagent

    Solution 1   2   ml

    Solution 2   2   ml

DEPC Water

    Add 0.01 ml of diethylpyrocarbonate (DEPC) in ddH_2O and mix thoroughly. Let the DEPC-mixed water incubate for overnight at room temperature. Autoclave at 121°C, 15 psi for 15 min.

70% Ethanol in DEPC water

    Absolute ethanol   70   ml

    DEPC water   30   ml

2% Agarose gel

    1X TAE buffer   20   ml

    Agarose gel   0.4   g

Ethidiumbromide solution

    Dissolve ethidiumbromide in 1X TAE buffer at final concentratiom 10 µg/ml and store in dark container or wrap container in aluminum foil.

Pencillin G and streptomycin solution

    Pencillin G and streptomycin were prepared at final concentration 10^6 U/ml and 500 mg/ml in sterile deionized water. The solution were sterilized by filtrating passed 0.45 µm filter, aliquoted and kept at -20°C.

VITA

My name is Mr. Jumpol Sopanaporn was born in Bagkok, Thailand on December 11, 1990. After graduation with Bachelor's degree of Science from Department of Biotechnology, Faculty of Science and Technology at Thammasat University in 2010. Next time, I enrolled in the Master' degree of Program in Industrial Microbiology, Faculty of Science at Chulalongkorn University in 2011.

Academic presentation

Sopanaporn, J., Apirattikul, N., Palaga, P., Yingyongnarongkul, B. and Yompakdee, C. Anti-proliferation activity of pinostrobin from Boesenbergia pandurata and its efficacy improvement using cationic liposome on human cancer cell lines. The 26th Annual Meeting of the Thai Society for Biotechnology and International Conference Mae Fah Luang University, Chiang Rai, Thailand 26-29 November, 2014.

"Best Proceeding Paper Award"

Preparing the manuscript in "Pinostrobin from fingerroot Boesenbergia pandurata, is a novel Wee1 inhibitor with synergistic effect when combine with some of DNA-damaging agents in some cancer cell lines"