EFFECT OF IMPERATORIN ON ANOIKIS IN H23 LUNG CANCER CELLS

Miss Kanuengnit Choochuay

A Thesis Submitted in Partial Fulfillment of the Requirements
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ผลของอิมเพอราโทรินต่อการตายแบบอะนอยคิสของเซลล์มะเร็งปอดชนิดเอช 23

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By	Miss Kanuengnit Choochuay
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Rataya Luechapudiporn, Ph.D.
Thesis Co-advisor	Assistant Professor Pithi Chanvorachote, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……..……Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

……..…..... Chairman

(Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph.D.)

…….. Thesis Advisor

(Assistant Professor Rataya Luechapudiporn, Ph.D.)

…….. Thesis Co-advisor

(Assistant Professor Pithi Chanvorachote, Ph.D.)

…….. Examiner

(Assistant Professor Suree Jianmongkol, Ph.D.)

…….. External Examiner

(Rawiwan Maniratanachote, Ph.D.)

คะนึงนิตย์ ชูช่วย : ผลของอิมเพอราโทรินต่อการตายแบบอะนอยคิสของเซลล์มะเร็งปอดชนิดเอช 23. (EFFECT OF IMPERATORIN ON ANOIKIS IN H23 LUNG CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.รัตยา ลือชาพุฒิพร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.ปิติ จันทร์วรโชติ, 72 หน้า.

งานวิจัยนี้เป็นงานวิจัยแรกที่ศึกษา ถึงคุณสมบัติของอิมเพอราโทริน ซึ่งเป็นสารประเภทฟูเรนโนคูมารินที่ได้จากรากของโกฐสอในการเพิ่ม ความไวต่อการตายแบบอะนอยคิสในเซลล์มะเร็งปอดชนิดเอช23 ในการศึกษานี้ใช้ อิมเพอราโทริน ที่ความเข้มข้นต่ำกว่าระดับความเป็นพิษ ในการเพิ่มการตายแบบอะพอพโทซิสหลังจากหลุดจากการยึดเกาะ ในการวิเคราะห์ โปรตีนหลักที่เกี่ยวข้องกับการตายแบบอะนอยคิส ด้วยวิธีเวสเทินบล็อตพบว่าอิมเพอราโทรินเพิ่มระดับโปรตีนพี 53 ซึ่งส่งผลต่อมาทำให้มีการลดลงของระดับโปรตีนเอ็มซีแอล-1และการเพิ่มขึ้นของโปรตีนแบกซ์ โดยมีผลเพียงเล็กน้อยต่อการแสดงออกของโปรตีนบีซีแอล-2 อีกทั้งพบว่าอิมเพอราโทรินยับยั้งการเจริญเติบโตในสภาวะไร้การ ยึดเกาะของเซลล์มะเร็ง ปอดชนิดเอช 23 เป็นการ ยืนยันผลของอิมเพอราโทรินต่อการยับยั้งการแพร่กระจายของเซลล์มะเร็ง นอกจากนั้นในการศึกษานี้ ยังพบว่าอิมเพอราโทรินเพิ่มความไวต่อการตายแบบอะนอยคิสในเซลล์มะเร็งปอดชนิดอื่นๆ คือ เอช 292 และเอ 549 อีกด้วย เนื่องจากการตายแบบอะนอยคิส เป็นกระบวนการที่ มีบทบาทสำคัญในการ ป้องกันการแพร่กระจายของเซลล์มะเร็ง ผลการวิจัยนี้จึงเป็นข้อมูลเกี่ยวกับกลไกในการออกฤทธิ์ของอิมเพอราโทรินเพื่อนำไปสู่การพัฒนาสารชนิดนี้ในการรักษาโรคมะเร็งต่อไป

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 255 5 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : IMPERATORIN / ANOIKIS / ANCHORAGE-INDEPENDENT GROWTH / LUNG CANCER CELLS

KANUENGNIT CHOOCHUAY: EFFECT OF IMPERATORIN ON ANOIKIS IN H23 LUNG CANCER CELLS. ADVISOR: ASST. PROF. RATAYA LUECHAPUDIPORN, Ph.D., CO-ADVISOR: ASST. PROF PITHI CHANVORACHOTE, Ph.D., 72 pp.

Anoikis-sensitization activity of imperatorin, an active furanocoumarin component of *Angelica dahurica* root, is reported herein for the first time in human lung cancer H23 cell. The present study demonstrated that the imperatorin treatment at sub-toxic concentrations enhanced human lung cancer H23 cell apoptosis after detachment. A western blot analysis of a major protein involved anoikis showed that imperatorin significantly increased p53 protein level which subsequently down-regulated Mcl-1 protein and up-regulated Bax while it had a minimal effect on Bcl-2 expression. In addition, imperatorin exhibited a strong inhibitory effect on the anchorage-independent growth of the cells supporting the anti-metastasis potential of imperatorin. Further, this study demonstrated that imperatorin sensitizes anoikis in another lung cancer cells, namely H292 and A549. Because anoikis was shown to be a critical hindrance preventing cancer cell metastasis, the knowledge regarding such an activity and an underlying mechanism may lead to the development of this compound for a cancer therapy.

Department : Pharmacology and Physiology Student's Signature .....
Field of Study :Pharmacology ..... Advisor's Signature .....
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LIST OF ABBREVIATIONS

%	= percentage
C	= degree Celsius
µg/ml	= microgram per milliliter
µM	= micromolar
µl	= microliter
ANOVA	= analysis of variance
Bcl-2	= B-cell lymphoma-2
CO2	= carbon dioxide
DMEM	= Dulbecco's Modified Eagle Medium
DMSO	= dimethyl sulfoxide
et al.	= et alibi, and others
FITC	= fluorescein isothicyanate
h	= hour, hours
Mcl-1	= Myeloid cell leukemia-1
nm	= nanometer
PBS	= phosphate buffer saline
PI	= popidium iodide
PolyHEMA	= poly(2-hydroxyethylmethacrylate)
RPMI	= Roswell Park Memorial Institutes medium
S.D.	= standard deviation
SDS-PAGE	= sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBST	= tris-buffered saline, 0.1 % Tween 20
XTT	= 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Chapter I

Introduction

Metastasis is the major cause of cancer-related death in many human cancers, including lung cancer (Jemal, *et al*., 2008; Herbst, Heymach and Lippman, 2008). All major histological type of lung cancer, adrenocarcinoma is the most common kind of lung cancer, both in smokers and non-smokers (Sun, Schliier and Gazdar, 2007). In particular, lung cancer patients are frequently found to have metastatic tumors at the time of diagnosis which may be the cause of a high rate of death in this type of cancer (Sun, Schiller and Gazdar, 2007; Mountzios, Fouret and Soria, 2008). Cancer metastasis is a complex process, consisting of cancer cell detachment, migration, extravasation, and adhesion to target sites. As an important barrier for cancer metastasis, apoptosis mediated by cell detachment termed "anoikis" has garnered increasing attentions in the cancer research domain (Frisch and Screaton, 2001; Christofori, 2003). The anoikis process of the cells initiates after the cells lose contact from neighboring cells or from their extracellular matrix (ECM) leads to the activation of p53 and the subsequent alteration of pro- and anti-apoptotic protein in Bcl-2 family (Frisch, 1999; Hanahan and Weinberg, 2000; Martin and Vuori, 2004). The balance between pro-apoptotic Bcl-2 proteins such as Bim, Bmf, Bax, and Bid and anti-apoptotic proteins such as Bcl-2 and Bcl-XL are found to be disturbed in response to cell detachment signals (Adams and Cory, 1998; Kroemer, Galluzzil and Brenner, 2007). The mitochondrial membrane is then disrupted, following by the release of cytochrome *c* and the activation of caspases (Scorrano and Korsmeyer, 2003; Grossmann, 2002). The loss of cell adhesion was shown to initiate p53-dependent mitochondrial apoptosis pathway (Benchimol, 2001; Schuler and Green, 2001). Certain cancer cells develop mechanisms to attenuate p53 activation and thus resulted in anoikis resistance (Derouet *et al*., 2007; Grossmann, 2002; Guadamillas, Cerezo , and Pozo, 2011; Ravid *et al*., 2005; Song et al., 2012; Zhang *et al*., 2004). Recently, the role of anti-apoptosis Mcl-1 protein in the inhibition of anoikis has been intensively pronounced. A high level of endogenous or the overexpression of Mcl-1 was showed to be tightly related with an anoikis response in many cancers (Kim,

2012; Woods, 2007) such as melanoma (Boisvert-Adamo, 2009) and lung cancers (Li *et al*., 2008). So far, it has been well accepted that the resistance to anoikis is a hallmark of successful metastatic cancers. In addition, an ability of cancer cells to grow in anchorage-independent condition was shown to be an important characteristic of cancer aggressiveness and to be related to metastasis potential in different types of human malignancies (Zhu *et. al*., 2000), including lung cancer (Ramachandra, *et al*., 2002).

Imperatorin is a major active furanocoumarin enriched in a root of *Angelica dahurica*(Baek *et al*., 2000), has been reported to possess a variety of pharmacological actions against cancers, including the inhibition of cancer cell proliferation (Kostova, 2005) and the induction of cancer cell apoptosis both *in vitro* and *in vivo*. In addition, previous studies showed that imperatorin decreased the cellular level of Bcl-2 and increased p53 and Bax expression in early stage of apoptosis response (Luo *et al*., 2011; Pae *et al.*, 2002). However, until now a role of imperatorin in inhibition of cancer cell metastasis as well as cancer cell anoikis is largely unknown. Because of its anti-proliferation properties and induction of apoptosis, imperatorin might have been a potential effect on detachment-induced apoptosis (anoikis) sensitization. As an ongoing research for anti-cancer drug development, the present study aimed to investigate the effects of imperatorin on adrenocarcinoma H23 cell anoikis, anchorage-independent growth and to explore a possible underlying mechanism. Furthermore, to prove that imperatorin might play a major role in lung cancer anoikis, then this study also investigated the effect of imperatorin on anoikis in other lung carcinoma cells, namely H292 (squmous cells) and A549 (adrenocarcinoma).

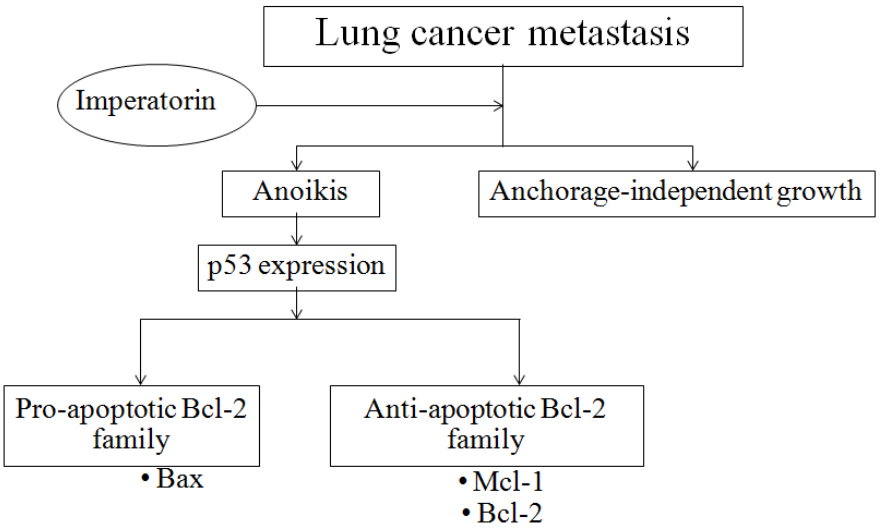
Research questions

1. Whether imperatorin have any effects on anoikis process in H23, H292 and A549 lung cancer cells.
2. Whether imperatorin have any effects on anchorage-independent growth condition in H23 lung cancer cells.
3. What are the underlying mechanisms of imperatorin on anoikis process in H23 lung cancer cells?

Hypothesis

1. Imperatorin might sensitize anoikis in H23, H292 and A549 lung cancer cells.
2. Imperatorin might inhibit anchorage-independent growth condition in H23 lung cancer cells.
3. Imperatorin might alter expression of p53, Mcl-1, Bcl-2 and Bax.

Conceptual framework



Objectives

1. To study the effect of imperatorin on anoikis process in H23, H292 and A549 lung cancer cells.
2. To study the effect of imperatorin on anchorage-independent growth condition in H23 lung cancer cells.
3. To identify underlying mechanism of imperatorin on major protein expression involved anoikis in H23 lung cancer cells, namely p53, Mcl-1, Bcl-2 and Bax.

Chapter II

Literature review

1. Lung cancer and cancer metastasis

Lung cancer is the leading cause of cancer deaths worldwide (Jemal *et al*., 2008; Herbst *et al*., 2008). There are two major forms of lung cancer, non-small cell lung cancer and small cell lung cancer. Non-small cell lung cancer can be divided into three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma and large-cell lung cancer (Figure 2.1). Almost of cases are non-small cell lung cancer (NSCLC) and most patients present with advanced stage or metastatic disease (Herbst *et al*., 2008).

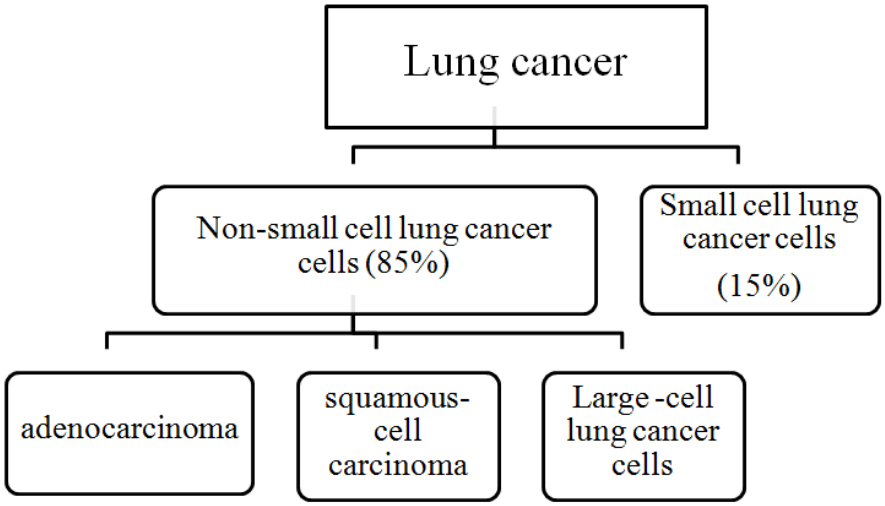


Figure 2.1 The histologic subtypes of non-small cell lung cancer cell.

Metastasis is the most common cause of death in human cancer especially lung cancer (Jemal *et al*., 2008; Herbst *et al*., 2008). Cancer metastasis is a complex steps consisted in cancer cell detachment, migration, extravasation, and adhesion of the detached cells to other target sites (figure 2.2). As a barrier to develop metastasis, apoptosis after lose contact from neighbouring cells or from extracellular matrix (ECM), this cell death process is termed anoikis (Christofori *et al*., 2003; Frisch and

Screaton, 2001; Hanahan *et al*., 2000). Resistance to anoikis is a hallmark of metastatic cancer cells, especially anchorage-independent growth of tumor cells (Ramachandra *et al*., 2002; Zhu *et al*., 2001). Furthermore, metastatic progression is modulated by apoptotic or anti-apoptotic factors. Two important groups of proteins orchestrate anoikis and the fate of the cells are the Bcl-2 family and p53. The Bcl-2 family proteins consist of structurally related proteins that can be either pro- or anti-apoptotic including Bcl-2 and Mcl-1. The mitochondrial pathway is a major cell death pathway that contributes to anoikis and anoikis resistance (Frisch and Screaton, 2001).

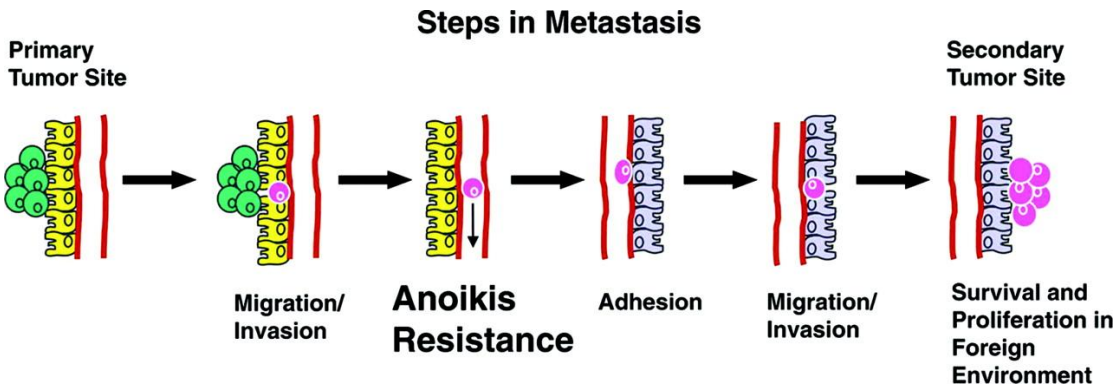


Figure 2.2 Metastasis involves several steps in which tumor cells disseminate from the primary site to secondary metastatic sites (Sandra *et al*., 2010).

2. Anoikis and anoikis resistance

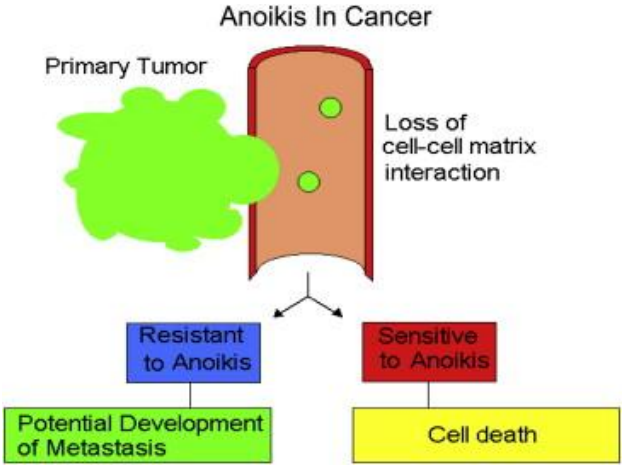


Figure 2.3 Anoikis or detachment-induced apoptosis (Coates, Galante and Bold, 2010)

Anoikis (figure 2.3) is detachment-induced apoptosis or loss of cell-matrix interactions (Frisch and Screaton, 2001). A number of recent studies have showed an important role for anoikis in various cancers, including prostate, breast and lung cancers (Fiucci *et al*.,2002; Jiang *et al*., 2001; Kim *et al*.,2012). The common pathway of anoikis involves the loss of integrins-related signaling, which triggers the mitochondrial death pathway. In the case of cancer cells, ability to resist to anoikis and survive in anchorage-independent conditions have potentiated their metastasis to the distant sites. The possible explanation of such a successful metastasis is that the cancer cells possess innate or adaptive mechanisms to overcome anoikis (Guadamillas, Cerezo and Pozo, 2011; Simpson, Anyiwe and Schimmer, 2008; Sakamoto and Kyprianou, 2010)**.** Moreover, in the animal models, tumors that are resistant to anoikis show a higher incidence of metastases and increase cell survival in the blood circulation. Interestingly, numerous studies have suggested that anoikis resistance involved in stimulation of pro-survival signals and suppression of death signals. Several evidences indicated that Bcl-2 and Mcl-1 proteins function in inhibition of anoikis in many cancers, and the cancer cells possess high basal levels of such proteins exhibited anoikis resistance characteristics (Boisvert-Adamo *et al*., 2009; Krajewska *et al*., 1996; Kim *et al*., 2012). Importantly, high level of Mcl-1

related with ability of the cancer cells to metastasis (Kim *et al*., 2012; Simpson *et al*., 2008; Krajewska *et al*., 1996; Wood *et al*., 2007; Zhuang *et al*., 2007).

3. Bcl-2 family proteins

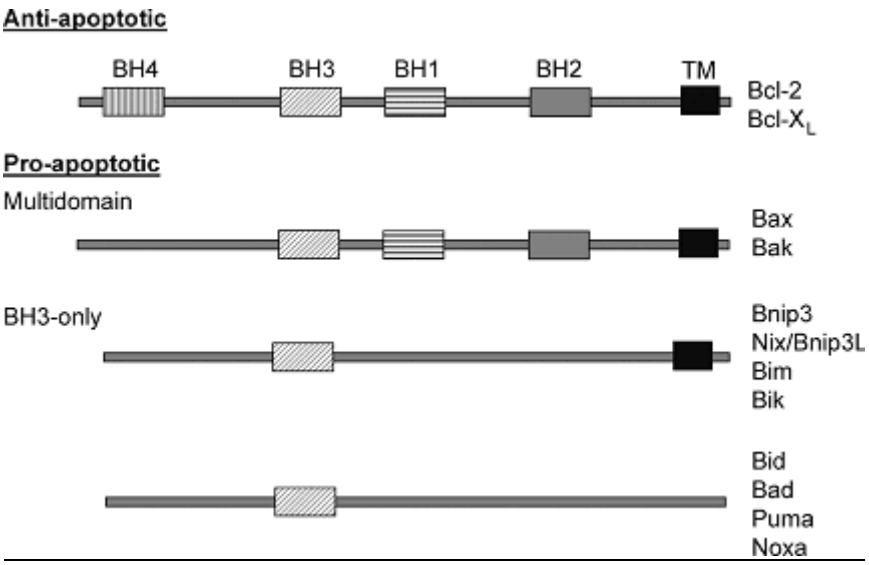


Figure 2.4 Bcl-2 family proteins (Gustafsson and Gottlieb, 2007).

There are more than 22 members in Bcl-2 protein family which are classified by Bcl-2 homology (BH) domains (Scorrano and Korsmeyer, 2003). The, Bcl-2 and Bcl-xL known as anti apoptotic proteins, have four BH domains, and support survival process by inhibiting pro-apoptotic Bcl-2 proteins function after binding with them.

There are 2 classes of pro-apoptotic Bcl-2 proteins, the first classes have BH1, BH2 and BH3 domain such as Bax and Bak. The second classes have only BH3 domain such as Bid, Bim, Bad, Bik and Bmf (Adams and Cory, 1998).

The anti apoptotic Bcl-2 proteins interact with pro apoptotic Bax/Bak or BH3-only proteins and preventing mitochondrial membrane permeabilization (Chipuk and Green, 2008). Bcl-2 proteins play a major role in cell death induced by disruption of extracellular matrix contact. Furthermore, Bcl-2 oncoprotein overexpression correlates with the progression and metastases of cancers (Vuori and Martin, 2004).

4. The Bcl-2 family proteins and anoikis

Cancer cells from the same tumor, normally exhibited various phenotypes and behaviors, which may cause by genetic mutations or adaptive mechanisms. Therefore, anoikis resistance could be developed by many mechanisms. Clinical as well as *in vitro* studies have indicated several ways that render cancer cells resistant to anoikis which are some cells exhibited high Bcl-2, and other may have high Mcl-1 protein. In addition, attenuated p53 activation was shown to be a possible mechanism of anoikis resistance (Derksen *et al*., 2006; Ravid *et al*., 2005; Zhang, 2004).

The Bcl-2 family proteins most commonly localize in the cytoplasm including mitochondria and endoplasmic reticulum (ER) (Gross, McDonnell and Korsmeyer**,** 1999; Krajewski *et al*., 1993; Portier and Taglialatela, 2006; Hoetelmans *et al*., 2000). Although these proteins localize in multiple organelles, their anti-apoptotic function has predominantly been investigated in the mitochondria, a site in which Bcl-2 is actively transported to *via* the mitochondrial chaperone protein (Krajewski *et al*., 1993; Hoetelmans *et al*., 2000). Once Bcl-2 is placed on the outer mitochondrial membrane, it interacts with pro-apoptotic family members as well as Bax to form heterodimers. Thus, Bcl-2 prevents the initiation of apoptosis through blocking the formation of mitochondrial transition pores, the release of cytochrome *c*, and subsequent the activation of a caspase cascade (Kuwana and Newmeyer, 2003; Finucane *et al*., 1999; Kroemer, Galluzzil and Brenner, 2007).

5. Role of Bcl-2 in cancer

Apoptosis is involved in multiple pathways, including mitochondrial-mediated pathway and death receptor pathway (Lowe and Lin, 2000; Evan and Vousden, 2001). Members of the Bcl-2 family can modulate the apoptotic pathway. The antiapoptotic members in Bcl-2 family are Bcl-2, Bcl-xl, and Mcl-1. Bcl-2 is one of the key regulators of apoptosis because it protects cells from apoptotic death and is crucial in cells survival (Chao and Korsmeyer, 1998). The development of some tumors is contributed by dysregulation of Bcl-2 expression, which results in abnormal cell growth (Adams and Cory, 2007). Abnormally high levels of Bcl-2, especially elevated expression of Bcl-2 in some tumors is often associated with shorter survival time and generally poorer clinical outcomes (Zhao *et al*., 2012). Furthermore, metastasis in cancers is related with overexpression of Bcl-2 (Martin *et al*., 2003; Miyake *et al*., 1999; Zuo *et al*., 2010). Zuo *et al*, have proved that overexpression of Bcl-2 in squamous carcinoma cells dramatically enhanced tumor metastasis to the lung and correlated with elevated cell motility and invasion. Furthermore, Bcl-2 altered the ability of squamous carcinoma cells to efficiently form cell-cell contacts, which led to increased cell scattering and inability to form strong multi-cell adhesions. Consequently, upregulation of Bcl-2 in advanced stage cancer may facilitate the evolution of cells into more aggressive and leading to an increase in metastatic potential (Zuo *et al*., 2010).

6. Role of Mcl-1 in cancer

Mcl-1 is a short-half-life protein in Bcl-2 family, which binds and inactivates pro-apoptotic proteins, including Bak, tBid, Bim and PUMA, following inhibits the destruction of mitochondrial membrane and cytochrome *c* releasing (Akgul, 2009; Michels, Johnson and Packham, 2005; Thomas, Lam and Edwards, 2010). In cancer cell, apoptotic resistance and apoptotic sensitivity correlated with overexpression and depletion of Mcl-1 levels, respectively (Boucher *et al*., 2000; Dash *et al*., 2010; Warr and Shore, 2008). The role of Mcl-1 in the anoikis response is related with short half-life characteristic, result in the reduction of anoikis. The prolonged viability may give the cells more time to reattach to the ECM at a distal site or for the accumulation of additive mutations that can disrupt anoikis execution. Mcl-1 serves at the convergence point of many resultant signals downstream of detachment from the ECM that mediates the initiation of an anoikis response and the prevention of metastasis (Woods *et al.,* 2007; Boisvert-Adamo *et.al*, 2009). Recently, the role of anti-apoptosis Mcl-1 protein in the inhibition of anoikis has been intensively pronounced. A high level of endogenous or the overexpression of Mcl-1 was showed to be tightly related with an anoikis response in many cancers (Boisvert-Adamo *et al*., 2012; Kim *et al*., 2012; Li *et al*., 2008; Woods *et al*., 2007). So far, it has been well accepted that the resistance to anoikis is a hallmark of successful metastatic cancers. Also, an ability of cancer cells to grow in anchorage-independent condition was shown to be an important characteristic of cancer aggressiveness and to be related to metastasis potential in different types of human malignancies, including lung cancer (Ramachandra *et al*., 2002; Zhu *et al*., 2000).

7. p53

As a major apoptosis regulator, p53 also plays a critical role in anoikis and metastasis. The p53-dependent anoikis has been demonstrated in many cell types (Kim *et al*., 2012), including lung cancer cells (Benchimol, 2001; Kim *et al*., 2012; Schuler and Green, 2001; Steels *et al*., 2001). Accumulative data suggest that lung cancer cells resistant to anoikis by attenuating the increase in p53 abundance that takes place in response to cell (Steels *et al*., 2001). As cellular p53 level represents total p53 that normally kept very low level in the cells. The p53 in normal condition will be degraded rapidly and continuously via ubiquitin-proteasomal pathway. In the present of DNA damaging signals, p53 will be stabilized and the level of the protein will increase. An increase in cellular p53 level has been shown in many studies to indicate the apoptosis response (Flores et al., 2002; Fridman and Lowe, 2003; Lozano *et al*., 2012; Oda *et al*., 2000).

8. Imperatorin

Figure 2.5 *Angelica dahurica*

Angelica dahurica known as Khot-saw in Thai, has been used as a traditional herbal medicine for increasing blood circulation. Imperatorin (9-(3-methylbut-2-enyloxy)-7*H*-furo [3, 2-g] chromen-7-one) is a bioactive furanocoumarin isolated from roots of *Angelica dahurica*. Furanocoumarin has been reported to possess a wide range of miscellaneous biological functions, including anti-platelet aggregation, anti-inflammatory, hepatoprotective activities and anticancer effects (Baek *et al*., 2000; Kostova, 2005; Luo *et al*., 2011; Pae *et al.*, 2002).

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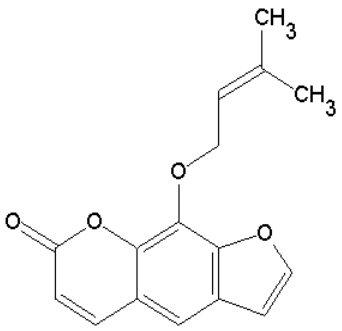


Figure 2.6 Chemical structure of imperatorin (Pae *et al.*, 2002)

9. Imperatorin and cancer cells study

Imperatorin exhibit strongly pharmacological activity it has been proved in anti-platelet aggregation, anti-inflammation, including antitumor property. Imperatorin was reported to anti-proliferative effect in many cancer cell lines including, colon cancer, liver cancer, leukemia, breast cancer, cervical cancer, gastric cancer, pancreatic cancer, human gastric cancer and human lung cancer with minimum effect on normal cells. A recent study suggested that the isopentenyl-group is clearly related with antiproliferative activity of simple coumarins especially, imperatorin. Furthermore, imperatorin has been shown to induce apoptosis of HL-60 cells at micromolar concentrations (Pae *et al.,* 2002) and it inhibited the skin cancer progression in an animal model. The structure-activity relationship established from the many studies indicated that the prenyl group has an important role in the cytotoxic effects. Because of its anti-proliferation properties and induction of apoptosis, imperatorin might have been a potential application as an anti-cancer agent. Imperatorin induced apoptosis in many cell lines through both intrinsic and extrinsic pathways. Furthermore, it significantly showed antitumor activity in vivo (Luo *et al*., 2011; Pae *et al.*, 2002).

More importantly, imperatorin exhibited anticancer effects in animal models. Nude mice treated with 50 and 100 mg/kg imperatorin for 2 weeks showed declines in tumor size. In addition, imperatorin-treated mice did not show any significant weight loss or toxicity in the heart and liver (Luo *et al*., 2011).

Chapter III

Material and Method

1. Materials and cells

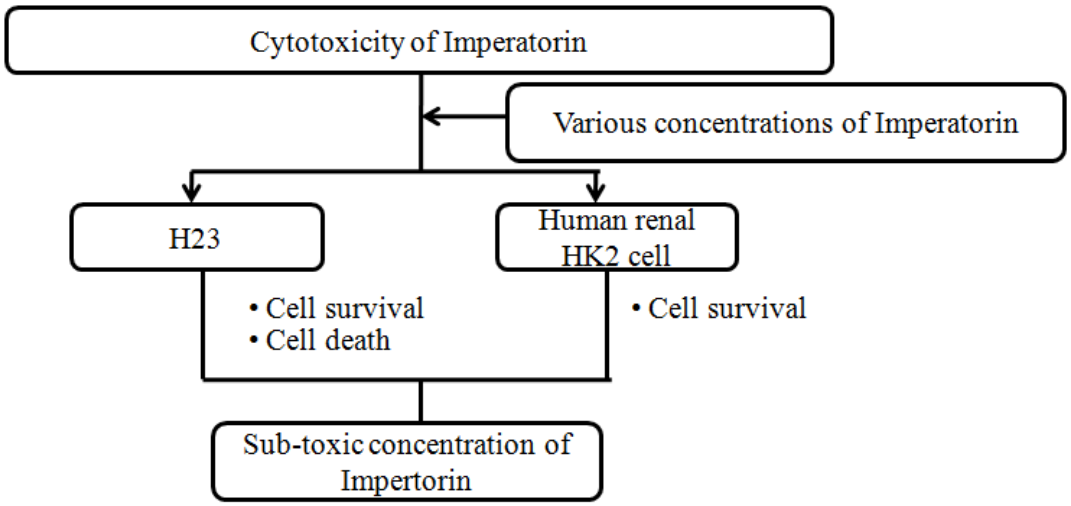
NCI-H23, NCI-H292, A549, and HK-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). RPMI 1640 medium, DMEM medium, 100 units/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum (FBS) and 2 mM L-glutamine were purchased from Gibco (NY, USA). Imperatorin, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), Hoechst 33342 and propidium iodide were purchased from Sigma (NY, USA). Antibodies for Mcl-1, Bcl-2, BAX, P53, β-actin and peroxidase-labeled secondary antibodies were obtained from Abcam Inc. (Cambridge, MA).

2. Equipments

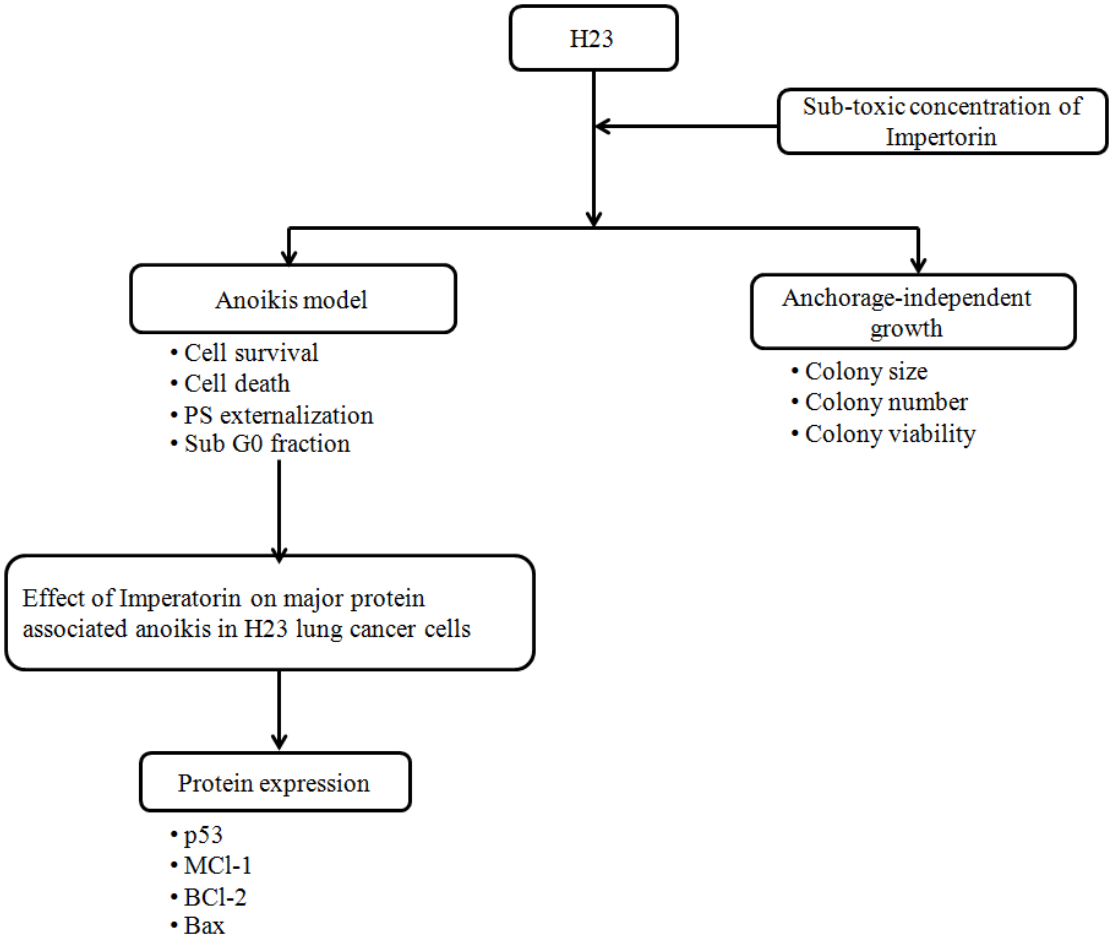
Laminar flow cabinet, carbon dioxide incubator, pH meter, centrifuge, microplate reader (Perskin Elmer, USA), fluorescence microscope (Olympus IX51 with DP70, Japan) and automated cell counter (Bio-Rad, USA).

3. Experimental design

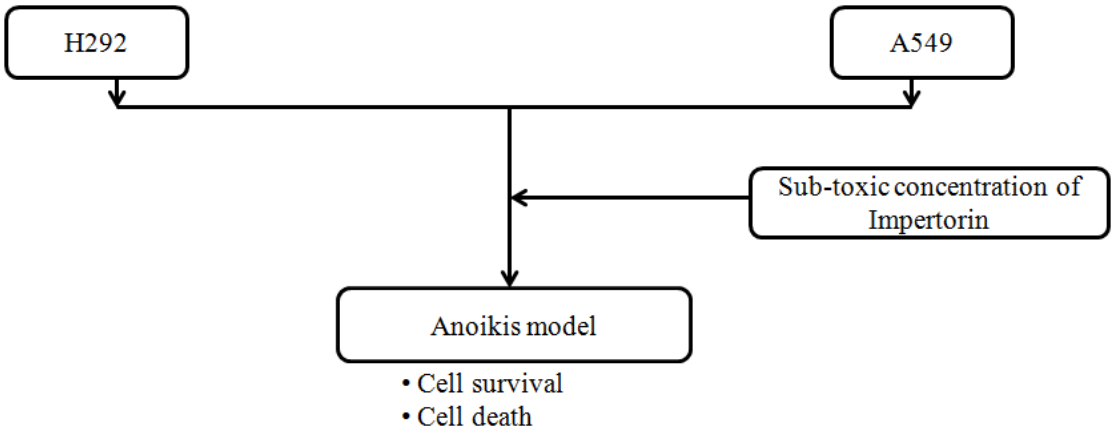
<u>Part I</u> Investigation on the cytotoxic effects of imperatorin in H23 lung cancer cells.



<u>Part II</u> Investigation effects of imperatorin on anoikisand anchorage independent growth and evaluation for major protein associated with anoikis in H23 lung cancer cells.



<u>Part III</u> Investigation effects of imperatorin on anoikis in other lung carcinoma cells.



This experimental study was designed as followed.

3.1 Investigation on the cytotoxic effects of imperatorin in H23 lung cancer cells.

The direct effects of imperatorin on H23 lung cancer cells were determined by cytotoxic assay in various concentrations of imperatorin used in further experiments. Then cell survival was analyzed by XTT assay.

To determine mode of cell death in response to imperatorin treatment in H23 cells were further identified by the fluorescence dye co-staining. Hoechst 33342 assay was performed for apoptosis detection, and propidium iodide staining assay was used for necrosis detection. Cells were treated or left untreated with various concentrations of imperatorin. After 24 h, cells were incubated with Hoechst 33342 and propidium iodide. The apoptotic cells with condensed chromatin and/or fragmented nuclei stained by Hoechst 33342 and PI-positive necrotic cells were visualized and counted under a fluorescence microscope.

3.2 Investigation effects of imperatorin on anoikis in H23 lung cancer cells.

To prove that imperatorin was able to sensitize anoikis in H23 lung cancer cells. Adherent H23 cells were trypsinized and suspended in RPMI medium after that seeded in ultra attach plate cluster, 6-well plate. In order to investigate the optimum time for imperatorin incubation in anoikis assay, cells were treated with imperatorin at

series time 0, 6, 12 and 24 h. Then, cells were incubated with XTT for cell survival assay.

To determine mode of cell death in response to imperatorin treatment in H23 cells were further identified by the fluorescence dye co-staining. Hoechst 33342 assay was performed for apoptosis detection, and propidium iodide staining assay was used for necrosis detection. Cells were treated or left untreated with imperatorin. After 12 h detachment, cells were stained with Hoechst 33342 and propidium iodide. The apoptotic cells with condensed chromatin and/or fragmented nuclei stained by Hoechst 33342 and PI-positive necrotic cells were visualized and scored under a fluorescence microscope.

The phosphatidylserine (PS) externalization to outer membrane surface was constituted in early apoptosis process. Then, annexin-V staining assay was performed in order to confirm the apoptosis response of H23 cells with imperatorin treatment after 12 h detachment. Furthermore, cell cycle analysis was determined sub-G0/G1 fraction in response to imperatorin treatment compare with detachment at time 0 h and detachment at time 24 in an absence of imperatorin.

3.3 Investigation effects of imperatorin on anchorage independent growth in H23 lung cancer cells.

To investigate whether imperatorin has an effect on cells growth and tumorigenesis by soft agar assay. First, H23 cells were suspended in RPMI complete medium with imperatorin containing 0.33% agar and spread over a lower layer containing with medium, but containing 1 % agar in a 6-well plate. After 14 days of incubation, colony number and colony size were photographed at ×10 magnifications with a IX51 microscope (Olympus, Japan) and DP70 Imaging system (Olympus, Japan). Colony survival was determined by resazurin-based assay.

3.4 Evaluation for major protein associated with anoikis on H23 lung cancer cell

To further prove the mechanism by which imperatorin sensitizes anoikis, we evaluated the level of proteins associating in an anoikis process, namely, p53, Mcl-1, Bcl-2, and Bax. H23 cells were seeded at density of $1x10^5$ cells per well in ultra attach plate cluster, 6-well plate. Then cells were treated with 0.1, 0.5 and 1 µg/ml of

imperatorin. After 12 h of incubation time, cells were subjected to perform western blot analysis and evaluated for p53, Mcl-1, Bcl-2 and Bax. The level of β-actin protein of each cell was also evaluated as control.

3.5 Investigation effects of imperatorin on anoikis in other lung carcinoma cells.

To prove that imperatorin was able to sensitize anoikis in other lung carcinoma cells. Adherent H292 and A549 cells were trypsinized and suspended RPMI medium or DMEM medium and then seeded in ultra attach plate cluster, 6-well plate. Suspended cells were treated with imperatorin and cell viability was analyzed by XTT assay.

To determine mode of cell death in response to imperatorin treatment in H292 and A549 cells were further identified by the fluorescence dye co-staining. Hoechst 33342 assay was performed for apoptosis detection, and propidium iodide staining assay was used for necrosis detection. Cells were treated or left untreated with imperatorin. After 24 h detachment, cells were incubated with Hoechst 33342 and propidium iodide. The apoptotic cells with condensed chromatin and/or fragmented nuclei stained by Hoechst 33342 and PI-positive necrotic cells were visualized and scored under a fluorescence microscope.

4. Methods

4.1 Sample preparation

The stock of 25 mg/ml of imperatorin dissolved in DMSO was diluted with deionized water to obtain the working concentrations. The final concentration of DMSO in culture medium was less than 0.5 %.

4.2 Cytotoxicity assay (XTT assay)

H23 cells were sampled 10 µl and added with 10µl Trypan blue. The cells were counted with the automated cell counter (Bio-Rad, USA) for cell density. Cells were adjusted density to $8x10^3$cell/well, seeded in a 96-well flat bottomed microplate and incubated with 0.1, 0.5, 1, 5 and 10 µg/ml of imperatorin for 24 h. Following incubation time, 100 µl of XTT solution (20µM) was added to each wells and the plate was incubated at 37 °C for additional 4 h. Then absorbance of each sample was

measured with microplate reader at 450 nm. All experimental were performed in independent replicate cultures (n=3). Optical density (OD) ratio of treated to non-treated control cells was calculated and presented as percent of cell survival.

$$\text{Percent cells survival} = \left(\frac{OD_{450} \text{ of treatment}}{OD_{450} \text{ of control}} \right) \times 100$$

4.3 Anoikis assay

An anoikis-sensitization effect of imperatorin was then evaluated using sub-toxic concentrations. Adherent H23, H292 and A549 cells in culture plates were trypsinized into a single-cell suspension in RPMI medium and then seeded in low attach plate cluster , 6-well plate at a density of $1x10^5$cells/well. Suspended cells were treated with 0.1, 0.5 and 1 µg/ml of imperatorin and then incubated at 37 °C for 0, 6, 12 and 24 h. Cells were seeded in 96-well plate. For cell survival assay, cells were incubated with 20µM XTT for 4 h at 37 °C. The absorbance of each sample was measured with microplate reader at 450 nm.

4.4 Annexin V detection and sub-G0/G1 fraction analysis by flow cytometry

Cell anoikis were evaluated by Annexin V-FITC staining assay. Cells were trypsinized and suspended in RPMI medium and then seeded in low attach plate cluster, 6-well plate at a density of $1x10^5$cells/well. Suspended cells were treated with 0.1 and 0.5 µg/ml of imperatorin and then incubated at 37 °C for 12 h. Cells were collected, re-suspended, and incubated with Annexin V-FITC for 30 min at 37 ºC. Apoptotic cells were scored by flow cytometry using a 485-nm excitation beam and a 538-nm band-pass filter (FACSort, Becton Dickinson, NJ). The fluorescence intensity was averaged and analyzed by Cell Quest software (Becton Dickinson). For sub-G0/G1 analysis, cells after detachment for 24 h with specific treatment were harvested, re-suspended, and incubated with propidium iodide (PI) buffer for 15 min at 37 ºC and determined for cell cycle profile by flow cytometry.

4.5 Nuclear staining assay

Apoptotic and necrosis cell death was analyzed by Hoechst 33342 and PI co-staining. After presence or absence of imperatorin treatments, cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml of PI for 30 min at 37 ºC. The cells having condensed chromatin and/or fragmented nuclei were counted as apoptosis. Necrotic cells were stained with PI. The apoptotic and necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

4.6 Soft Agar Colony Forming Assay

H23 cells (2,000 cells/wells) were suspended in 1 ml of RPMI complete medium with specific treatment containing 0.33% agar and spread over a lower layer containing with 0.75 μl of the same medium, but containing 0.75 μl of 0.5% agar in a 6-well plate. After 14 days of incubation at 37 °C in a humidified CO_2 incubator, colony survival determined by resazurin-based assay staining and photographed at × 10 magnification with a IX51 microscope (Olympus, Japan) and DP70 Imaging system(Olympus, Japan).

4.7 Western Blot

Adherent H23 cells were trypsinized and suspened in RPMI medium and then seeded in low attach plate cluster, 6-well plate at a density of $1x10^5$ cells/ml. Suspended cells were treated with 0.1, 0.5 and 1 μg/ml of imperatorin and then incubated at 37 °C for 12 h. Cells were lysed with lysis buffer containing a commercial protease inhibitor mixture (Roche Applied Science), 1% Triton X-100, 100 mM phenylmethylsulfonyl fluoride, 150 mM sodium chloride, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 50 mM sodium fluoride, and 1 mM sodium orthovanadate at 4 °C for 20 min. Supernatants were collected and calculated by Bradford method (Bio-Rad, USA) for protein adjustment. Proteins (40 μg) were separated under denaturing conditions by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4,125 mM sodium chloride, 0.05% Tween 20) for 1 h and incubated with specific primary antibodies for 10 h at 4 °C. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-labeled isotype-specific

secondary antibodies for 1 hour at room temperature. The chemiluminescence detection system (Amersham Biosciences, Australia) were indicated immune complexes and analyzed by using analyst/PC densitometry software (Bio-Rad, USA).

5. Statistical Analysis

Datas were presented as the means±S.D. of independent triplicate experiments (n = 3). Statistical analysis was performed by analysis of variance (ANOVA) and Tukey's test at a significance level of $p<0.05$.

Chapter IV

Results

4.1 Cytotoxic effect of imperatorin on lung cancer H23 and normal renal HK-2 cells

To investigate the cytotoxic effect of imperatorin, this study first examined the effect of imperatorin on H23 cell by cytotoxicity, apoptosis, and necrosis assays. Cells were left untreated or treated with imperatorin at the concentrations 0.1, 0.5, 1, 5, 10, 25, 50 and 10µg/ml. After 24 h, cell survival and cell death detection were determined. Figure 4.1 showed that a significant cytotoxic effect of imperatorin was found at the concentration of 10 µg/ml with the approximately 85% of the cells remained viable, whereas the concentrations less than 5 µg/ml had no significant effect on H23 cell survival. In this study, sub-toxic concentrations of imperatorin ranges 0.1-1 µg/ml were treated in all experimenatal. Figure 4.2 (a) showed that imperatorin was not toxic for lung cancer cells in the applied concentration range. The percentage of apoptosis cells at 24 h was found to be 2, 4, 5, 8, 9, 12, 20 and 28 % in response to imperatorin at the concentrations of imperatorin at 0.1, 0.5, 1, 5, 10, 25, 50 and 100 µg/ml, respectively. In addition, the nuclear morphology study supported the above findings that no apoptotic and necrotic cell death was detected in response to less than 5 µg/ml imperatorin (Figure 4.2 (b)). These results suggested that concentrations less than 5 µg/ml of imperatorin had neither cytotoxic nor proliferative effects on H23cells.

From the above result, sub-toxic concentration of imperatorin was chosen for all study at concentrations 0.1, 0.5 and 1 µg/ml. Anti-cancer drugs are frequently found to cause toxic to the normal cells and such cytotoxic effect may become an important hindrance of successful chemotherapy. This study also provided the supportive data regarding the cytotoxic effect of imperatorin on normal renal cells. Human renal HK-2 cells were treated with various concentrations of imperatorin and cell survival was determined as described. Interestingly, the sub-toxic concentrations

of imperatorin obtained from lung cancer H23 testing were found to be non-toxic to HK-2 cells (Figure 4.3). Even though further investigations were needed, these data supported the safety for human renal HK-2 cells in the use of imperatorin for an anti-cancer aspect.

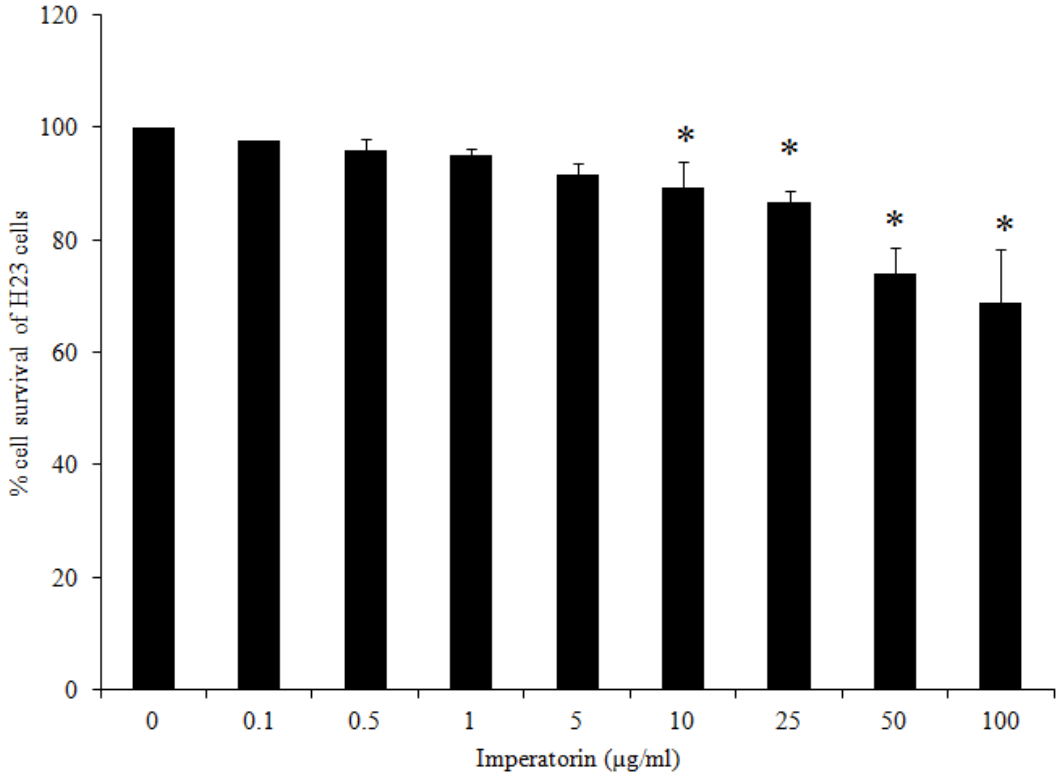
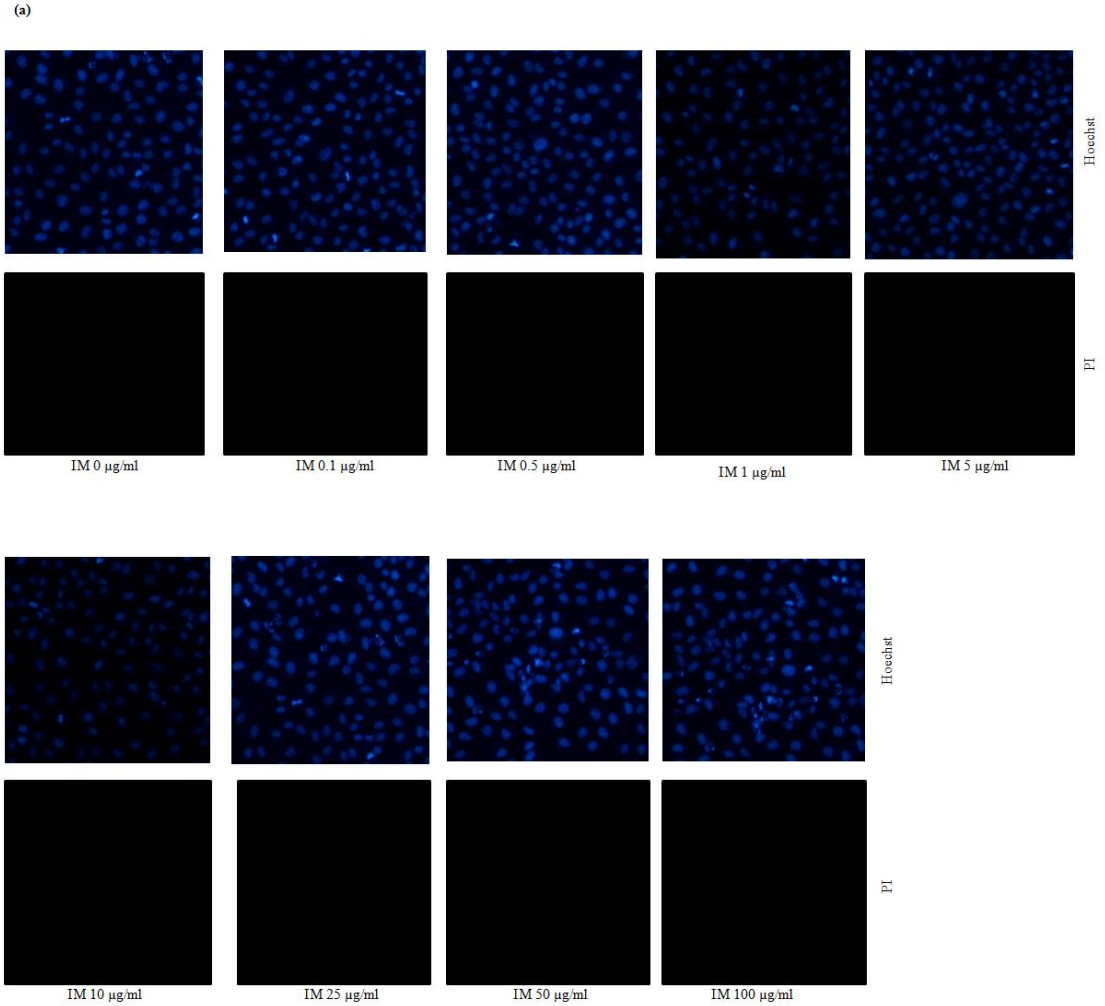


Figure 4.1 Effect of imperatorin on cytotoxicity in lung cancer H23 cells, cells were treated with various concentrations of imperatorin (0.1-100 µg/ml) for 24 h. Cell survival was determined by XTT assay. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.


(a)
Hoechst
PI
IM 0 µg/ml
IM 0.1 µg/ml
IM 0.5 µg/ml
IM 1 µg/ml
IM 5 µg/ml
Hoechst
PI
IM 10 µg/ml
IM 25 µg/ml
IM 50 µg/ml
IM 100 µg/ml

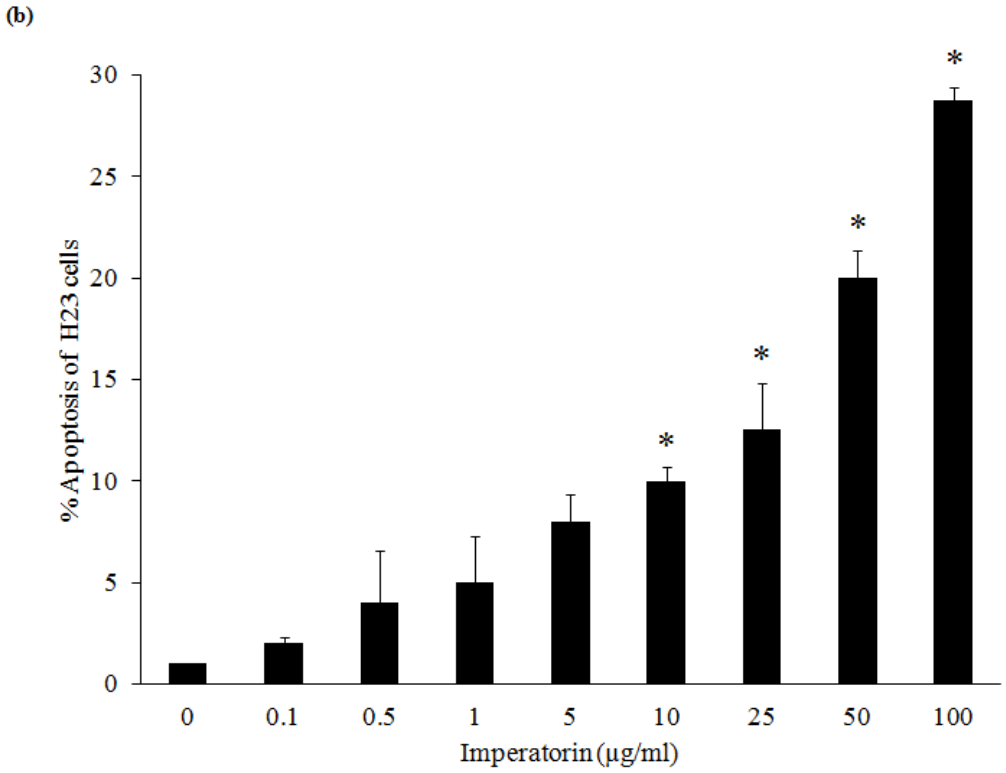


Figure 4.2 Effect of imperatorin on cytotoxicity in lung cancer H23 cells, cells were treated with various concentrations of imperatorin (0.1-100 µg/ml) for 24 h. (a) Morphology of cell nuclei after 24 h attachment was visualized under a fluorescence microscopy after Hoechst 33342/PI co-staining. (b) Percentage of apoptotic detection by scoring DNA condensed and/or fragmented nuclei by Hoechst 33342 at 24 h after attachment. Data are mean±S.D. of independent triplicate experiments (n = 3), *p < 0.05 versus non-treated control.

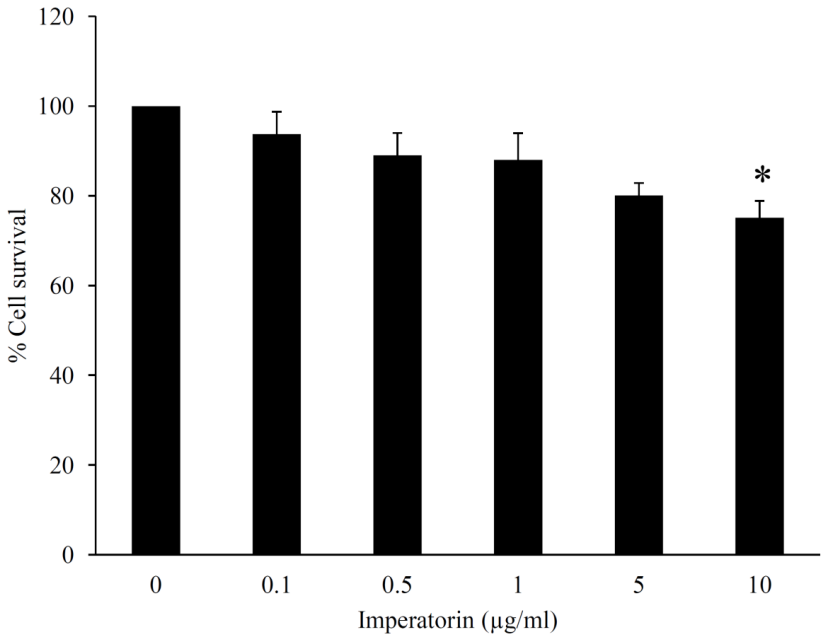


Figure 4.3 Cytotoxic effect imperatorin on normal renal HK-2 cells. Cell survival was determined by XTT assay. Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

4.2 Imperatorin sensitizes lung carcinoma H23 cells to detachment-induced cell death.

An anoikis-sensitization effect of imperatorin was then evaluated using sub-toxic concentrations. Cells were detached and cultured in the attachment-resistant poly-HEMA-coated plates in the presence or absence of sub-toxic concentrations of imperatorin (0.1-1 µg/ml) and the cell survival was determined at indicated times by XTT assay. In order to investigate the optimum time for imperatorin incubation in anoikis assay, cells were treated with sub-toxic concentration of imperatorin at series time (0, 6, 12 and 24 hour). Figure 4.4 showed that in the absence of imperatorin, cell detachment induced a time-dependent decrease in cell survival and the decrease was first detected at 6 h after cell detachment with approximately 70% of the cells remained survival. Importantly, the addition of the detached cells with imperatorin caused a significant reduction of viable cells after detachment in a concentration-dependent manner. After treatment with imperatorin at the concentration of imperatorin 0.1µg/ml could reduce the survival of the cells to approximately 65%,

40% and 35% at the time of 6, 12 and 24 h, respectively. At the concentration of imperatorin 0.5µg/ml could reduce the survival of the cells to approximately 55%, 40 % and 30 % at the time of 6, 12 and 24 h, respectively. Imperatorin at the concentration of 1 µg/ml could reduce the survival of the cells to approximately 50%, 30% and 25% at the time of 6, 12 and 24 h, respectively.

The mode of cell death in response to imperatorin was investigated by Hoechst33342 and PI staining assay. Figures 4.5 showed that the addition of imperatorin to the detached cells significantly enhanced an anoikis response indicated by the increase of cells containing condensed DNA. The percentage of apoptosis cells at 12 h after cell detachment was found to be 54, 58 and 69 % in response to imperatorin at the concentrations of 0.1, 0.5 and 1 µg/ml, respectively. Also, annexin-V staining assay was performed in order to confirm the apoptosis response of the cells (Figure 4.6). Consistent with previous findings, annexin-V stained cells detected by flow cytometry were found to be increase relative Annexin-V positive cell in a dose-dependent manner in response to imperatorin treatment at 0.1 and 0.5 µg/ml. Additional cell cycle analysis showed relative sub G0/G1 fraction significantly increased to 0.52 and 0.54, respectively (Figure 4.7). It is interesting to note that there was no necrotic cell death detected in the present study. These results indicated that the apoptosis induced by cell detachment or anoikis is the primary mode of cell death.

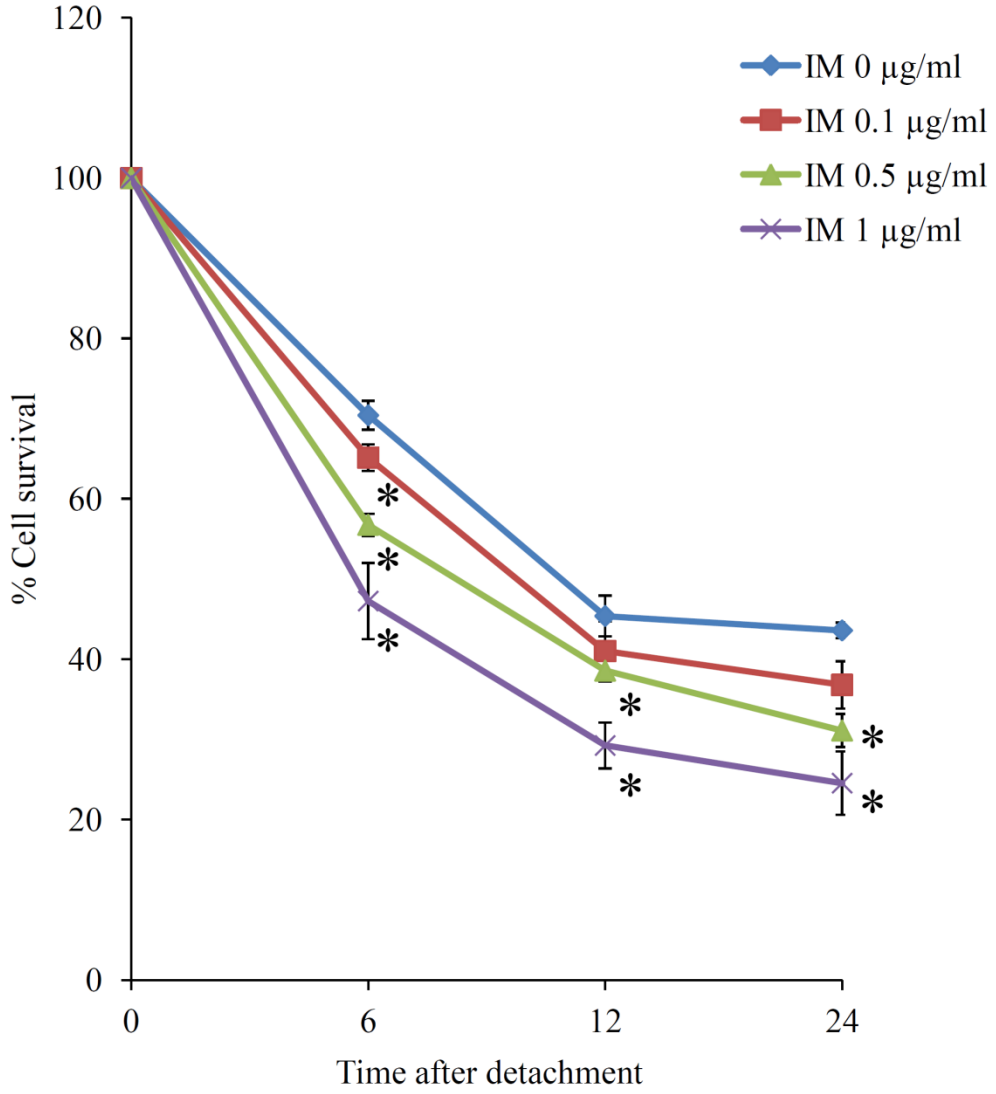


Figure 4.4 Imperatorin sensitizes detachment-induced cell death in H23 cells. Cells were detached and treated with sub-toxic concentrations of imperatorin (0.1-1 μg/ml). Cell survival at indicated times was determined by XTT assay. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

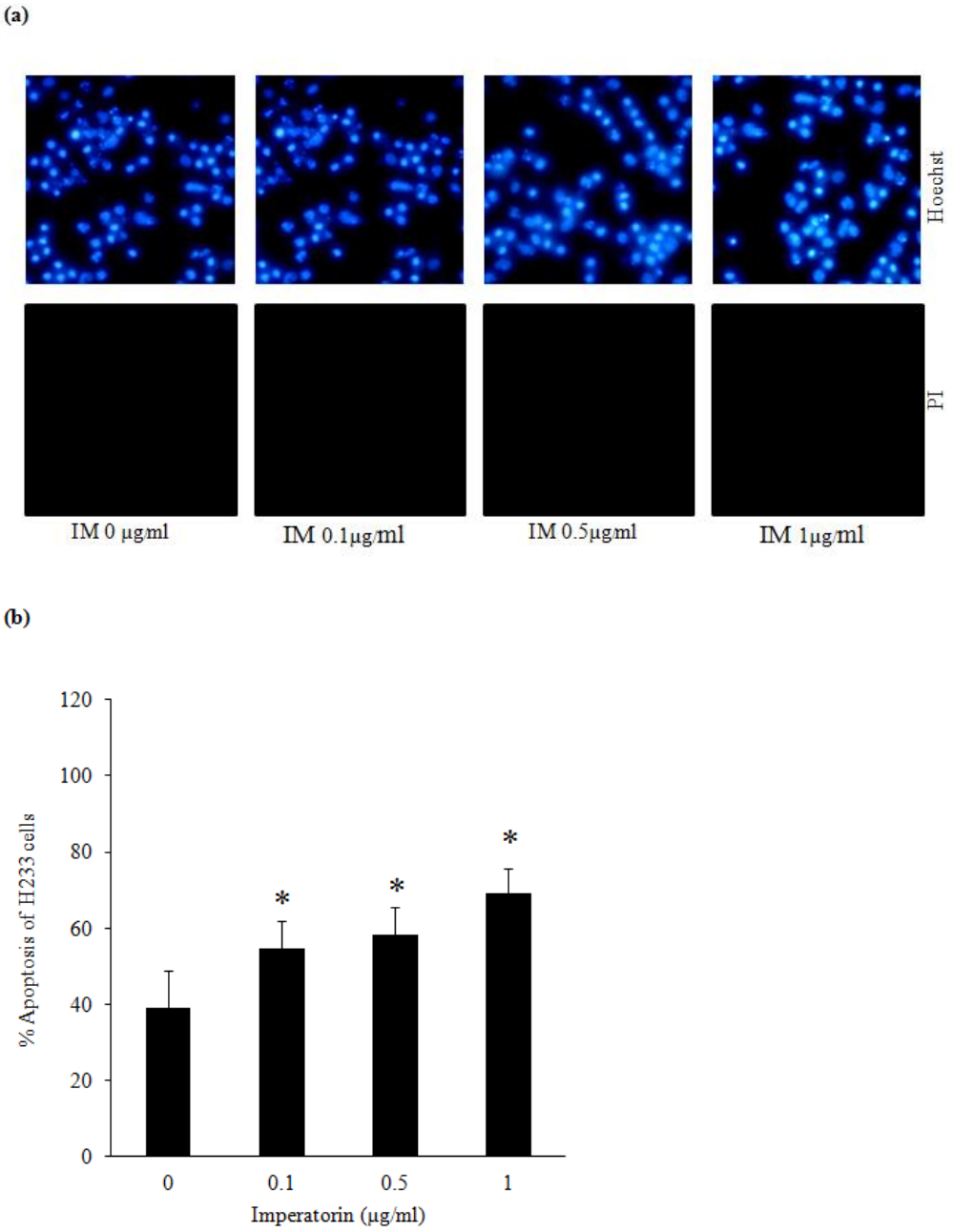


Figure 4.5 Imperatorin sensitizes detachment-induced cell death in H23 cells. Cells were detached and treated with sub-toxic concentrations of imperatorin (0.1-1 μg/ml). (a) Morphology of cell nuclei after 12h detachment was visualized under a fluorescence microscopy after Hoechst 33342/PI co-staining. (b) Percentage of apoptotic detection by scoring DNA condensed and/or fragmented nuclei by Hoechst 33342 at 12h after detachment. Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

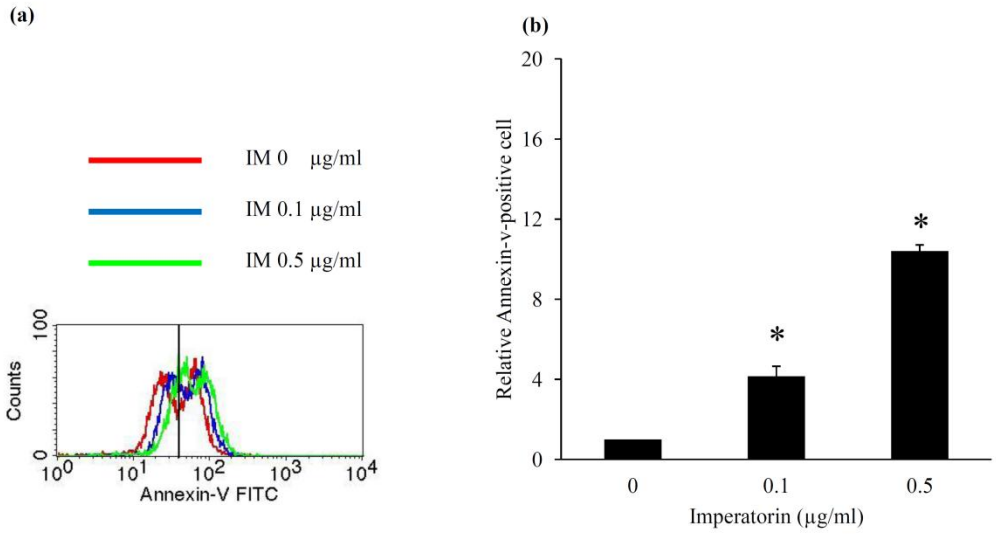


Figure 4.6 Imperatorin sensitizes detachment-induced cell death in H23 cells. (a) Apoptosis was evaluated by Annexin V-FITC and at 12 h after detachment. (b) Relative annexin-v- positive cell detected by flow cytometry. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus detachment time 0, #$p < 0.05$ versus detachment time 12 in an absence of imperatorin.

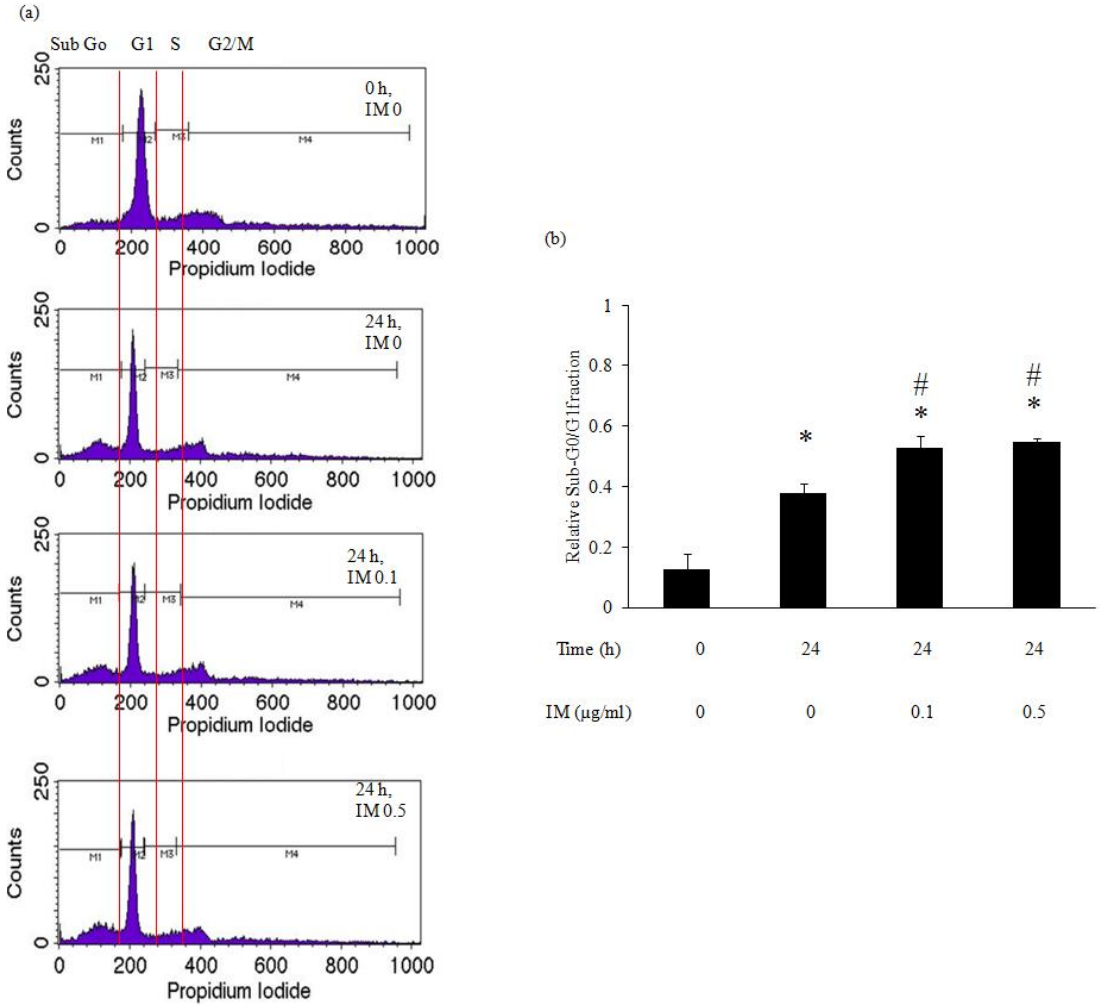


Figure 4.7 Imperatorin sensitizes detachment-induced cell death in H23 cells. Sub G0/G1 fraction and cell cycle analysis. (a) At the indicated time, cells were harvested, re-suspended and incubated with PI buffer for 15 min at 37 ^{0}C and determined for cell cycle profile by flow cytometry. (b) sub-G0/G1 fraction was analyzed from histogram. Data are mean±S.D. of independent triplicate experiments (n = 3), *p < 0.05 versus detachment time 0, #p < 0.05 versus detachment time 24 in an absence of imperatorin.

4.3 Imperatorin inhibits anchorage-independent growth of H23 cells

Having shown the effect of imperatorin in sensitizing anoikis of lung cancer cells, next experimental to prove whether such a compound could influence the growth of cancer cells in the anchorage-independent condition. As a well-accepted characteristic of metastatic cancer cells, the anchorage-independent growth or the cell growth in the detached condition was shown to be a potentiating factor presenting in highly aggressive cancers (Ramachandra *et al*., 2002; Zhu *et al*., 2000). H23 cells were subjected to soft agar assay in the presence or absence of sub-toxic concentrations of imperatorin and cultured for 2 weeks. Cell colony number as well as colony size was determined by a microscopy. Figures 4.8 showed that in the absence of imperatorin H23 cells was able to survive, grow under anchorage-independent conditions, and form large cell colonies. In contrast, the addition of imperatorin resulted in both reduction in colony number and colony size (Figure 4.9). In order to quantify cell survival in anchorage-independent assay, resasurin-based cell survival assay was performed. Figure 4.10 showed the consistent results with the above mentioned that viable cells in colony formation assays decreased in response to the imperatorin treatment in a concentration-dependent manner. Together, the results indicated that imperatorin was able to inhibit the cancer cell growth in the anchorage-independent condition.

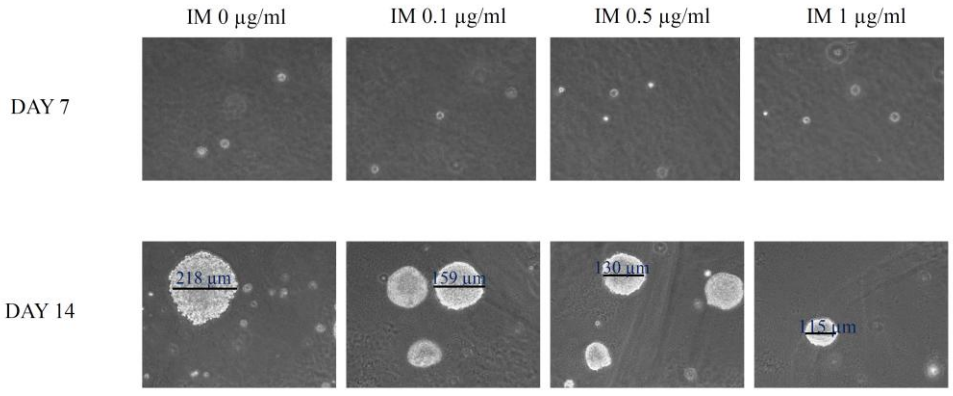


Figure 4.8 Effect of imperatorin on anchorage-independent growth of H23 cells. Cells were subjected to soft agar colony formation assay. Colonies were attained by a microscopy at × 10 magnification.

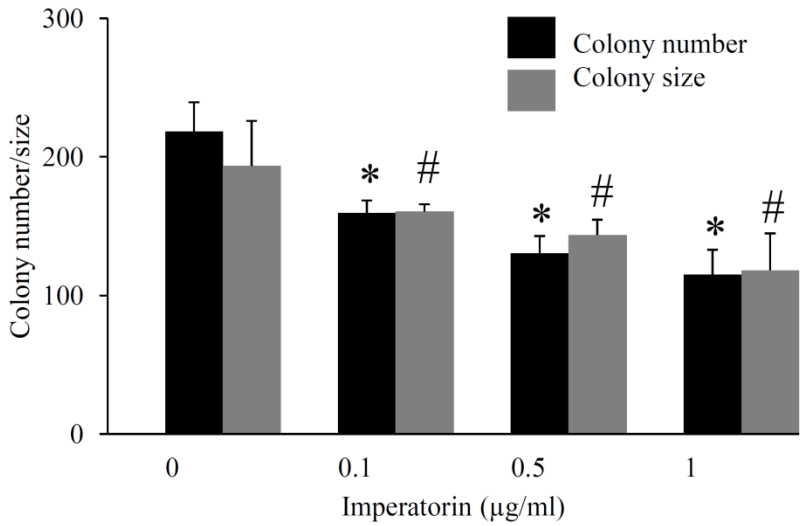


Figure 4.9 Effect of imperatorin on anchorage-independent growth of H23 cells. Colony number and colony size were determined by using image analyzer. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control (colony number) and #$p < 0.05$ versus non-treated control (colony size).

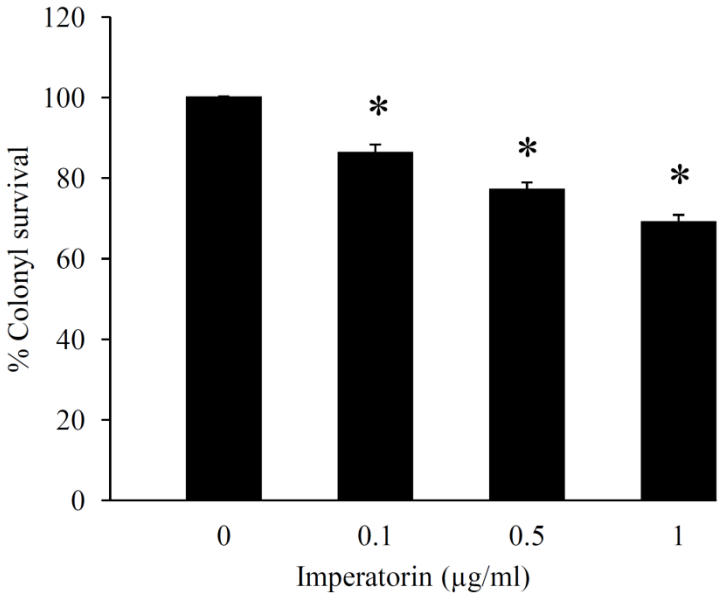


Figure 4.10 Effect of imperatorin on anchorage-independent growth of H23 cells. Cell survival was determined with resasurin-based assay. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

4.4 Effect of imperatorin on protein associated with anoikis on H23 non-small cell lung cancer cell

To further clarify the mechanism by which imperatorin sensitizes detachment-induced apoptosis, this experimental evaluated the level of proteins associating in an anoikis process, namely p53, Mcl-1, Bcl-2, and BAX. Cells were detached and treated with 0-1 µg/ml imperatorin and the cells were subjected to western blotting as described in Materials and Methods. Figure 4.11 showed that imperatorin significantly enhanced an increase of p53 level in a concentration-dependent manner in comparison to that of the non-treated control. Further, imperatorin was found to decrease the anti-apoptotic Mcl-1 level in this lung cancer cells whereas it exhibited only minimal effect on Bcl-2 protein (figure 4.11). The pro-apoptotic Bax was found to be significantly up-regulated in response to 1 µg/ml imperatorin. As Mcl-1 was shown to be a key regulator for anoikis resistance and p53 function was found to be attenuated in many cancer cells, these findings have highlighted the possible use of imperatorin for a cancer therapy.

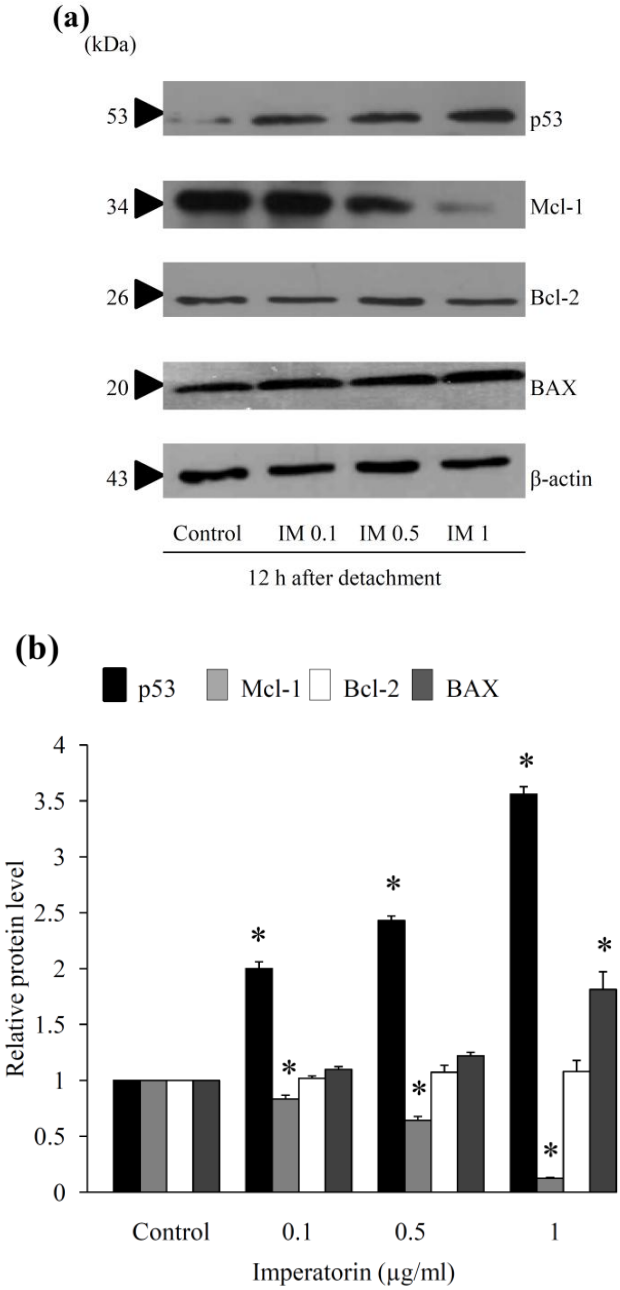


Figure 4.11 Effect of imperatorin on proteins associating anoikis process. . (**a**) Cells were detached and treated with various concentrations of imperatorin (0-1 μg/ml) for 12 h and the expression of p53, Mcl-1, Bcl-2, and BAX proteins was determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. (**b**) The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the results. Data are mean± S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

4.5 Anoikis-sensitization effect of imperatorin on other lung carcinoma cells

This study further investigated the effect of imperatorin on anoikis in other lung cancer cells. Human lung cancer H292 and A549 cells were treated with various concentrations of imperatorin for 24 h. Cell survival assay indicated that imperatorin at the concentration less than 10 µg/ml had neither cytotoxic nor proliferative effect on H292 and A549 cells (Figure 4.12 and 4.13). To test the effect of imperatorin on anoikis response of these cells, cells were incubated with 0.1-1 µg/ml imperatorin in detached condition and cell survival was determined after 24 h by XTT assay. Figure 4.15 and 4.16 showed that cell detachment induced a gradual decrease in cell survival and imperatorin significantly sensitized both H292 and A549 cells to anoikis in a dose-dependent manner. After 24 h post-detachment, H292 cells exhibited approximately 60, 50, and 40 % survival in response to 0.1, 0.5 and 1 µg/ml of imperatorin treatments, respectively (Figure 4.14). Besides, the imperatorin treatment decreased the survival of A549 cells to 50, 40 and 35% at the concentrations of 0.1, 0.5 and 1 µg/ml, respectively (Figure 4.15). The mode of cell death in response to imperatorin was investigated by Hoechst33342 and PI staining assay. Figures 4.16 and 4.17 showed that the addition of imperatorin to the detached cells significantly enhanced an anoikis response indicated by the increase of cells containing condensed DNA. The percentage of apoptosis cells at 24 h after cell detachment was found to be 40, 45 and 60 % in response to imperatorin in H292 at the concentrations of 0.1, 0.5 and 1 µg/ml, respectively. Similar to H292, A549 increased in percentage of apoptosis cells at 24 h after cell detachment to be 45, 50 and 60 % in response to imperatorin at the concentrations of 0.1, 0.5 and 1 µg/ml, respectively. The morphology of apoptotic nuclei were observed in the imperatorin-treated cells while PI-positive necrotic cells were not detected. These data supported the sensitizing effect of imperatorin on lung cancer cell anoikis.

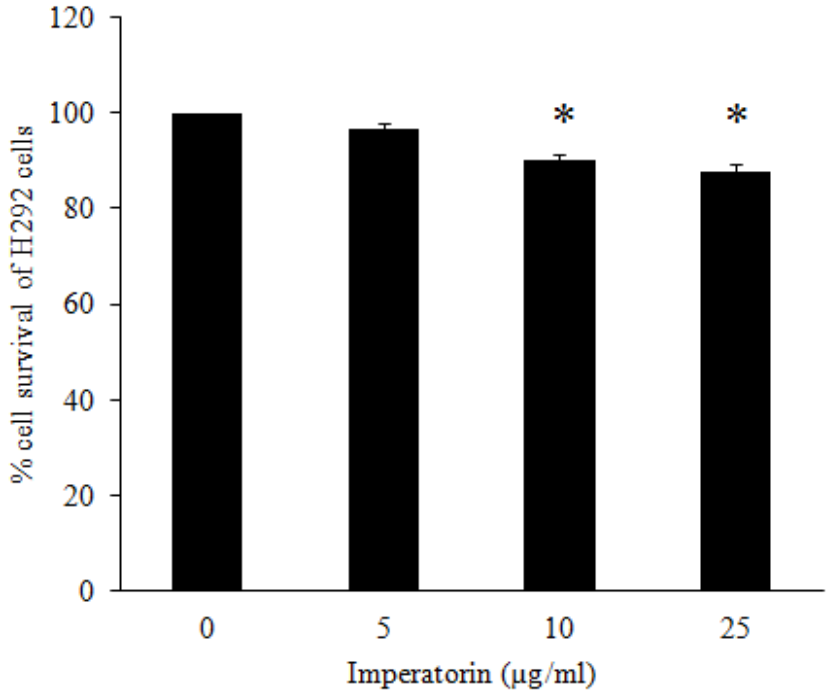


Figure 4.12 Effect of imperatorin on cytotoxicity in lung cancer H292 cells, cells were treated with various concentrations of imperatorin (0-25 µg/ml) for 24 h. Cell survival was determined by XTT assay. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

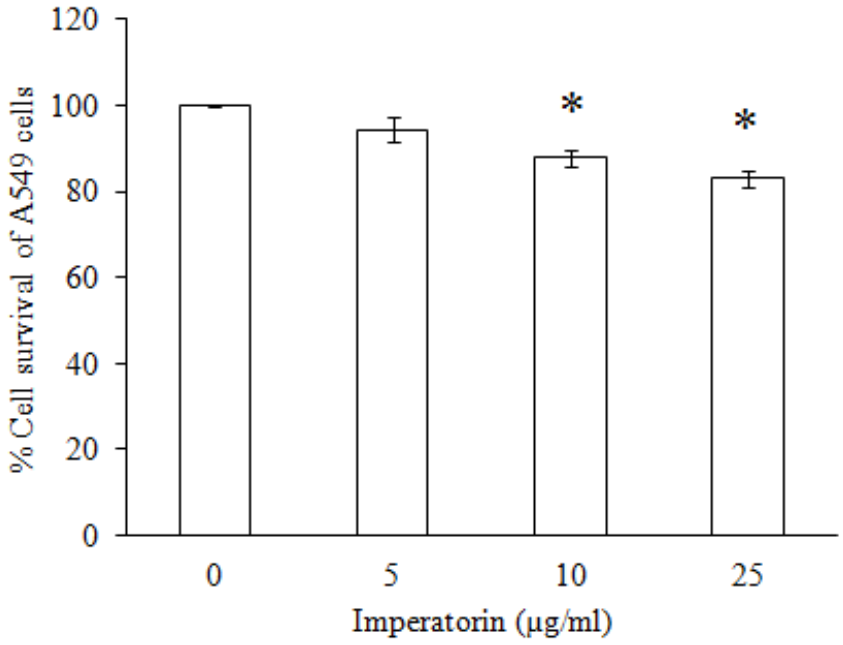


Figure 4.13 Effect of imperatorin on cytotoxicity in lung cancer A549 cells, cells were treated with various concentrations of imperatorin (0-25 µg/ml) for 24 h. Cell survival was determined by XTT assay. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

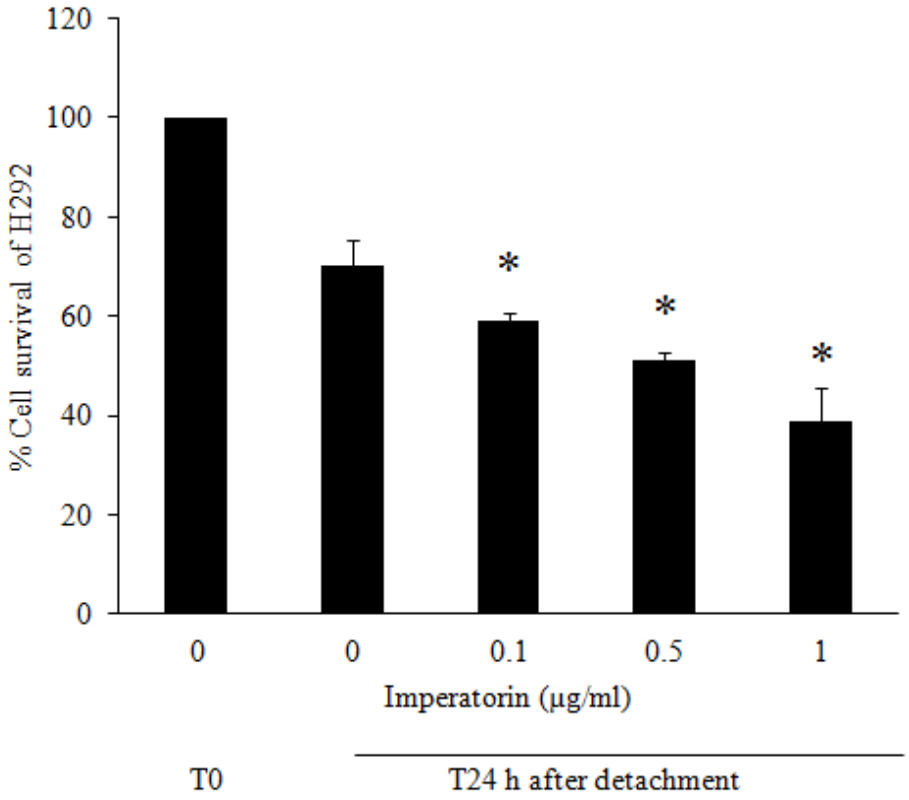


Figure 4.14 Anoikis-sensitization effect imperatorin on H292 lung cancer cells. H292 cells were detached and incubated with various concentrations of imperatorin (0-1 μg/ml) for 24 h, and cell survival was determined by XTT assay. Data are mean±S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control at 24 h after cell detachment.

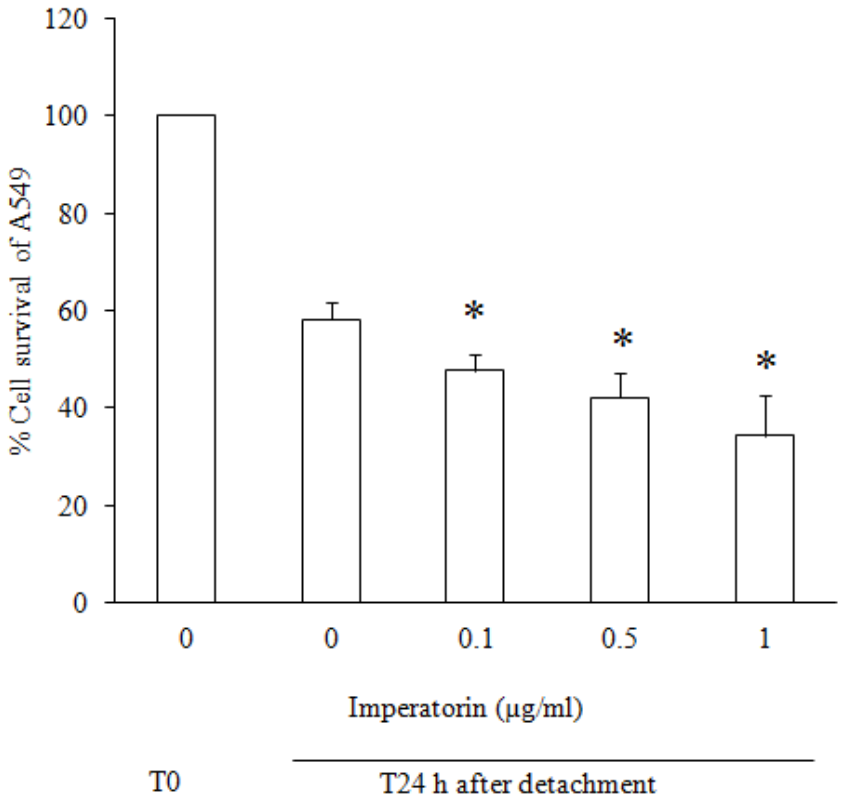


Figure 4.15 Anoikis-sensitization effect imperatorin on A549 lung cancer cells. A549 cells were detached and incubated with various concentrations of imperatorin (0-1 µg/ml) for 24 h, and cell survival was determined by XTT assay. Data are mean± S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control at 24 h after cell detachment.

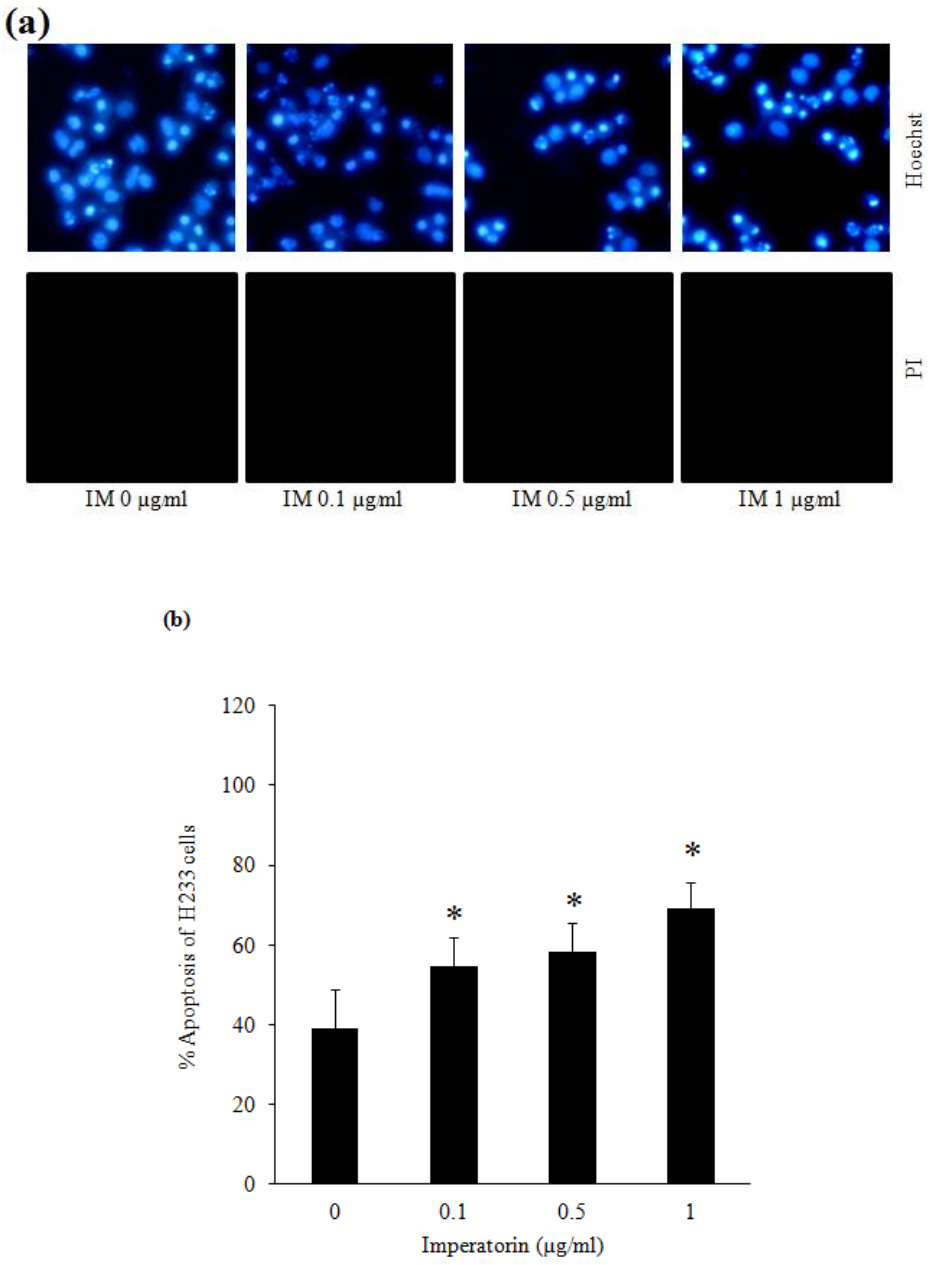


Figure 4.16 Anoikis-sensitization effect imperatorin on H292 lung cancer cells. (a) Nuclear Morphology of apoptotic cells was visualized under a fluorescence microscopy. (b) Percentage of apoptotic detection by scoring DNA condensed and/or fragmented nuclei by Hoechst 33342 at 24 h after detachment. Data are mean± S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control at 24 h after cell detachment.

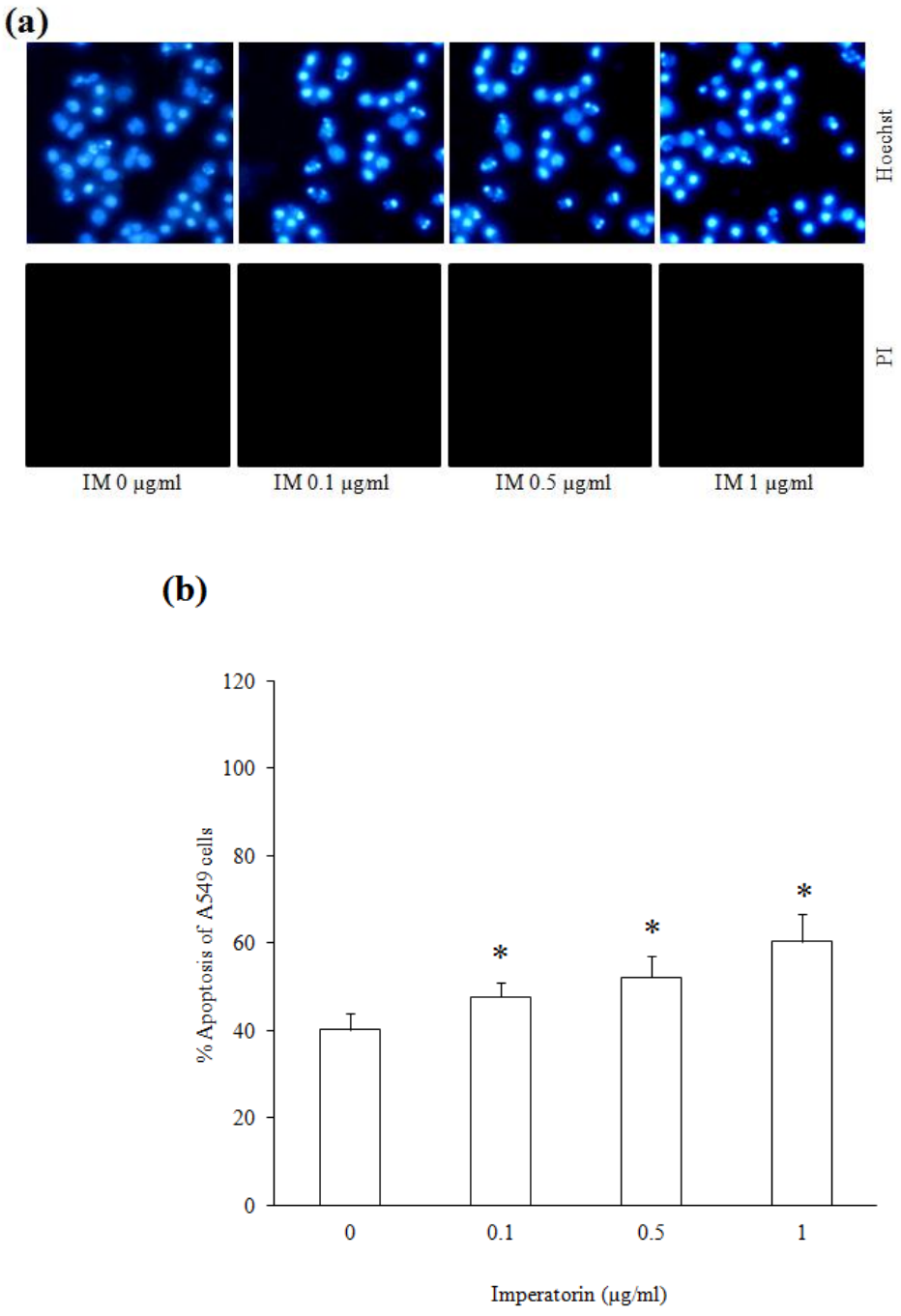


Figure 4.17 Anoikis-sensitization effect imperatorin on A549 lung cancer cells. (a) Nuclear Morphology of apoptotic cells was visualized under a fluorescence microscopy. (b) Percentage of apoptotic detection by scoring DNA condensed and/or fragmented nuclei by Hoechst 33342 at 24 h after detachment. Data are mean± S.D. of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control at 24 h after cell detachment.

Chapter V

Discussion and conclusion

Among various human cancers, lung cancer is accepted as the leading cause of the cancer mortality worldwide, and most death is associated with cancer metastasis (Jemal *et al*., 2008; Herbst *et al*., 2008). To metastasize, a malignant cell must detach from its primary tumor, invade the nearby circulatory or lymphatic system, and establish itself in a new site. An anoikis, a detachment-induced apoptosis has been shown to play a critical role in induction of most cancer cell death during travelling in the blood or lymphatic circulations. Indeed, the resistance to anoikis is a prerequisite capability of metastatic cancer cells (Boisvert-Adamo et al., 2009; Cao et al., 2009; Kodama et al., 2005; Wei et al., 2002). Anoikis has primarily been described as an intrinsic apoptotic pathway mainly through a mitochondrial death pathway and was shown to be tightly related with the disturbance in the balance of proteins in Bcl-2 family (Kroemer et al., 2007). The previous studies have suggested that anti-apoptotic Bcl-2 and Mcl-1 proteins play an important role in mediating anoikis resistance as well as the aggressive behaviors of lung cancer cells (Chunhacha *et al*., 2012; Song *et al*., 2005; Yang *et al*., 2009; Zuo *et al*., 2010).

Increasing attention is paid upon natural substances as a source for novel anti-cancer drugs. Imperatorin is a furanocoumarin isolated from the root of *Angelica dahurica*, which was previously reported to have several pharmacological effects including anti-cancer effect (Baek *et al*., 2000; Kostova, 2005; Luo *et al*., 2011; Pae *et al*., 2002). The present study has further provided the evidence supporting the potential of this natural compound to be use for anti-metastasis aspects. The results were reported herein for the first time that imperatorin at sub-toxic concentrations could sensitize lung cancer cell anoikis in concentration dependent manner (Figure 4.4). Cell death in response to imperatorin was investigated by Hoechst33342 and PI staining assay. Detached cells with imperatorin treatment significantly enhanced an anoikis response indicated by the increase of cells containing condensed DNA (Figures 4.5). The percentage of apoptosis cells after cell detachment was significantly in concentration dependent pattern. It is interesting to note that there was

no necrotic cell death detected in the present study. These results indicated that the apoptosis induced by cell detachment or anoikis is the primary mode of cell death.
In addition, the externalization of phosphatidylserine (PS) to outer membrane surface was constituted in early apoptosis process. Then, annexin-V staining assay was performed in order to confirm the apoptosis response of the cells (Figure 4.6). Consistent with previous findings, annexin-V stained cells detected by flow cytometry were found to be increase relative Annexin-V positive cell in a concentration-dependent manner in response to imperatorin treatment. Furthermore, cell cycle analysis showed relative sub-G0/G1 fraction significantly increased to imperatorin treatment (Figure 4.7) (Luo *et al*., 2011). Therefore, the cell death assays showed that imperatorin sensitized apoptosis after detachment upon evidenced by cell survival, DNA condensation, and PS externalization.

As a well-accepted characteristic of metastatic cancer cells, the anchorage-independent growth or the cell growth in the detached condition was shown to be a potentiating factor presenting in highly aggressive cancers (Ramachandra et al., 2002; Zhu et al., 2000). In soft agar assay corresponded with anoikis assay, imperatorin significantly inhibit the growth of cancer cells in the anchorage-independent condition. Taken together, the results supporting imperatorin potentiated in sensitizing anoikis of lung cancer cells. Furthermore, in experimental imperatorin at the concentrations used for anoikis sensitization was not toxic to normal renal HK-2 cells.

As an increase in Mcl-1 protein in certain cancer cells was reported to be a mechanism by which such cells resist to anoikis (Kim *et al*., 2012; Zuo *et al*., 2010). Moreover, Mcl-1 protein was shown to be a key regulator for anoikis in many cell systems (Boisvert-Adamo *et al*., 2009; Kim *et al*., 2012; Zuo *et al*., 2010). Imperatorin which was reported herein to down-regulate Mcl-1 may be able to be developed for anti-metastasis approaches.

The loss of cell adhesion was shown to initiate p53-dependent mitochondrial apoptosis pathway (Benchimol, 2001; Schuler and Green, 2001). Certain cancer cells develop mechanisms to attenuate p53 activation and thus resulted in anoikis resistance (Derouet *et al*., 2007; Grossmann, 2002; Guadamillas, Cerezo, and Pozo, 2011; Ravid *et al*., 2005; Song et al., 2012; Zhang *et al*., 2004). The results show that the treatment with imperatorin significantly enhanced p53 activation in H23 lung cancer cells and

influenced the cell anoikis response. Subsequently, the up-regulation of Bax concomitant with the down-regulation of Mcl-1 protein was detected in the present study and was shown to be a mechanism by which imperatorin sensitized H23 cell anoikis. In previous studies imperatorin at micromolar dose had a significant effect on p53 levels and downregulated Bcl-2 proteins (Luo *et al*., 2011; Pae *et al.,* 2002). However, in this study, the sub-toxic concentrations of imperatorin caused no effect on the Bcl-2 expression. High level of Mcl-1 are detected in the more differentiated apical layers of epithelia including prostate, breast, colon and lung epithelia while Bcl-2 expression tend to be higher in the basal cell layer. The differences suggest unique roles of Mcl-1 and Bcl-2 in apoptosis control. Moreover Mcl-1 expression is also downregulated during apoptosis in many cells system, often in contrast to the anti-apoptosis Bcl-2 protein (Michels, Johnson and Packham, 2005).

In conclusion, This study demonstrated that imperatorin at sub-toxic concentrations sensitized human lung carcinoma H23 cell anoikis and inhibited the growth of the cells in anchorage-independent condition. The results also revealed the roles of imperatorin treatment on increased p53 protein level and downregulated Mcl-1 which could be important in understanding the mechanism of action of imperatorin and benefit the development of this compound. This new finding suggests the possible anti-metastasis role of imperatorin which may be beneficial for cancer therapy.

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APPENDIX

Table 1 The percentage of cell survival was determined by XTT assay after treatment with various concentration of imperatorin in lung cancer cell H23.

Imperatorin (μg/ml)	% Cell survival
0	100.00±0.00
0.1	97.65±0.02
0.5	96.03±1.87
1	94.99±1.17
5	91.46±2.12
10	89.16±4.65*
25	86.54±2.16*
50	74.01±4.66*
100	68.75±9.66*

Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

Table 2 The percentage of cell apoptosis was determined by Hoechst 33342/PI co-staining assay after treatment with various concentration of imperatorin in lung cancer cell H23.

Imperatorin (μg/ml)	% Apoptosis
0	1.00±1.12
0.1	2.16±1.19
0.5	4.54±0.25
1	5.01±0.72
5	8.75±0.36
10	9.16±0.68*
25	12.54±2.27*
50	20.01±1.36*
100	28.75±0.68*

Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

Table 3 The percentage of cell survival was determined by XTT assay after treatment with various concentration of imperatorin in normal renal HK-2 cell.

Imperatorin (μg/ml)	% Cell survival
0	100.00±0.00
0.1	93.79±4.98
0.5	89.01±4.98
1	88.04±5.91
5	80.07±2.79
10	75.12±3.73*

Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

Table 4 The percentage of cell survival was determined by XTT assay after detactment at optimum times and treatment with various concentration of imperatorin in lung cancer cell H23.

Imperatorin (µg/ml)	Time after detachment (h)			
	0	6	12	24
0	100.00±0.00	70.41±1.82	45.38±2.55	43.58±0.97
0.1	100.00±0.00	65.13±1.66*	41.01±3.69	36.79±2.96
0.5	100.00±0.00	56.75±1.38*	38.57±1.34*	31.10±2.06*
1	100.00±0.00	47.25±4.74*	29.23±2.86*	24.54±3.95*

Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

Table 5 The percentage of cell apoptosis was determined by Hoechst 33342/PI co-staining assay after 12 h detachment and treatment with various concentration of imperatorin in lung cancer cell H23.

Imperatorin (μg/ml)	% Apoptosis
Control	38.92±10.01
0.1	54.51±7.30*
0.5	58.27±7.19*
1	69.16±6.67*

Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

Table 6 The Relative Annexin-v-positive cell, apoptosis was evaluated by Annexin v-FITC and flow cytometry at 12 h after detachment with sub-toxic concentration of imperatorin in lung cancer cell H23.

Imperatorin (μg/ml)	Relative Annexin-v-positive cell
0	1
0.1	$4.15\pm0.5^{*}$
0.5	$10.41\pm0.3^{*}$

Data are mean±S.D. of independent triplicate experiments (n = 3), $*p < 0.05$ versus detachment time 0, $\#p < 0.05$ versus detachment time 12 in an absence of imperatorin.

Table 7 Sub G0/G1 fraction and cell cycle analysis determined for cell cycle profile by flow cytometry.

Time after detachment	Imperatorin (μg/ml)	Sub G0/G1 fraction
0	0	$0.13\pm0.05^{*}$
24	0	$0.38\pm0.03^{*}$
24	0.1	$0.53\pm0.04^{*,\#}$
24	0.5	$0.55\pm0.01^{*,\#}$

Data are mean±S.D of independent triplicate experiments (n = 3), $*p < 0.05$ versus detachment time 0, $\#p < 0.05$ versus detachment time 24 in an absence of imperatorin.

Table 8 Colony number and size of H23 cell in anchorage-independent growth assay determined by image analyzer.

Imperatorin (μg/ml)	Colony number	Colony size (μM)
0	218.00±21.00	193.00±13.00
0.1	159.00±9.00*	161.00±5.00#
0.5	130.00±12.00*	144.00±11.00#
1	115.00±17.00*	118.00±2.00#

Data are mean±S.D of independent triplicate experiments (n = 3), *p < 0.05 versus non-treated control (colony number) and #p < 0.05 versus non-treated control (colony size).

Table 9 The percentage of colony survival was determined by resazurin-based assay after 14 days of anchorage-independent growth assay and treatment with various concentration of imperatorin in lung cancer cell H23.

Imperatorin (µg/ml)	% Colony survival
0	100.00±0.00
0.1	87.52±1.83*
0.5	77.43±1.51*
1	69.38±1.49*

Data are mean±S.D of independent triplicate experiments (n = 3), *$p < 0.05$ versus non-treated control.

Table 10 Level of protein associated anoikis in H23 cell, qualified for protein expression by Western blotting analysis.

Imperatorin (μg/ml)	Relative protein level			
	p53	Mcl-1	Bcl-2	BAX
0	1	1	1	1
0.1	2.01±0.06*	0.83±0.03*	1.02±0.02	1.10±0.03
0.5	2.43±0.04*	0.65±0.04*	1.07±0.06	1.22±0.03
1	3.56±0.07*	0.13±0.01*	1.08±0.10	1.81±0.16*

Data are means±S.D. (n=3), $*p < 0.05$ versus non-treated control.

Table 11 The percentage of cell survival was determined by XTT assay after treatment with various concentration of imperatorin in lung cancer cell H292.

Imperatorin (μg/ml)	% Cell survival
0	100.00±0.00
5	96.81±1.09
10	90.09±1.10*
25	87.75±1.64*

Data are represents means ±S.D. (n=3), $*p < 0.05$ versus non-treated control after 24 h attachment.

Table 12 The percentage of cell survival was determined by XTT assay after treatment with various concentration of imperatorin in lung cancer cell A549.

Imperatorin (μg/ml)	% Cell survival
0	100.00±0.00
5	94.43±2.85
10	87.77±2.11*
25	83.04±1.92*

Data are means±S.D. (n=3), $*p < 0.05$ versus non-treated control after 24 h attachment.

Table 13 The percentage of cells survival was determined by XTT assay after 24 h detachment and treatment with various concentration of imperatorin in lung cancer cell H292.

Time after detachment	Imperatorin (μg/ml)	% Cell survival
0	0	100.00±0.00
24	0	58.16±3.68
24	0.1	47.72±3.37*
24	0.5	42.15±4.96*
24	1	34.43±8.26*

Data are means±S.D. (n=3), $*p < 0.05$ versus non-treated control after 24 h detachment.

Table 14 The percentage of cells survival was determined by XTT assay after 24 h detachment and treatment with various concentration of imperatorin (concentration dependency) in lung cancer cell A549.

Time after detachment	Imperatorin (μg/ml)	% Cell survival
0	0	100.00±0.00
24	0	70.11±5.39
24	0.1	58.63±1.83*
24	0.5	50.93±1.77*
24	1	38.99±6.59*

Data are means±S.D. (n=3), $*p < 0.05$ versus non-treated control.

Table 15 The percentage of cell apoptosis was determined by Hoechst 33342/PI co-staining assay after 12 h detachment and treatment with various concentration of imperatorin in lung cancer cell H292.

Imperatorin (μg/ml)	% Apoptosis
0	29.18±5.39
0.1	38.67±1.83*
0.5	45.65±1.77*
1	59.54±3.59*

Data are means±S.D. (n=3), $*p < 0.05$ versus non-treated control after 24 h detachment.

Table 16 The percentage of cell apoptosis was determined by Hoechst 33342/PI co-staining assay after 12 h detachment and treatment with various concentration of imperatorin in lung cancer cell A549.

Imperatorin (μg/ml)	% Apoptosis
0	40.16±3.68
0.1	47.72±3.37*
0.5	52.15±4.96*
1	60.43±6.26*

Data are means±S.D. (n=3), $*p < 0.05$ versus non-treated control after 24 h detachment.

Vita

Miss Kanuengnit Choochuay was born on October 1, 1986 in Trang. She received her B. Pharm from the Faculty of Pharmacy, Chiangmai University in 2010.