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BIOLOGICAL ACTIVITY SCREENING OF THAI MEDICINAL PLANTS: STRUCTURAL IDENTIFICATION OF POTENT COMPOUNDS WITH ANTICANCER ACTIVITY

Miss Chutima Kaewpiboon

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	BIOLOGICAL ACTIVITY SCREENING OF THAI MEDICINAL PLANTS: STRUCTURAL IDENTIFICATION OF POTENT COMPOUNDS WITH ANTICANCER ACTIVITY
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Screening medicinal plants

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PART I

THESIS CONTENT

1.1 Overview of the publications

Medicinal plants have played an important role in maintaining and improving human health for a long time. Several evidences have revealed numbers of potential medicinal plants used in various folklore medicine systems to promote human health and cure illness such as anticancer, antioxidant, antidiabetic, antimicrobial, anti-inflammatory, antimalarial and antiviral activities [1].

There are 4 publications for the research in this dissertation. The objective was to obtain certain Thai medicinal plants which can represent the promising sources of natural products with potential biological activity for further pharmacological and phytochemical investigations. The first preliminary study involved the screening for plants with the *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities from 52 species of Thai medicinal plants

(Publication 1; Chutima Kaewpiboon, Kriengsak Lirdprapamongkol, Chantragan Srisomsap, Pakorn Winayanuwattikun, Tikamporn Yongvanich, Preecha Phuwaprisirisan, Jisnuson Svasti and Wanchai Assavalapsakul. Studies of the *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities of selected Thai medicinal plants, BMC Complementary and Alternative Medicine 2012, 12:217).

Since cancer is the disease causing the eventual death resulting from the uncontrollable growth of the cells, therefore publications 2, 3 and 4 were mainly focused on the anticancer activity of the obtained potent compounds and their mechanisms. Unlike regular cells, cancer cells do not experience programmatic death, but instead continue to grow and divide [2]. This leads to a mass of abnormal cells growing out of control and spreading to the other parts of the body. Moreover it can grow further, invade and destroy other healthy tissues, resulting in a serious condition difficult to treat [3]. Although chemotherapeutic agents can treat various types of cancers, anticancer drugs are not suitable for a long term therapy since the cancer cells can eventually develop the multidrug resistance (MDR) [4]. MDR, the main principal mechanism of many cancers, is a major factor in the failure of cancer chemotherapy [5] from the decreased uptake of

drugs, alterations in cellular pathways and increased active efflux of drugs [2]. The most common mechanism of MDR cancer is often the result of efflux increase by the overexpression of the membrane transporter protein, P-glycoprotein (P-gp) [3]. The function of P-gp in cancer-cell membranes is energy-dependent transporter which can pump out cytotoxic drugs from cancer cells, leading to lower intracellular concentrations of chemotherapeutic drugs, thereby decreasing the cytotoxic effects of the chemotherapeutic agents [6]. In addition, P-gp also plays a role in the inhibition of caspase activation in the apoptotic pathway, programmed cell death, in MDR cancer cells [7, 8] thus leading to inhibition of cell death. In publication 2, A549RT-eto, which is etoposide resistant in human lung A549 cancer cells was used as the MDR model to study the resistance mechanism to chemotherapy drugs. In this work, the overexpression of proteins, STAT1-HDAC4 other than P-gp in A549RT-eto was studied. Based on the results, we proposed that the inhibition of these proteins in A549RT-eto should represent the potential therapeutic targets for the treatment of chemotherapeutic resistant lung cancer cells overexpressing P-gp.

(Publication 2; Chutima Kaewpiboon, Ratakorn Srisuttee, Waraporn Malilas, Jeong Moon, Sangtaek Oh, Hye Gwang Jeong, Randal N. Johnston, Wanchai Assavalapsakul and Young-Hwa Chung. Up-regulation of STAT1-HDAC4 confers resistance to etoposide through enhanced multidrug resistance (MDR)1 expression in human A549 lung cancer cells, Accepted in: Molecular medicine reports, 2014).

Therefore, to overcome the MDR cancer by using our proposal mechanism, the potent natural products from plants were further isolated and investigated in publications 3 and 4.

In publication 3, the bioactive compounds from selected Thai medicinal plant extracts with cytotoxicity were isolated and finally tested for *in vitro* cytotoxic activity against MDR cancer. From publication 1, the leaf extract of *Bryophyllum pinnata* exerted strong *in vitro* cytotoxicity in both of normal lung (A549) and its MDR cancer cell lines (A549RT-eto). However, since the antitumor compounds of *B. pinnata* were already isolated and identified [9], in this work , the bioactive compounds which can reverse the etoposide resistance in human lung A549 cancer cells by the down-regulation of NF-**K**B leading to apoptosis cell-death were isolated from *Bryophyllum laetivirens*.

(Publication3; Chutima Kaewpiboon, Ratakorn Srisuttee, Waraporn Malilas, Jeong Moon, Sirichat Kaowinn, Il-Rae Cho, Randal N. Johnston, Wanchai Assavalapsakul and Young-Hwa Chung. Extracts of *Bryophyllum laetivirens* reverse etoposide resistance in human lung A549 cancer cells by down-regulation of NF-κB, Oncology reports 2014, 31(1):161-168).

From the obtained bioactive compound and its mechanism in publication 3, we further hypothesized whether there were other types of mechanisms which can reverse the etoposide resistance such as autophagy induction. Therefore, the other 7 purified potent compounds were later screened and tested in human lung A549 cancer cells, leading to apoptosis cell-death evident in publication 4.

(Publication 4; Chutima Kaewpiboon, Serm Surapinit, Waraporn Malilas, Jeong Moon, Preecha Phuwapraisirisan, Santi Tip-pyang, Randal N. Johnston, Sang Seok Koh, Wanchai Assavalapsakul and Young-Hwa Chung. Feroniellin A-induced autophagy causes apoptosis in multidrug-resistant human A549 lung cancer cells, International journal of oncology 2014, 44:1233-1242).

All these four manuscripts attached in Part II were already published as partial fulfillments for the requirements of the Degree of Doctor of Philosophy.

1.2 Background and significance of the study

Recent investigations of plants that have been the basis of traditional medicine for a long time have revealed a significant number of novel metabolites with potent pharmacological properties [10-12]. For example, the chemotherapeutic drug, pacitaxel (Taxol) was initially discovered from the bark of the Himalayan Yew tree by screening for anticancer activity in plant extracts [13]. Various types of plants have been used not only for dietary supplements but also as traditional folk treatments for many health problems [14]. As a matter of fact, the long history of folk medicine has demonstrated the potential of plants as the sources of bioactive compounds [15]. A number of Thai medicinal plants have been reported to provide the foundation for modern pharmaceuticals and lead structure of drugs [16] since Thailand has a great diversity of indigenous (medicinal) plant species and hence is a potential source for bioactive compounds, including those with potential antitumor [17, 18], antioxidant [19, 20], anti-lipase (and so potential antiobesity)

[17, 21] and antimicrobial activities [22, 23]. Since current pharmacological reagents are restricted by the increasing spread and evolution of resistance and/or their undesired side effects, and are often difficult to synthesize as the pure bioactive stereoisomer, the investigations to explore novel drugs or those that can act as templates for the development of new therapeutic agents appear imperative. This work focused mainly on *in vitro* cytotoxicity in cancer cell lines from medicinal plants since cancer is one of the most common and severe problems of clinical medicine that has been the leading cause of death in Thailand [24]. Cancer is a general term applied to a series of malignant diseases which may affect many different parts of the body. If the process is not arrested, it may progress until it causes the death of the organism [25]. Nowadays, much commonly used anticancer therapeutics shows broadly cytotoxic agents which have been discovered using cell-based cytotoxicity assays. Drug discovery from medicinal plants has played an important role in the treatment of cancer [26-28]. Many cancer researches of medicinal plants for chemotherapeutic potential have been used as an alternative treatment for various human cancerous cell lines. The well-known anticancer agents from medicinal plants are for example, paclitaxel from *Taxus brevifolia*, camptothecin from *Camptotheca acuminata,* vinblastine from *Catharanthus roseus* and epipodophyllotoxin from *Podophyllum peltatum* [28, 29]. Therefore, plant natural products are attractive sources of new cancer therapeutic candidate compounds. Although chemotherapeutic agents can treat various types of cancers, anticancer drugs are not suitable for a long term therapy since it can lead to multidrug resistant (MDR) cancer cells which is a major reason for the failure of cancer therapy [30, 31]. MDR cancer does not only resist to a cytotoxic drug used, but also to all of drugs with different structures and cellular targets [32]. Various cellular pathways and molecular mechanisms lead to MDR which represent adaptations to cellular stress and toxic insults. The major mechanisms of MDR are divided into five categories (1) increased drug efflux, (2) decreased drug influx, (3) DNA repair activation, (4) activation of detoxifying systems and (5) blockage of apoptosis [33]. However, the most extensively studied mechanism is the increased drug efflux by the overexpression of the energy-dependent ATP-binding cassette (ABC) drug efflux transporters [5]. Various transport proteins of the ABC superfamily have been characterized, including P-glycoprotein (P-gp), multidrug resistance-associated protein-1

(MRP1), its homologues MRP2-6 and the breast cancer resistance protein (BCRP) [34-37]. They are overexpressed in MDR cancer cells and transport anticancer drugs out of the cell, resulting in reduction of intracellular levels of the anticancer drug necessary for effective therapy. Although these resistant proteins belong to the ABC superfamily, they are quite different with respect to gene locus, amino acid sequence, structure and substrate [38]. P-gp, encoded by the MDR1 gene is the first discovered, the most studied and well characterized multidrug transporter [39]. The overexpression of P-gp reduces the intracellular concentrations of the drug causing the decreased cytotoxicity [40]. Recently, several studies have documented the ability of natural compounds to reverse the MDR cancer by the inhibitory effect on drug efflux transporter, P-gp [41-44]. These compounds are known as MDR-reversing agents, chemosensitizers or modulators with a broad spectrum of chemical structures [45, 46]. The co-administration of P-gp modulators with the chemotherapy drugs should result in higher uptake of the drugs. They may necessitate a reduction in drug dose to prevent toxic adverse effects in cancer chemotherapy treatment [47]. The examples of the protein modulators are such as capsaicin, a pungent component of hot red chili pepper (*Capsicum annuum*), 6-gingerol, a phenolic substance responsible for the spicy taste of ginger (*Zingiber officinale*), resveratrol, a phytoalexin present in grapes (*Vitis vinifera*), sesamin, a lignan existing exclusively and abundantly in sesame (*Sesamum indicum*) seeds, matairesinol in soybean (*Glycine max*), glycyrrhetinic acid and glabridin in licorice (*Glycyrrhiza glabra*) extract, ginsenosides and their hydrolyzed metabolites from the roots of *Panax ginseng* and polyphenol epigallocatechin gallate (EGCG) in tea (*Camellia sinensis*) have been shown for their inhibitory effects on the human P-glycoprotein [48-51].

Besides the P-gp mechanisms, previous researches demonstrated the overexpression of other proteins which are regulated and involved in MDR cancer such as STAT1, HDAC and NF-**K**B. Therefore, the inhibition of these proteins is necessary for the sensitization of MDR cancer.

1. STAT1

Signal transducer and activator of transcription (STAT) or STAT proteins are signaling molecules with dual functions discovered during studies on interferon (IFN)-dependent gene expression. Seven mammalian STAT family members have been molecularly cloned and share common structural elements [52]. They can be activated by phosphorylation through Janus kinase (JAK) or cytokine receptors, G-protein-coupled receptors, or growth factor receptors (such as EGFR); by platelet-derived growth factor receptors that have intrinsic tyrosine kinase activity; or by intracellular non-receptor tyrosine kinase recruitment [53, 54]. STAT is comprised of a family of transcription factors which translocate from the cytoplasm to the nucleus of cells [55]. Its seven members, STAT1, 2, 3, 4, 5A, 5B and 6, influence different signal transduction pathways to influence normal physiological cell processes such as differentiation, proliferation, apoptosis and angiogenesis [56]. High levels of constitutively active STAT1 have been reported to be necessary to support the cell survival in several different types of cancer (Figure 1.1) [57, 58]. In addition, previous studies demonstrated that STAT1 expression could develop drug resistance in cancer cells. For example, the overexpression of STAT1-regulated genes contributes to doxorubicin resistance observed in 8226/Dox40 cells [59, 60]. Hence, targeting this pathway could be a potential therapeutic of MDR cancer.

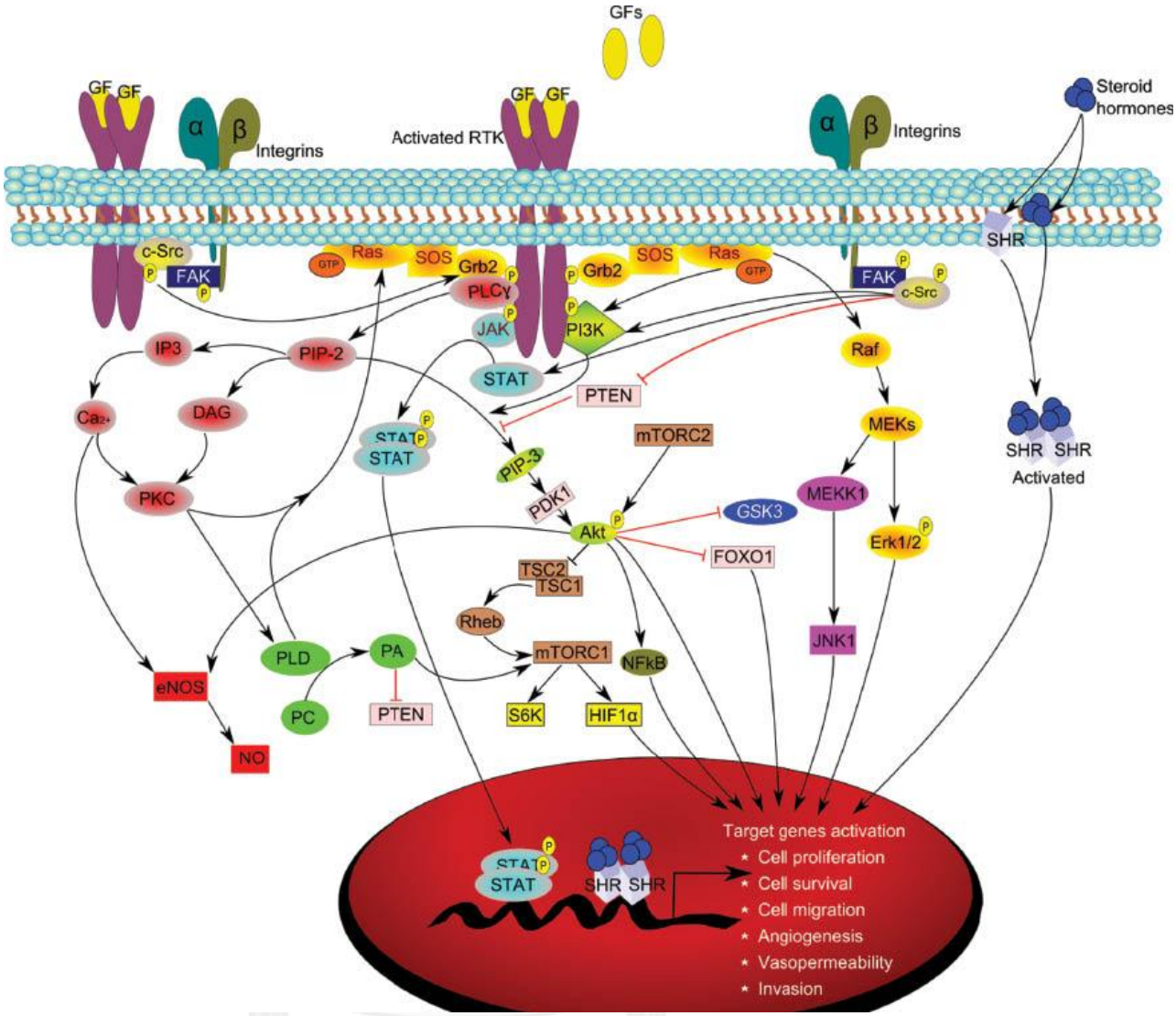

Figure 1.1 The JAK/STAT pathway also plays significant role in cell growth, survival and differentiation. Activated RTK dimers allow phosphorylation of JAK proteins, which will activate STATs to form dimers. These dimers then get translocated into the nucleus and activate transcription of specific genes, related to survival and proliferation (taken from Sheeba *et. al*., 2013) [61].

2. HDAC

Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from an **ε**-N-acetyl lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly (Figure 1.2) [62]. This activity is generally associated with transcriptional repression of tumor suppressors, such as p21 and E-cadherin [63]. Through these mechanisms, HDACs act as critical regulators of cell growth, differentiation and apoptotic programs. The family of HDACs has 18 isoenzymes that can be subclassified into 4 classes. HDAC4, the class-II deacetylase, is a key role in platinum resistant cells that overexpress this HDAC [64]. In Figure 1.3, recent research has been focusing on the potential of HDAC inhibitors (HDACis) as anti-cancer agents in a broad variety of tumors [65, 66]. HDACis have been shown to change the expression pattern of genes involved in differentiation, cell cycle arrest, and apoptosis and thus considered as the candidate for cancer therapy [67]. Since HDAC4 function has also been found in MDR cancer, inhibitors of HDAC4 have gained importance as potential therapeutics.

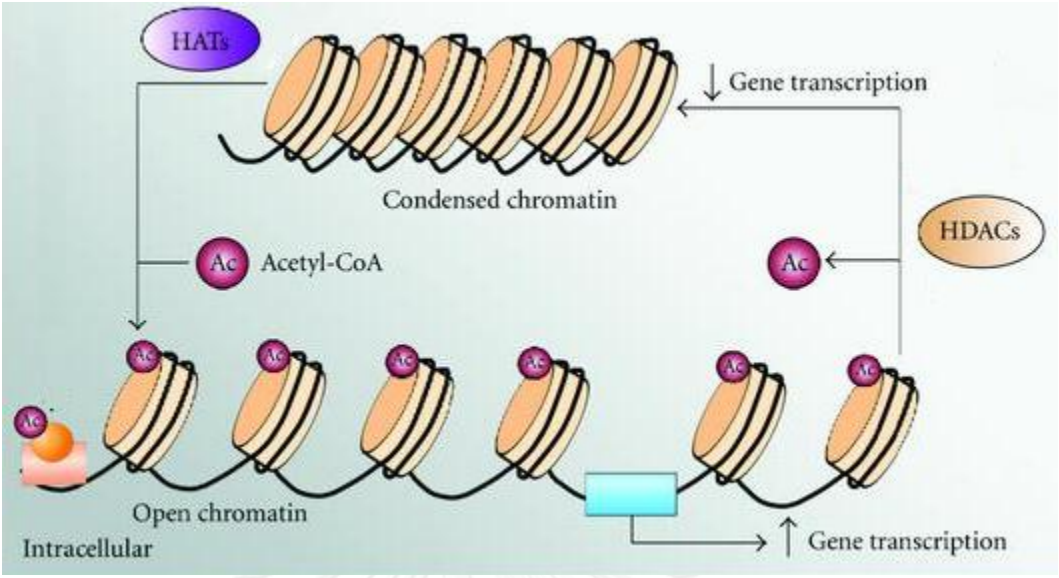

Figure 1.2 The pathways of histone acetylation is regulated by the addition of acetyl-CoA via the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) in lysine resides on core histones. This addition of the acetyl group results in a more open transcriptionally and correlates with transcriptional activity. Removal of acetyl groups by HDACs leads to a condensed, transcriptionally repressive chromatin conformation and correlates with gene silencing. HDACs are also involved in the reversible acetylation of non-histone proteins. Altered HDAC and/or HAT activities are present in many types of cancers. (modified from Royce *et. al*., 2012) [68].

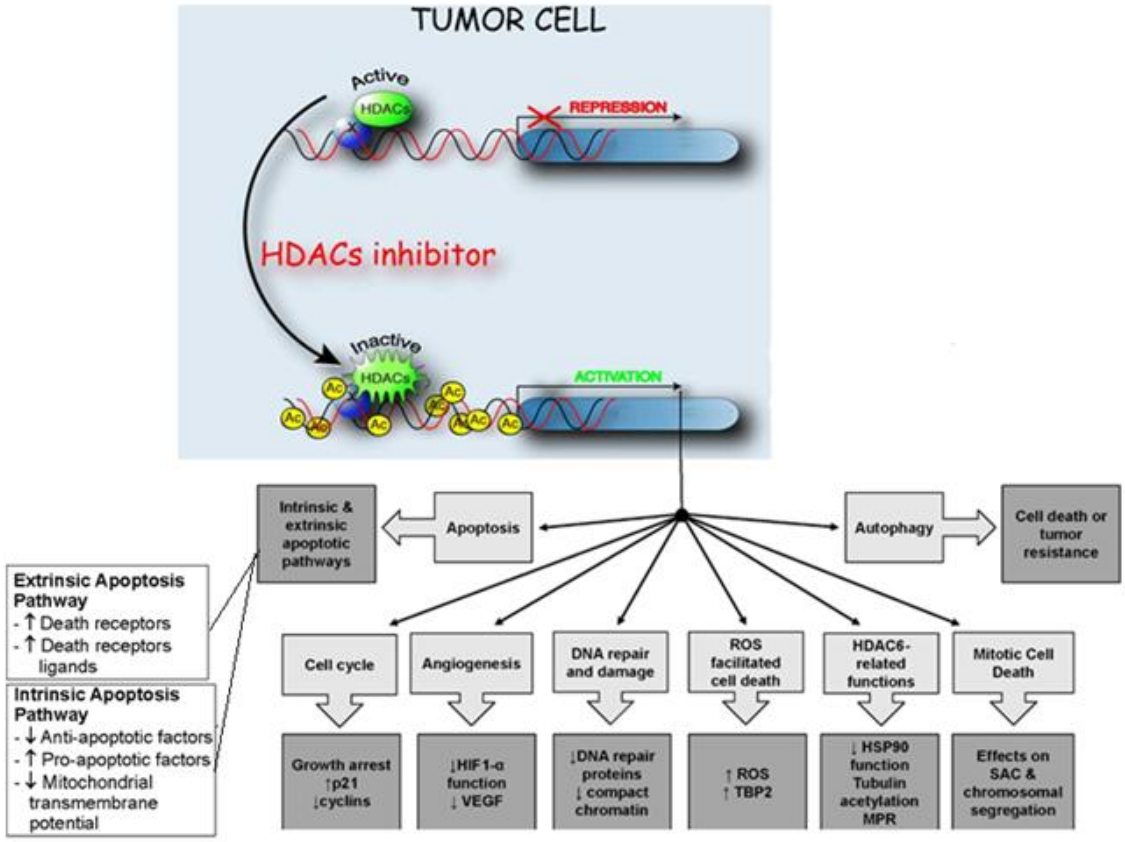

Figure 1.3 Inhibition of HDACs causes accumulation of acetylated forms of these proteins, altering their function. Acetylation of histone provides an important level of epigenetic control on gene expression by altering chromatin activity. HDACs inhibitor (HDACis) induce different phenotypes in various transformed cells, including growth arrest, activation of the extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, reactive oxygen species (ROS)-induced cell death, mitotic cell death and senescence. Consequently HDACis can be targeted as targets for cancer therapy (modified from Kim *et. al*., 2011) [69].

3. NF-**K**B

Nuclear factor **K**-light-chain-enhancer of activated B cells (NF-**K**B), a transcription factor, is key regulators of a variety of genes involving in inflammatory responses, growth, differentiation, apoptosis, and development. In most non-stimulated cells, the five members of the mammalian NF-**K**B family, p65 (RelA), RelB, c-Rel, p50/p105 (NF-**K**B1), and p52/p100 (NF-**K**B2), exist inside the cytoplasm of the cells as homo- or heterodimers. They bound to the NF-**K**B dimers and are maintained by an inhibitor (I**K**B) inside the cytoplasm (Figure 1.4). Following different extracellular stimuli, such as tumor necrosis factor **α** (TNF**α**), cytokines or DNA damaging agents including chemotherapeutic drugs, cause rapid phosphorylation of I**K**B by the IKK kinase complex, polyubiquitination and degradation [70]. Then, the NF-**K**B proteins translocate to the nucleus by nuclear localization signal (NLS) and activate the transcription factor induction of its target genes. These target genes code for cellular survival, growth, differentiation, proinflammatory molecules as well as antiapoptotic proteins (e.g., Bcl-2 and Bcl-xL) [71].

Previous studies suggested that NF-**K**B could participate in resistance to cancer treatment. For example, NF-**K**B regulated antiapoptotic gene expression thus it could inhibit cancer cell death in chemotherapy treatment. The NF-**K**B pathway responds actively to MDR1 induction due to its activation by the generation of reactive oxygen species, the activation of I**K**B kinase and the degradation of I**K**B [72]. Furthermore, NF-**K**B is bound at nucleotide position -6902 of the human MDR1 promoter mediating the transcription of MDR1 leading to MDR cancer [73]. Therefore, the inhibition of NF-**K**B activity can sensitize resistant cancer cells to anticancer drug through a decreased MDR1 expression [74]. Moreover, the transcription factor NF-**K**B suppressing the apoptotic potential of chemotherapeutic drugs also plays an important role in drug resistance of cancer cells [75]. As such, it is believed that inhibitors of NF-**K**B might promote apoptosis in cancer cells and can be helpful to overcome MDR cancer via apoptosis [75-77].

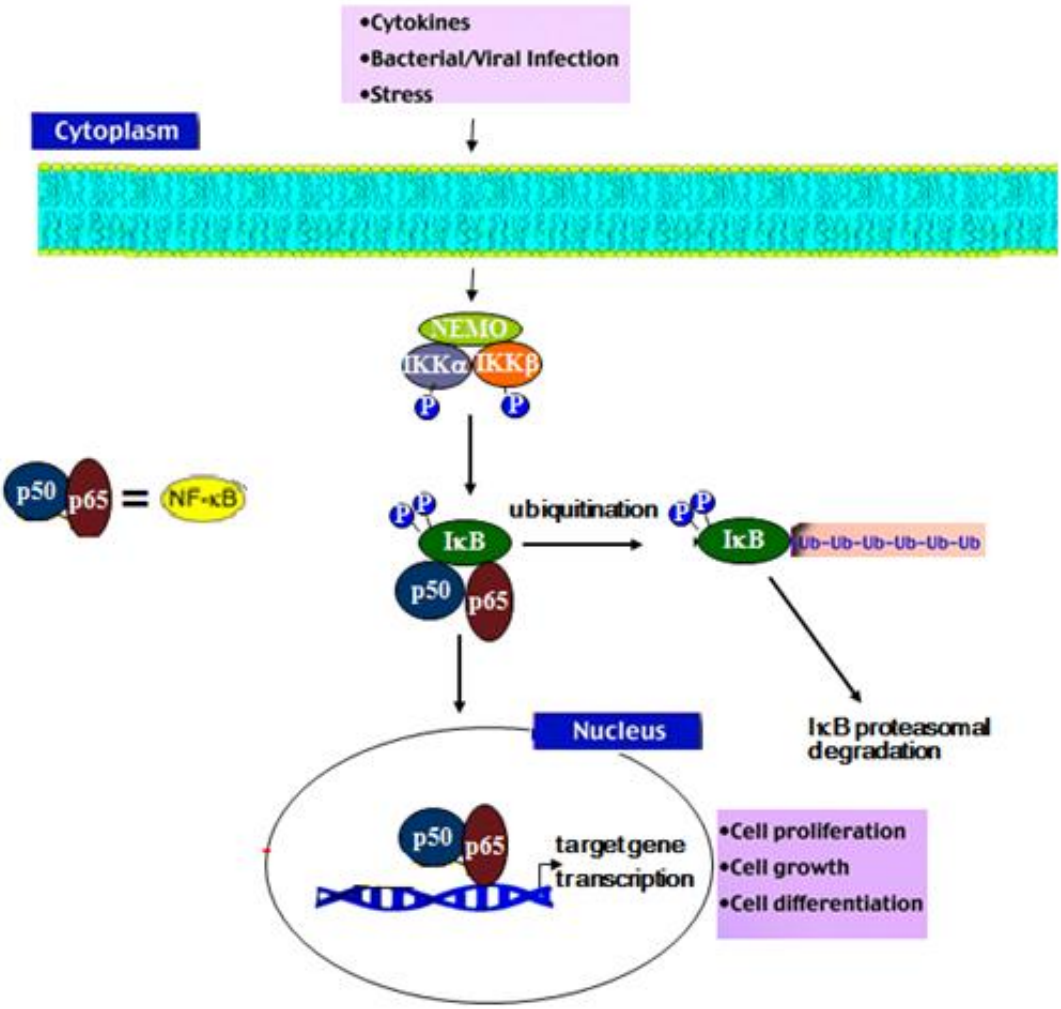

Figure 1.4 Mechanism of NF-**κ**B in most non-stimulated cells, NF-**κ**B dimers are maintained by an inhibitor (I**κ**B) inside the cytoplasm. Following different extracellular stimuli, such as tumor necrosis factor **α** (TNF**α**), cytokines or DNA damaging agents including chemotherapeutic drugs, cause rapid phosphorylation of I**κ**B by the IKK kinase complex, consisting of catalytically active kinases (e.g., IKK**α**, IKK**β**, and IKK**γ**) and non-catalytic regulatory proteins (e.g., NEMO and ELKS), polyubiquitination and degradation [70]. Then, the NF-**κ**B proteins translocate to the nucleus by nuclear localization signal (NLS) and activate the transcription factor induction of its target genes. These target genes code for cellular survival, drugs resistance, growth, differentiation, proinflammatory molecules as well as antiapoptotic proteins (e.g., Bcl-2 and Bcl-xL) (modified from Yamamoto *et. al*., 2001) [77].

Moreover, the other type of mechanism to induce cell-death such as autophagy induction has been reported from previous studies [78, 79]. Autophagy, an ancient system necessary to maintain homeostasis in eukaryotic cells, degrades long-lived cytoplasmic proteins and organelles and provides nutrients during starvation or stress conditions [80] through programmed processing involving the sequential activity of autophagy related gene (ATG) products (Figure 1.5). As autophagy is necessary for cellular homeostasis, it is involved in biological processes including development, aging and degeneration [81]. However, aberrant regulation of autophagy is related to many diseases, including cancer and neurodegenerative disorders [82]. As a specific example, the first report connecting autophagy to cancer showed that allelic loss of the essential autophagy gene *Beclin-1 (BECN1)* is prevalent in human breast, ovarian, and prostate cancers [83], and that *Becn1+/-* mice develop mammary gland hyperplasia and lymphomas as well as lung and liver tumors [84]. Subsequent studies demonstrated that *ATG5-/-* and *ATG7-/-* livers give rise to adenomas [85]. These lines of evidences suggest that autophagy acts as a tumor suppressor in cancer development. On the contrary, many other reports have shown that autophagy exerts a pro-survival function in tumor cells [86-88]. Additional studies have demonstrated that the inhibition of autophagy by pharmaceutical drugs sensitized cells to apoptotic cell death, and that combination therapies using autophagy inhibitors plus chemotherapy led to faster tumor cell death than did chemotherapy alone [89]. These findings indicate that pro-survival autophagy may represent a major hindrance to successful cancer therapy.

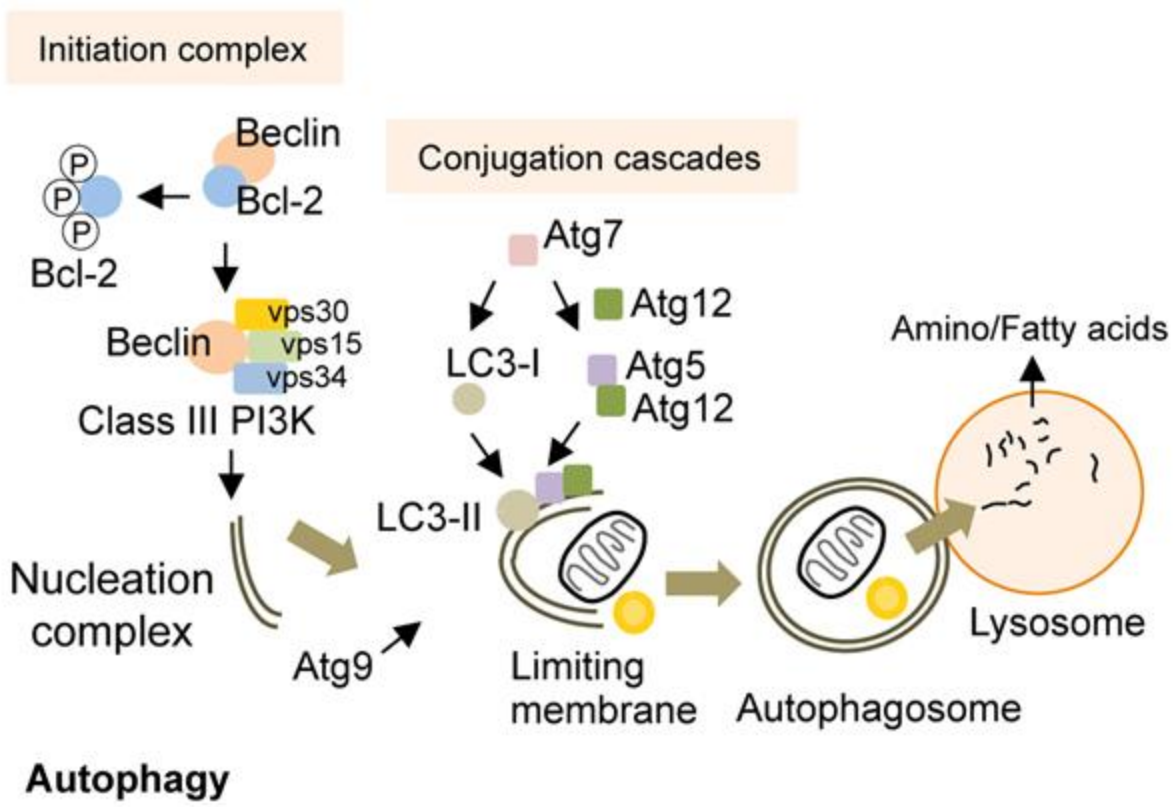

Figure 1.5 The molecular regulators of autophagy induction require the release of Beclin from Bcl-2 contributes to the formation of the nucleation complex. Two independent conjugation cascades, the LC3-II and the ATG5-12 cascades, serve to elongate the nucleation complex to generate the limiting membrane. The sole transmembrane atg, ATG9, delivers additional membranes for limiting membrane formation. The limiting membrane then sequesters cytosolic cargo and seals upon itself to form an autophagosome. The fusion of autophagosomes to lysosomes results in cargo degradation and release of nutrients into the cytosol (taken from Singh *et. al.,* 2011) [90].

1.6 Purposes of the study

Publication 1: The aim of this study was to screen for potential *in vitro* cytotoxic against cancer cells, antioxidant, lipase inhibitory and antimicrobial activities from the crude extracts of 52 species of Thai medicinal plants.

Publication 2: Since we focused on the cytotoxicity of the cancer cells, the aim of this work was to study one of the mechanisms that resist the chemotherapy, namely the overexpression of 2 proteins, STAT1 and HDAC4, in cancer cells resistant to the drug, etoposide called A549RT-eto. We proposed that the inhibition of these proteins was potential therapeutic targets for treatment of A549RT-eto cells, overexpressing P-gp.

Publication 3: The objective was to investigate the efficiency of an extract of *Bryophyllum laetivirens* to induce cytotoxicity in lung cancer resistant to etoposide, A549RT-eto cells. The possible molecular mechanisms, down regulation of NF-**K**B to reverse of MDR cancer leading to apoptosis were also examined.

Publication 4: The objective was to study the 7 purified compounds from 2 types of plants in order to reverse A549RT-eto cells. Possible molecular mechanisms, down regulation of NF-**K**B including autophagy, to reverse the MDR cancer leading to apoptosis were also investigated.

1.7 Scope of study

In the initial work, the 52 Thai medicinal plant extracts were screened for various biological activities, namely *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities. The work focused mainly on *in vitro* cytotoxicity of plant extracts against multidrug resistant (MDR) cancer cell lines. Then, the mechanism of MDR cancer using the etoposide resistance in human lung A549 cancer cells (A549RT-eto) by the overexpressed proteins such as STAT1, HDAC4, NF-**K**B and P-gp comparing with normal lung cancer (A549) cells was studied as a model. Furthermore, the active compounds of potential plant extracts were later isolated and further investigated for the molecular

mechanisms such as inhibition of 3 proteins STAT1, HDAC4 and NF-κB resulting in down regulation of P-gp to reverse MDR cancer leading to cell death.

1.8 Expected advantages

Thai medicinal plant extracts showed the broad spectrum of the biological activities. They can be applied as the guideline for the selection of Thai medicinal plant species for further pharmacological and phytochemical investigations. The bioactive compounds from these plants are important sources to overcome multidrug resistant (MDR) cancer which can lead to the development of anti-cancer therapeutic drug against MDR cancer.

PART II

THE MANUSCRIPTS OF DISSERTATION

Publication 1

Studies of the *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities of selected Thai medicinal plants

Published in: BMC complementary and alternative medicine 2012, 12:217.

Publication 2

Up-regulation of STAT1-HDAC4 confers resistance to etoposide through enhanced multidrug resistance (MDR)1 expression in human A549 lung cancer cells

Accepted in: Molecular medicine reports 2014

Publication 3

Extracts *of Bryophyllum laetivirens* reverse etoposide resistance in human lung A549 cancer cells by down-regulation of NF-κB

Published in: Oncology reports 2014, 31(1):161-168.

Publication 4

Feroniellin A-induced autophagy causes apoptosis in multidrug-resistant human A549 lung cancer cells

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CHAPTER I

STUDIES OF THE *in vitro* CYTOTOXIC, ANTIOXIDANT, LIPASE INHIBITORY AND ANTIMICROBIAL ACTIVITIES OF SELECTED THAI MEDICIANAL PLANTS

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Abstract

Background: Traditional folk medicinal plants have recently become popular and are widely used for primary health care. Since Thailand has a great diversity of indigenous (medicinal) plant species, this research investigated 52 traditionally used species of Thai medicinal plants for their *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities.

Methods: The 55 dried samples, derived from the medicinally used parts of the 52 plant species were sequentially extracted by hexane, dichloromethane, ethanol and water. These 220 extracts were then screened for *in vitro* (i) cytotoxicity against four cell lines, derived from human lung (A549), breast (MDA-MB-231), cervical (KB3-1) and colon (SW480) cancers, using the MTT cytotoxicity assay; (ii) antioxidant activity, analyzed by measuring the scavenging activity of DPPH radicals; (iii) lipase inhibitory activity, determined from the hydrolytic reaction of *p*-nitrophenyllaurate with pancreatic lipase; and (iv) antimicrobial activity against three Gram-positive and two Gram-negative bacteria species plus one strain of yeast using the disc-diffusion method and determination of the minimum inhibitory concentration by the broth micro-dilution assay.

Results: The crude dichloromethane and / or ethanol extracts from four plant species showed an effective *in vitro* cytotoxic activity against the human cancer cell lines that was broadly similar to that of the specific chemotherapy drugs (etoposide, doxorubicin, vinblastine and oxaliplatin). In particular, this is the first report of the strong *in vitro* cytotoxic activity of *Bauhinia strychnifolia* vines. The tested tissue parts of only six plant species (*Allium sativum*, *Cocoloba uvifera, Dolichandrone spathacea, Lumnitzera littorea, Sonneratia alba* and *Sonneratia caseolaris*) showed promising potential antioxidant activity, whereas lipase inhibitory activity was only found in the ethanol extract from *Coscinum fenestratum* and this was weak at 17-fold lower than Orlistat, a known lipase inhibitor. The highest antimicrobial activity was observed in the extracts from *S. alba* and *S. caseolaris* against *Pseudomonas aeruginosa* and *Candida albicans*, respectively.

Conclusion: The Thai medicinal plant *B. strychnifolia* is first reported to exert strong *in vitro* cytotoxic activities against human cancer cell lines and warrants further enrichment and characterization. The broad spectrum of the biological activities from the studied plant extracts can be applied as the guideline for the selection of Thai medicinal plant species for further pharmacological and phytochemical investigations.

Key words; Antimicrobial; Antioxidant; Cytotoxic; Lipase inhibitory; Thai medicinal plants

1.1 Introduction

Recent investigations into plants that have been the basis of traditional medicine for a long time have revealed a significant number of novel metabolites with potent pharmacological properties [10-12]. For example, the chemotherapeutic drug, pacitaxel (Taxol) was initially discovered from the bark of the Himalayan Yew tree by screening for anticancer activity in plant extracts [13]. Various types of plants have been used not only for dietary supplements but also as traditional folk treatments for many health problems [14]. Indeed, the long history of folk medicine demonstrates the potential of plants as sources of bioactive compounds [15]. Traditional medicine is widely used throughout Thailand [18], with both ready-made preparations and herbal drugs being used, and a number of these Thai medicinal plants have provided the foundation for modern pharmaceuticals and drug leads [16]. Thailand has a great diversity of indigenous (medicinal) plant species and hence is a potential source for bioactive compounds, including those with potential antitumor [17, 18], antioxidant [19, 20], anti-lipase (and so potential antiobesity) [17, 21] and antimicrobial activities [22, 23].

Since current pharmacological reagents are restricted by the increasing spread and evolution of resistance and/or their undesired side effects, and are often difficult to synthesize as the pure bioactive stereoisomer, investigations to explore novel drugs, or those that can act as templates for the development of new therapeutic agents appears imperative. Therefore, the aim of this study was to screen for potential *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities from the crude extracts of the folk medicinally used parts of 52 species of Thai medicinal plants.

1.2 Material and Methods

1.2.1 Chemicals and reagents

Ascorbic acid, chloramphenicol, dimethyl sulfoxide (DMSO), doxorubicin, etoposide, *p*-iodonitrotetrazolium violet, *p*-nitrophenyllaurate (*p*-NPL) vinblastine, oxaliplatin, porcine pancreatic lipase (PPL; Type II: from porcine pancreas), 2,2-diphenyl-1-picryhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT) were purchased from Sigma (USA). Amphotericin B was purchased from Bristol-Myers Squibb (France). *Orlistat* (*Xenical*®) was purchased from Roche (Italy). Cell culture media and antibiotics were purchased from Gibco (USA). Fetal bovine serum (FBS) was purchased from Hyclone (USA). All other chemicals were of analytical grade.

1.2.2 Plant materials

Fifty two types of Thai medicinal plants were selected based on traditional medicinal uses. These plants were grown in Pom Phra Chulachomklao Mangrove Forests, Phra Chulachomklao Fort, Samut Prakarn (Table 1.1). The taxonomic identification was done by the members of the Mangrove Forest Restoration and Regeneration Project during 2004 to 2008 from the Metropolitan Electricity Authority, using the available taxonomic key with the aid of the relevant literature (e.g. the ecology of mangrove plants [91] and flora of Thailand). The specimens, collected during August to October 2010, were dried, ground into a fine powder and then extracted as previously described in Mothana *et al*. [92] with minor modification. Twenty grams of the sample powder was sequentially extracted with hexane, dichloromethane (DCM) and ethanol (300 mL each), respectively, by Soxhlet extraction for 8 h. The residues were dried overnight and then extracted with 300 mL water in a shaking water-bath at 60°C for 8 h. The obtained crude hexane, DCM and ethanol extracts were evaporated to dryness on a rotary evaporator while the crude

water extract was freeze dried. The dried samples were then stored at -20 °C until use.

1.2.3 *In vitro* cytotoxic activity assay

The *in vitro* cytotoxic activity of the crude extracts was determined from the mitochondrial activity of cell lines which represent the number of viable cells after the treatment, by using the MTT cytotoxic assay as previously described [93] on four different human cell lines in tissue culture. The non-small cell lung adenocarcinoma (A549) and breast cancer (MDA-MB-231) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cervical (KB3-1) and colon (SW480) cancer cell lines were kindly provided by Professor Gottesman (Laboratory of Cell Biology, National Cancer Institute, National Institute of Health, MD, USA) and Dr. Chanida Vinayanuwattikun (Faculty of Medicine, Chulalongkorn University), respectively.

Cell suspensions in complete medium (CM) (either RPMI (A549 and SW480) or DMEM (MDA-MB-231 and KB3-1), supplemented in both cases with 10% (v/v) FBS, 100 units/mL penicillin and 100 µg/mL streptomycin), were seeded into each well of a 96-well plate (5×10^3 cells per well) and incubated at 37°C with 5% (v/v) CO_2. After 24 h, the crude extracts at five different concentrations in DMSO (two-fold serial dilutions from 100 to 6.25 µg/mL) dissolved in the respective CM were then added into the wells and further incubated for 72 h. Thereafter, the media in the wells were removed and replaced with fresh CM containing 5 mg/mL MTT and incubated at 37°C for 2 h to allow the formation of the insoluble formazan crystal by the mitochondrial active (viable) cells. The media were then removed, 100 µL DMSO was added to lyse the cell membranes and solubilize the formazan crystals and the absorbance was measured at 550 nm using a Biochrom Asys UVM 340 Microplate Reader (Holliston, MA, USA). The percentage of cell survival was calculated from Eq. (1).

$$\% \text{ Cell survival} = (OD_T / OD_C) \times 100 \quad (1)$$

where OD_T and OD_C are the mean absorbance of the treated and the control cells, respectively.

The concentration of the extract which caused a half maximal inhibition of cell proliferation (IC_{50}), as determined by the MTT assay, was obtained from a semilog plot of the crude extract concentrations against the percentage of cell survival. Etoposide (200-0.39 µg/mL), doxorubicin (50-0.1 µg/mL), vinblastine (100-0.2 µg/mL) and oxaliplatin (100-0.2 µg/mL) were used as the specific positive controls for the A549, MDA-MB-231, KB3-1 and SW480 cell lines, respectively.

1.2.4 DPPH radical scavenging (antioxidant activity) assay

The DPPH free radical scavenging assay was used for the evaluation of the antioxidant activity of the crude extracts, as previously described [92]. The dried crude hexane, DCM, ethanol and water extracts were each dissolved to five different concentrations in ethanol (10, 50, 100, 500 and 1000 µg/mL). The reaction mixture, containing 100 µL of the desired extract concentration in ethanol, 25 µL of 1 mM DPPH and 75 µL of ethanol were added into a 96-well plate and incubated at 37°C for 30 min. The absorbance at 517 nm was then monitored from the yellowish solution in a Biochrom Asys UVM 340 Microplate Reader. The DPPH radical scavenging activity was then calculated from Eq. (2).

$$\% \text{ of DPPH radical scavenging activity} = ((A_0-(A_1-A_2))/A_0) \times 100 \quad (2)$$

where A_0, A_1 and A_2 are the absorbance of the DPPH (no crude extract), the crude extract with DPPH and the crude extract without DPPH, respectively.

The concentration which caused a half-maximal reduced DPPH radical level (EC_{50}) was then determined. Extracts with an EC_{50} < 10 µg/mL were considered as having a high level of antioxidant activity [94]. Ascorbic acid (10, 50, 100, 500 and 1000 µg/mL) was used as a positive control.

1.2.5 Determination of the lipase inhibitory activity

The lipase inhibitory activity of the crude extracts was estimated from the ability to inhibit the *in vitro* porcine pancreatic lipase activity as previously described [95] with slight modification. Briefly, the dry crude ethanol and water extracts were dissolved in 50 mM Tris-HCl pH 8.5 containing 50% (v/v) DMSO to a concentration of 50 mg/mL. The assay mixture contained 10 µL of one of five different concentrations (two-fold serial dilutions from 2.5 to 0.156 mg/mL) of the crude extracts, 12 µL of 20 mg/mL of PPL (type II) in 50 mM Tris-HCl pH 8.5 and 10 µL of 5.1 mM *p*-NPL in ethanol. The lipase activity was determined from the hydrolysis of *p*-NPL by measuring the absorbance at 410 nm of the *p*-nitrophenol product formed at 37°C for 5 min using a FLUOstar OPTIMA micro reader (BMG LABTECH, Offenburg, Germany). The lipase inhibitory activity was expressed as the percentage of the decrease in A_{410} when PPL was incubated with the crude extracts compared to the negative control (solvent only), and was calculated from Eq. (3).

$$\% \text{ of enzyme inhibition} = (E\text{-}T/\ E) \times 100 \quad (3)$$

where E and T are the absorbance of the reaction without and with the crude extract, respectively.

The concentration of the extract which caused a half maximal inhibition of the lipase activity (IC_{50}) was obtained from a semilog plot of the crude extract concentrations against the percentage of enzyme inhibition. *Orlistat* (250 - 0.49 µg/mL) was used as the positive control.

1.2.6 Antimicrobial assay

Three Gram-positive (*Bacillus subtilis* (MCCU0351), *Micrococcus luteus* (MCCU0350) and *Staphylococcus aureus* (MCCU0352)) and two Gram-negative (*Escherichia coli* (MCCU0349) and *Pseudomonas aeruginosa* (MCCU0359)) bacterial strains, plus the yeast (*Candida albicans*) were used for the antimicrobial screening.

The antimicrobial activities of the crude ethanol and water extracts were determined using the disc-diffusion and broth micro-dilution assays [92]. For the disc-diffusion assay, the various microbes were spread at 1.5 x 10^7 cells/plate on 9-cm diameter nutrient agar petri dishes. Sterile filter paper discs of 6 mm in diameter (Whatman, cat. NO. 1442125, lot 577125) were impregnated with 2 mg of the crude extract dissolved in 5% (v/v) ethanol, dried and then placed on the surface of the spread agar plates. They were kept for 2 h in a 4°C refrigerator to enable pre-diffusion of the crude extracts and then further incubated at 37°C for 18 h, except for *M. luteus* (room temperature for 48 h) and *C. albicans* (30°C for 48 h) [96]. At the end of the incubation, the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone. An inhibition zone of 10 mm in diameter or more was regarded as representing a high antimicrobial activity. Chloramphenicol (20 µg/disc) and amphotericin B (40 µg/disc) were used as positive controls for the bacteria and yeast, respectively.

The crude extracts that revealed a positive antimicrobial activity in the disc-diffusion assay were then evaluated by the broth micro-dilution assay to determine the minimal inhibitory concentration (MIC) against the microorganisms as previously reported [92], except with minor modifications. Within sterile 96-well plates, two-fold serial dilutions of the selected crude extracts were prepared (in duplicate) in the appropriate broth containing 5% (v/v) DMSO to produce a concentration range of 1,000 to 7.8 µg/mL. The bacterial cell suspension, at 0.5 McFarland standards (approximately 1.5 × 10^8 colony forming units/mL) [97] was added into each well, except for the blank wells which served as extract and media sterility controls.

Control cultures for bacterial growth without the crude extract were also included in each plate. The plates were then incubated at 37°C for 18 h, where after 20 µL of a 0.04% (w/v) *p*-iodonitrotetrazolium violet solution was then added to each well and incubated for 30 min. A change in color from yellow to pink, indicating the reduction of the dye from the bacterial growth, was observed. The MIC of the crude extract was determined from the lowest concentration at which no growth of microorganism, as determined by the color change, was observed. Similarly, two-fold dilutions of chloramphenicol (250-7.81 µg/mL) or amphotericin B (500-15.63 µg/mL) were used as a positive control for the bacteria and yeast, respectively.

1.3 Results

1.3.1 Plant extraction

Table 1.1 lists the ethnobotanical data of the investigated plant species illustrating their botanical names, the part(s) of the plant used for extraction, the traditional folk medicinal uses of these plant tissues, and the yield obtained from each extract (as % by dry weight of the total dry weight) (Table 1.2). A total of 220 extracts, representing 52 plant species (since two tissue parts were used for each of *Bauhinia strychnifolia*, *Moringa oleifera* and *Solanum trilobatum*), were screened for *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities.

Table 1.1 Ethnobotanical data of the investigated Thai plant (page 28-31)

Plant species	Family	Part tested	Traditional uses [98]	Thai name
1. *Acrostichum aureum* Linn.	Pteridaceae	Leaves	Rheumatism	ปรงทะเล
2. *Acanthus ebracteatus* Vahl.	Acanthaceae	Leaves	Rheumatism and snake bites	เหงือกปลาหมอดอกขาว
3. *Acanthus ilicifolius* Linn.	Acanthaceae	Leaves	Diabetes, diuretic, dyspepsia, hepatitis, leprosy, neuralgia, paralysis, ringworms, rheumatism, skin diseases, snake bites and stomach pains	เหงือกปลาหมอดอกม่วง
4. *Allium sativum* Linn.	Alliaceae	Bulbs	Wounds, ulcers, skin infections, flu, athlete's foot, some viruses, strep, worms, respiratory ailments, high blood pressure, blood thinning, cancer of the stomach, colic, colds, kidney problems, bladder problems and ear aches	กระเทียม
5. *Anacardium occidentale* Linn.	Anacardiaceae	Leaves	Antiseptic, antidysenteric, antibacterial, ulcers and astringent	มะม่วงหิมพานต์
6. *Avicennia alba* Bl.	Avicenniaceae	Leaves	Antifertililty, skin disease, tumor and ulcers	แสมขาว
7. *Avicennia officinalis* Linn.	Avicenniaceae	Leaves	Aphrodisiac, diuretic and hepatitis	แสมดำ
8. *Azima sarmentosa* (Blume) Benth.	Salvadoraceae	Leaves	Unknown	พุงดอ
9. *Barleria lupulina* Lindl.	Acanthaceae	Whole plant	Anti-inflammatory for insect bites	เสลดพังพอน
10. *Barringtonia asiatica* (Linn.) Kurz	Lecythidaceae	Leaves	Stomachache and rheumatism	จิกทะเล
11. *Bauhinia strychnifolia* Craib.	Caesalpiniaceae	Leaves Vines	Antipyretic	ย่านางแดง

Plant species	Family	Part tested	Traditional uses [98]	Thai name
12. *Bruguiera sexangula* Poir.	Rhizophoraceae	Leaves	Antitumor	พังกาหัวสุมดอกขาว
13. *Celosia argentea* Linn.	Amaranthaceae	Flower	Antioxidant , inflammation, antivirus and antibacterial	หงอนไก่ไทย
14. *Centella asiatica* Urban.	Umbelliferae	Leaves	Hypertension, diarrhea and urinary tract infections	บัวบก
15. *Ceriops tagal* (Perr.) C.B.Rob	Rhizophoraceae	Leaves	Unknown	โปรงแดง
16. *Clerodendrum inerme* Gaertn.	Verbenacceae	Leaves	Antiseptic, arrests bleeding, asthma, hepatitis, ringworm, stomach pains and uterine stimulant	สำมะงา
17. *Coccoloba uvifera* (L.) Jacq.	Polygonaceae	Leaves	Throat ailments and dysentery	องุ่นทะเล
18. *Colocasia esculenta* (L.) Schott var. aquatilis Hassk.	Araceae	Roots	Colorectal cancer and digestive disorders	บอน
19. *Coscinium fenestratum* (Gaertn.) Colebr.	Menispermaceae	Stem	Ophthalmopathy, wounds, inflammations, ulcers, skin diseases, abdominal, disorders, jaundice, diabetes, tetanus, fever and general debility	แฮ่ม
20. *Derris trifoliata* Lour.	Leguminosae	Leaves	Arrests hemorrhage, antispasmodic and stimulant	ถอบแถบน้ำ
21. *Dolichandrone spathacea* (L.f.) K. Schum.	Bignoniaceae	Leaves	Antitumor and antiseptic	แคทะเล
22. *Eurycoma longifolia* Jack.	Simaroubaceae	Roots	Malaria, aches, persistent fever, dysentery, glandular swelling, bleeding (as a coagulant), edema, hypertension, syphilitic sores and ulcers	ปลาไหลเผือก
23. *Excoecaria agallocha* Linn.	Euphorbiaceae	Leaves	Epilepsy, conjunctivitis, dermatitis, haematuria, leprosy and a purgative	ตาตุ่มทะเล

Plant species	Family	Part tested	Traditional uses [98]	Thai name
24. *Flagellaria indica* Linn.	Flagellariaceae	Leaves	Traditional shampoo	หวายลิง
25. *Ipomoea batatas* Lamk.	Convulvulaceae	Roots	Tonic during pregnancy and to induce lactation	มันเทศ
26. *Kalanchoe pinnata* Pers.	Crassulaceae	Leaves	Treat ailments such as infections, rheumatism and inflammation	ควั่ำตายหงายเป็น
27. *Lumnitzera littorea* Voigt.	Combretaceae	Leaves	Headaches, boils, ulcers and diarrhea	ฝาดดอกแดง
28. *Lumnitzera racemosa* Willd.	Combretaceae	Leaves	Antifertility, asthma, snake bite and diabetes	ฝาดดอกขาว
29. *Mangifera foetida* Lour.	Anacardiaceae	Leaves	Antipyric	มะมุด
30. *Melientha suavis* Pierre.	Opiliaceae	Leaves	Neoplasm, cardiovascular diseases, inflammation, neurodegenerative pathologies, cataracts, diabetes and anti-aging process	ผักหวานป่า
31. *Momordica cochinchinensis* Spreng.	Cucurbitaceae	Seed salve	Relief of dry eyes	ฟักข้าว
32. *Moringa oleifera* Lam.	Moringaceae	Leaves Seeds	Anti-hyperlipidemic,anti-inflammatory and antioxidant	มะรุม
33. *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy	Commelinaceae	Leaves	Chronic bronchitis and cancer	หญ้าปักกิ่ง
34. *Nypa fruticans* Wurmb.	Palmae	Leaves	Asthma, diabetes, leprosy, rheumatism and snake bite	จาก
35. *Orthosiphon grandiflorus* (Blume) Miq. Bold ing.	Labiatae	Whole plant	Treatment of arthritis, gout and rheumatism	หญ้าหนวดแมว
36. *Peperomia pellucida* (L.) Humb; Bonpl & Kunth	Piperaceae	Leave	Treating abdominal pain, abscesses, acne, boils, colic, fatigue, gout, headache, renal disorders and rheumatic joint pain	กระสัง

Plant species	Family	Part tested	Traditional uses [98]	Thai name
37. *Pereskia grandifolia* Haw.	Cactaceae	Leaves	Cancer, high blood pressure, diabetes and diseases associated with rheumatism and inflammation	กุหลาบเมาะลำเลิง
38. *Phyllanthus amarus* Schum&Thonn.	Euphorbiaceae	Leaves	Astringent, cooling, diuretic, stomachic, febrifuge and antiseptic	หญ้าลูกใต้ใบ
39. *Pluchea indica* (Linn.) Less.	Compositae	Leaves	Fever, gangrenous ulcers and rheumatism	ขลู่
40. *Pseuderatherum platiferum* (Nees) Radlk.	Acanthaceae	Leaves	Diarrhea, diabetes and cancer	พญาวานร
41. *Rhizophora apiculata* Blume.	Rhizophoraceae	Leaves	Hepatitis	โกงกางใบเล็ก
42. *Rhizophora mucronata* Poir.	Rhizophoraceae	Leaves	Hepatitis	โกงกางใบใหญ่
43. *Sandoricum koetjape* Burm.f. Merr.	Meliaceae	Bark	Cancer	กระท้อน
44. *Sesuvium portulacastrum* Linn.	Aizoaceae	Leaves	Hepatitis	ผักเบี้ยทะเล
45. *Sonneratia alba* j. Smith.	Sonneratiaceae	Leaves	Poultice in swellings and sprains	ลำพูทะเล
46. *Sonneratia caseolaris* Gaerth.	Sonneratiaceae	Leaves	Cough	ลำแพนทะเล
47. *Solanum torvum* Swartz.	Solanaceae	Leaves	Colds and cough, pimples, skin diseases and leprosy	มะเขือพวง
48. *Solanum trilobatum* Linn.	Solanaceae	Flower, Leaves	Cough	มะแว้งเครือ
49. *Suaeda maritima* (L.) Dumort.	Chenopodiaceae	Leaves	Hepatitis	ชะคราม
50. *Thespesia populnea* (L.) Soland ex Corr.	Malvaceae	Leaves	Inflammation and swollen joints	โพทะเล
51. *Trichosanthes cucumerina* Linn.	Cucurbitaceae	Fruits	Inflammation	บวบขม
52. *Vernonia cinerea* Less.	Asteraceae	Whole plant	Colds and fever, cough dysentery, hepatitis, neurasthenia, furunculosis, snake bites, body ache, dizziness and hypertension	หญ้าดอกขาว

Table 1.2 Ethnobotanical data and percent yield of the investigated Thai plant extracts (page 32-35)

Plant species	Part tested	Extract yield (%)			
		Extracts			
		Hexane	DCM	Ethanol	Water
1. *Acrostichum aureum* Linn.	Leaves	2.2	5.6	19.5	9.2
2. *Acanthus ebracteatus* Vahl.	Leaves	2.6	1.6	19.3	5.6
3. *Acanthus ilicifolius* Linn.	Leaves	0.3	0.7	3.6	7.5
4. *Allium sativum* Linn.	Bulbs	9.7	10.0	9.7	9.7
5. *Anacardium occidentale* Linn.	Leaves	1.8	0.6	1.8	3.4
6. *Avicennia alba* Bl.	Leaves	1.4	4.3	12.6	7.4
7. *Avicennia officinalis* Linn.	Leaves	5.5	3.4	38.2	18.2
8. *Azima sarmentosa* (Blume) Benth.	Leaves	0.4	1.8	6.3	8.4
9. *Barleria lupulina* Lindl.	Whole plant	3.5	2.2	3.2	6.4
10. *Barringtonia asiatica* (Linn.) Kurz	Leaves	9.6	2.1	20.4	10.3
11. *Bauhinia strychnifolia* Craib.	Leaves	2.6	1.1	12.1	3.4
	Vines	1.1	1.8	13.5	2.6
12. *Bruguiera sexangula* Poir.	Leaves	3.1	4.6	18.4	8.6
13. *Celosia argentea* Linn.	Flower	1.5	1.1	6.1	25.9

Plant species	Part tested	Extract yield (%)			
		Extracts			
		Hexane	DCM	Ethanol	Water
14. *Centella asiatica* Urban.	Leaves	9.2	9.9	6.9	9.7
15. *Ceriops tagal* (Perr.) C.B.Rob	Leaves	1.4	3.3	6.4	10.2
16. *Clerodendrum inerme* Gaertn.	Leaves	1.8	4.2	12.6	9.2
17. *Coccoloba uvifera* (L.) Jacq.	Leaves	0.4	1.3	5.6	4.2
18. *Colocasia esculenta* (L.) Schott var. aquatilis Hassk.	Roots	1.4	4.6	3.6	8.3
19. *Coscinium fenestratum* (Gaertn.) Colebr.	Stem	0.5	1.1	1.0	1.0
20. *Derris trifoliata* Lour.	Leaves	0.3	2.2	10.2	7.4
21. *Dolichandrone spathacea* (L.f.) K. Schum.	Leaves	3.1	6.9	24.9	7.7
22. *Eurycoma longifolia* Jack.	Roots	0.0	0.5	0.3	1.9
23. *Excoecaria agallocha* Linn.	Leaves	1.1	0.3	7.3	3.7
24. *Flagellaria indica* Linn.	Leaves	1.3	2.7	30.8	10.7
25. *Ipomoea batatas* Lamk.	Roots	1.2	3.4	5.1	10.4
26. *Kalanchoe pinnata* Pers.	Leaves	1.2	1.9	20.1	9.6

Plant species	Part tested	Extract yield (%)			
		Extracts			
		Hexane	DCM	Ethanol	Water
27. *Lumnitzera littorea* Voigt.	Leaves	2.6	3.6	10.2	25.4
28. *Lumnitzera racemosa* Willd.	Leaves	4.5	3.6	2.6	18.1
29. *Mangifera foetida* Lour.	Leaves	8.8	3.4	30.6	18.4
30. *Melientha suavis* Pierre.	Leaves	2.8	2.7	7.7	9.9
31. *Momordica cochinchinensis* Spreng.	Seed salve	ND	ND	31.1	3.7
32. *Moringa oleifera* Lam.	Leaves	2.0	0.7	2.0	8.5
33. *Murdania iroiformis* (Hassk.) Rolla Rao et Kammathy	Seeds	16.4	0.3	0.9	3.4
	Leaves	9.7	9.9	9.2	9.4
34. *Nypa fruticans* Wurmb.	Leaves	2.9	2.1	5.8	10.4
35. *Orthosiphon grandiflorus* (Blume) Miq. Bolding.	Whole plant	4.0	1.1	0.8	4.8
36. *Peperomia pellucida* (L.) Humb; Bonpl & Kunth	Leaves	4.5	2.3	8.9	12.9
37. *Pereskia grandifolia* Haw.	Leaves	2.4	1.3	30.3	12.4
38. *Phyllanthus amarus* Schum&Thonn.	Leaves	1.4	2.2	10.6	12.4
39. *Pluchea indica* (Linn.) Less.	Leaves	0.2	0.2	5.8	6.5
40. *Pseuderatherum platiferum* (Nees) Radlk.	Leaves	4.5	3.4	18.6	9.3
41. *Rhizophora apiculata* Blume.	Leaves	3.2	2.1	12.4	7.9
42. *Rhizophora mucronata* Poir.	Leaves	3.4	2.7	16.3	12.1

Plant species	Part tested	Extract yield (%)			
		Extracts			
		Hexane	DCM	Ethanol	Water
43. *Sandoricum koetjape* Burm.f. Merr.	Bark	2.6	4.3	15.1	23.3
44. *Sesuvium portulacastrum* Linn.	Leaves	0.3	0.3	4.3	10.1
45. *Sonneratia alba* j. Smith.	Leaves	4.1	2.6	15.4	7.3
46. *Sonneratia caseolaris* Gaerth.	Leaves	3.2	2.9	11.6	8.2
47. *Solanum torvum* Swartz.	Leaves	2.5	0.5	13.0	8.7
48. *Solanum trilobatum* Linn.	Flower	2.5	1.1	2.1	5.8
	Leaves	5.3	2.3	2.9	3.3
49. *Suaeda maritima* (L.) Dumort.	Leaves	1.3	0.8	10.5	4.2
50. *Thespesia populnea* (L.) Soland ex Corr.	Leaves	4.7	5.4	30.1	24.3
51. *Trichosanthes cucumerina* Linn.	Fruits	1.6	0.8	0.6	1.9
52. *Vernonia cinerea* Less.	Whole plant	0.2	0.3	0.5	11.1

1.3.2 *In vitro* cytotoxic activity

From table 1.3, the extracts from only four species (*B. strychnifolia* vines, *Coscinium fenestratum* stems, *Eurycoma longifolia* roots and *Kalanchoe pinnata* leaves) displayed potential *in vitro* cytotoxicity against the four human cancer cell lines tested. In terms of the deduced IC_{50} values, the crude water extracts had only a weak (> 20 µg/mL) to essentially no activity in all cases, but for the other less polar extracts they varied between the cell lines, plant species and solvents used in the extraction. Nevertheless, typically the crude DCM and ethanol extracts were more active suggesting the bioactive components have a moderate to low polarity. Overall, the vine extracts from *B. strychnifolia* were the most effective against three of the four human cancer cell lines. However, it was still effective against the fourth (SW480) cell line. The highest cytotoxic activity (IC_{50} of 0.28 µg/mL) was obtained against the MDA-MB-231 cell line by the crude hexane extract of *B. strychnifolia* vines, with the crude DCM extract being strongly cytotoxic against the A549 and KB3-1 cell lines (IC_{50} value of 1.16 and 1.86 µg/mL, respectively). In fact, this is the first report of the *in vitro* cytotoxic activity of this interesting plant against human transformed cell lines. For the remaining three plant species with positive *in vitro* cytotoxicity, the crude DCM and ethanol extracts of *K. pinnata* leaves showed a better overall *in vitro* cytotoxicity than the other two plant species, although these *K. pinnata* leaf extracts were not that effective against the A549 cell line. The crude DCM and ethanol extracts from *C. fenestratum* stems were effective against the KB3-1 cell line (IC_{50} values of 3.25 and 2.18 µg/mL, respectively). Although the extracts from *E. longifolia* roots were in general the least effective of the four positive plant species, its DCM and ethanol extracts were strongly cytotoxic against the MDA-MB-231 cell line (IC_{50} values of 1.6 and 1.2 µg/mL, respectively).

Table 1.3 *In vitro* cytotoxic activity of crude extracts against human cancer cell lines

Plant species	Cell lines															
	A549				MDA-MB-231				KB3-1				SW480			
	IC_{50} (µg/mL)[a]				IC_{50} (µg/mL)[a]				IC_{50} (µg/mL)[a]				IC_{50} (µg/mL)[a]			
	Hexane	DCM	Ethanol	Water	Hexane	DCM	Ethanol	Water	Hexane	DCM	Ethanol	Water	Hexane	DCM	Ethanol	Water
B. strychnifolia.(Vine)	5.95 ± 0.10	**1.16 ± 0.12**	16.62 ± 1.11	> 100	**0.28 ± 0.01**	14.94 ± 3.27	> 100	> 100	1.63 ± 0.21	**1.86 ± 0.15**	> 100	68.50 ± 3.00	7.25 ± 1.23	41.25 ± 4.53	19.00 ± 3.53	> 100
C. fenestratum	> 100	42.50 ± 10.11	> 100	> 100	> 100	15.13 ± 7.19	40.00 ± 7.29	> 100	> 100	**3.25 ± 0.24**	**5.15 ± 1.2**	> 100	> 100	17.45 ± 4.37	> 100	> 100
E. longifolia	80.00 ± 17.90	16.00 ± 1.30	5.50 ± 1.10	50.00 ± 8.60	10.40 ± 0.10	**1.60 ± 0.40**	**1.20 ± 0.20**	24.30 ± 6.50	35.14 ± 10.50	13.12 ± 1.58	15.78 ± 2.03	45.65 ± 11.50	52.14 ± 10.54	32.14 ± 15.58	21.15 ± 7.25	> 100
K. pinnata	35.00 ± 5.36	18.33 ± 4.15	15.00 ± 6.32	34.17 ± 7.66	87.50 ± 12.58	4.32 ± 0.31	3.18 ± 0.04	> 100	7.53 ± 1.04	**2.18 ± 0.23**	**1.03 ± 0.07**	> 100	44.06 ± 4.22	**5.23 ± 0.98**	**6.12 ± 2.14**	> 100
Positive control	Etoposide = 0.95 ± 0.06 µg/mL				Doxorubicin = 0.48 ± 0.02 µg/mL				Vinblastine = 1.13 ± 0.10 µg/mL				Oxaliplatin = 3.56 ± 0.62 µg/mL			

[a]Data are shown as the mean ± 1 SD and are derived from three independent determinations. Figures in bold font represent the highest activity/activities against that cell line and are referred to in the text.

1.3.3 Antioxidant and lipase inhibitory activities

With respect to the antioxidant activity, an EC_{50} value of less than 10 μg/mL in the DPPH radical scavenging assay is generally considered to be effective in this work. However, the positive control of ascorbic acid under these assay conditions was outside this limit (EC_{50} = 12 ± 1.29 μg/mL). Of the 220 plant extracts, only some of the crude water and ethanol extracts, but not the hexane and DCM extracts, from six plant species (see below) revealed potential DPPH radical scavenging activity. Therefore, it can be speculated that the principal bioactive components are relatively polar compounds and different to those with cytotoxic activities. Of the 110 crude ethanol and water extracts evaluated from the 52 plant species, extracts from only six plant species were found to have an effective DPPH radical scavenging activity, ranging in the order (lowest to highest EC_{50} value) of *Sonneratia caseolaris* leaves, *Coccoloba uvifera* leaves, *Sonneratia alba* leaves, *Lumnitzera littorea* leaves, *Allium sativum* bulbs and *Dolichandrone spathacea* leaves (EC_{50} values of 1.92 ± 0.38, 3.08 ± 1.01, 3.27 ± 0.53, 4.00 ± 0.25, 4.23 ± 0.67 and 5.17 ± 0.29 μg/mL, respectively). With respect to the crude water extracts, three (*S. alba*, *S. caseolaris* and *L. littorea*) of the six plant species with effective ethanol extracts also had effective DPPH radical scavenging activity (EC_{50} values of 6.43 ± 2.29, 7.25 ± 1.52 and 7.27 ± 0.64 μg/mL, respectively), but no other extracts were effective. Although the water extracts were less effective than the corresponding ethanolic ones, it should be born in mind that these are derived from sequential (ethanol before water), potentially non-exhaustive extractions that will vary in their compositions and so it cannot be inferred that they represent different component(s) or specific activities. With respect to the lipase inhibition, the crude ethanol extract from *C. fenestratum* was the only one found to display effective activity. Nevertheless, this was weak (IC_{50} value of 160 ± 0.02 μg/mL), compared to that for the positive control of Orlistat (IC_{50} value of 9.25 ± 1.25 μg/mL).

1.3.4 Antimicrobial activity

Using the disc-diffusion assay and taking an inhibition zone of ≥10 mm diameter as the indication of a strong antimicrobial activity [99], from the 110 crude ethanol and water extracts screened from 52 plant species, those from only four plant species were found to exhibit a strong antimicrobial activity (Table 1.4). The ethanol extracts of *C. fenestratum* stems showed the broadest range of activity, inhibiting all of the three tested Gram-positive bacteria (*S. aureus, B. subtilis* and *M. luteus*), and one (*E. coli*) of the two Gram-negative bacteria as well as the yeast strain (*C. albicans*). However, the crude ethanol extract from *Anacardium occidentale* leaves and *S. caseolaris* leaves were also effective against *E. coli*. For the aqueous extracts, that from *S. alba* leaves was found to inhibit on (*M. luteus*) of the three tested Gram-positive bacteria, both tested Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and the yeast strain (*C. albicans*), whilst that from *S. caseolaris* leaves could inhibit *E. coli* and *C. albicans*. The extracts from *A. occidentale* leaves, therefore, appeared to potentially be specific for Gram-negative bacteria.

To obtain the MIC of the crude extracts, the broth micro-dilution assay was utilized to screen those crude water and ethanol extracts that were positive by the disc-diffusion assay. The results (Table 1.4) showed that the crude ethanol extract of *C. fenestratum* stems inhibited the three tested Gram-positive and one (*E. coli*) of the two tested Gram-negative bacteria strains plus *C. albicans* equally and effectively (MIC values of 500 µg/mL), whilst the only other extract to be active against Gram-positive bacteria (water extract of *S. alba* on *M. luteus*) also had a MIC value of 500 µg/mL. However, a lower MIC was obtained against the Gram-negative bacteria *P. aeruginosa* and the yeast *C. albicans* from the aqueous extracts of *S. alba* and *S. caseolaris* (125 µg/mL). These MIC values are typically higher than those for the positive control of chloramphenicol for bacteria (MIC of ~8 to 32 µg/mL, except for *P. aeruginosa* at 125 µg/mL) but broadly comparable to that for amphotericin B for the yeast (MIC of 250 µg/mL). However, these crude extracts are likely to contain many non-bioactive compounds (in mass) and so the actual specific activities after enrichment would potentially be higher.

Table 1.4 *In vitro* antimicrobial activity and minimal inhibitory concentrations (MIC) of the investigated crude extracts

Plant species / Antimicrobial agent	Gram-positive bacteria												Gram-negative bacteria								Fungal strain			
	Staphylococcus aureus				*Bacillus subtilis*				*Micrococcus luteus*				*Escherichia coli*				*Pseudomonas aeruginosa*				*Candida albicans*			
	Inhibition zone* (mm)		MIC** (µg/mL)		Inhibition zone* (mm)		MIC** (µg/mL)		Inhibition zone* (mm)		MIC** (µg/mL)		Inhibition zone* (mm)		MIC** (µg/mL)		Inhibition zone* (mm)		MIC** (µg/mL)		Inhibition zone* (mm)		MIC** (µg/mL)	
	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water	EtOH	Water
A. occidentale	–	–	–	–	–	–	–	–	–	–	–	–	15	–	250	–	13	–	500	–	–	–	–	–
C. fenestratum	10	–	500	–	15	–	500	–	13	–	500	–	10	–	500	–	–	–	–	–	10	–	500	–
S. alba	–	–	–	–	–	–	–	–	–	13	–	500	–	10	–	250	–	10	–	125	–	10	–	250
S. caseolaris	–	–	–	–	–	–	–	–	–	–	–	–	15	13	500	250	–	–	–	–	–	15	–	125
Chloramphenicol (20 µg/disc)	30		7.81		30		31.3		24		31.3		30		31.3		15		125					
Amphotericin B (40 µg/disc)																					17		250	

* 2 mg of crude extract was used in each the disc-diffusion assay.

** 1,000-7.8 µg/mL of samples were used in the broth micro-dilution assay to determine the minimal inhibitory concentration (MIC).

1.4 Discussion

1.4.1 *In vitro* cytotoxic activity

According to the United States National Cancer Institute plant screening program, a plant extract is generally considered to have an active cytotoxicity effect if the IC_{50} value following incubation between 48 to 72 h, is 20 µg/mL or less [100]. The relevant tissue(s) (in terms of the tissue(s) used in folk medicine) from 52 species of medicinal plants in Thailand were screened as their crude hexane, DCM, ethanol and water extracts for *in vitro* cytotoxic activity against four human transformed (cancer) cell lines. The highest cytotoxic activity was obtained from the crude hexane extract obtained from the vines (but not leaves) of *B. strychnifolia* against the MDA-MB-231 cell line. Moreover, this extract also had potential strong activity against the KB3-1 cell line, whilst the crude DCM extract exhibited cytotoxic activity against the A549 and KB3-1 cell lines. From the results, the derived IC_{50} values for the crude hexane and DCM extracts from the vines of *B. strychnifolia* are not that different on each cell line from the respective positive controls (etoposide, doxorubicin, vinblastine and oxaliplatin). Assuming no strong synergy among all components in the extract, it can be implied that the bioactive component(s) in these fractions could be far more potent than the standard reference drugs and so merits their enrichment and further characterization.

Clinically, *B. strychnifolia* has been applied for the treatment of human food poisoning diarrhea [101] and also in 2011 as an anti-HIV-1 agent [102]. Nevertheless, the antitumor activity has never been reported. As a consequence, this work appears to be the first report on the antitumor activity of *B. strychnifolia*.

The crude DCM extract of *C. fenestratum* showed *in vitro* cytotoxicity against the KB3-1 cell line, which is consistent with the reported antiproliferative activity against the human colorectal carcinoma (HCT-116) cell line [103], where it apparently

induces expression of the peroxisome proliferator-activated receptor γ and pro-apoptotic genes.

The crude DCM and ethanol extracts of *E. longifolia* roots exhibited cytotoxic activity against the MDA-MB-231 cancer cell line, which has been reported previously on the human breast cancer cell line MCF-7 where the inhibition was linked to the induction of apoptotic cell death [104, 105]. In addition, from nearly 65 compounds isolated from the roots of *E. longifolia*, eight were found to demonstrate strong cytotoxicity towards the human lung cancer (A549) cell line and some of these were also strongly cytotoxic against the MCF-7 cell line [106]. Thus, it appears that the crude alcohol extracts from *E. longifolia* roots may exhibit a preferential or specific cytotoxicity against breast cancer.

The bufadienolide isolated from the methanol extract of *K. pinnata* has been reported to be a potential cancer chemotherapeutic agent since it inhibits the tumor promoting activity of Epstein-Barr Virus [107]. Here, we found that the crude ethanol extract from the leaves of this plant inhibited the growth of the KB3-1 cell line, which was developed from human papilloma virus infected cells. Hence, it is possible that the ethanol extracts affect the regulation of some viral proteins that control cell division.

Overall, the crude hexane and DCM extracts were more active than the corresponding aqueous and ethanol ones, suggesting that the active compound(s) against the cell lines in these plants are of low polarity. In addition, as mentioned before, since these are crude extracts and may contain many non-active components then the IC_{50} values reported here may in fact be far higher than those of the actual bioactive component(s) in the extracts, assuming no strong synergy between different components. Therefore, these results support that the bioactivity-guided enrichment of these fractions is merited.

1.4.2 Antioxidant and lipase inhibitory activity

With respect to the antioxidant activity, the crude ethanol and water extracts from only six of the 52 investigated plants showed any effective free radical scavenging activity in the DPPH assay when compared to that of the ascorbic acid reference standard. The hexane and DCM extracts did not exert any detectable antioxidant activity in this study. This result is similar to the study on Fenugreek seeds (*Trigonella foenumgraecum*) which showed that the highest antioxidant activity was found in the ethanol and methanol extracts followed by the aqueous extract with only low activities in the hexane and DCM extracts [108]. Moreover, most of the 52 plants investigated in this study naturally occur (and were obtained from) within mangrove forest areas, which are typically rich sources of phenolic compounds, such as flavonoids [98], to protect the plants from UV radiation [109, 110]. As a matter of fact, a linear relationship between the flavonoid content and the antioxidant activity has already been reported [111]. With respect to the lipase inhibitory activity, only the crude ethanol and water extracts were evaluated. The low polarity compounds including the natural lipids in the tissue(s) could be extracted by hexane and DCM at sufficiently high concentrations as to interfere in the assay by acting as alternative but unquantified substrates for the lipase providing false positives and potentially masking genuine weak and moderate positives. However, all but one of the 110 water and ethanol extracts were found to be inactive. The exception was the ethanol extract from *C. fenestratum* stems that exhibited a weak lipase inhibitory activity (IC_{50} value of 160 µg/mL) that had a 17.3-fold lower IC_{50} value than that for Orlistat, a known lipase inhibitor. Nevertheless, it is a crude extract, and so the actual IC_{50} of the active component(s) may be significantly higher. The lipase inhibitory activity from this plant (the first report) gives a suggestion of the potential to screen for novel plant compounds with anti-lipase activity. These may be of clinical dietary use in countering the problems of human obesity. Thus, it may be interesting for further studies to fractionate the crude hexane and DCM extracts to remove the natural lipid content and screen the other fractions for anti-lipase activity.

1.4.3 Antimicrobial activity

Unfortunately, the crude hexane and DCM extracts, although soluble in DMSO were not soluble in the 5% (v/v) DMSO-nutrient broth media used for the broth micro-dilution assay, where higher DMSO levels are themselves inhibitory in the assay. Therefore, only the 110 crude ethanol and water extracts were screened for antimicrobial activity. Significant antimicrobial activities (inhibition zones ≥ 10 mm) were obtained from the extracts of only four plant species, with MIC values of ≤ 500 µg/mL (as determined by subsequent broth assays).

Of the four plants with antimicrobial activity, the crude ethanol extract of *C. fenestratum* stems displayed a broad range of antimicrobial effects against all tested microorganisms except *P. aeruginosa*. Moreover, it has been previously reported to inhibit the growth of *Propionibacterium acnes* and *Staphylococcus epidermidis* [112], whereas the methanol extract of this plant was reported to inhibit the growth of *S. aureus, B. subtilis* and *E. coli* [113].

The aqueous extract from the leaves of *S. alba* displayed an antimicrobial activity against both tested Gram-negative bacteria (*E. coli* and *P. aeruginosa*), one (*M. luteus*) of the three tested Gram-positive bacteria (*M. luteus*), and the yeast *C. albicans*. In agreement with this is that both the methanol and ethyl acetate extracts have been reported to have antimicrobial activity against Gram-positive (*Bacillus cereus* and *S. aureus*) and Gram-negative (*E. coli*) bacteria [114], but the inhibition activity against *P. aeruginosa* and *C. albicans* is newly reported here.

Although the results of this work are consistent with those reported by Tao et al. [115] in that the ethanol extract of *S. caseolaris* had no antimicrobial activity against *B. subtilis, S. aureus*, *P. aeruginosa* and *E. coli*, our ethanol extract did inhibit the growth of *E. coli*. In addition, we found that the crude water extract of *S. caseolaris* leaves could inhibit the growth of *C. albicans*, which is consistent with the report that not only the ethanol leaf extract but also the methanol extract of the

cork of this plant could inhibit *C. albicans* [116]. Thus, these two species from the family *Sonneratiaceae* merit further investigation for antimicrobial agents. Finally, the ethanol extract from the leaves of *A. occidentale* demonstrated antimicrobial activity against *E. coli* and *P. aeruginosa*, which is similar to that previously reported [117-119], but is in contrast with the previous report that the ethanol extract of this plant's leaves also inhibited *M. luteus*, *S. aureus* and *B. subtilis* [117, 118], which was not observed in the present study.

Overall, the ethanol and aqueous extracts exhibited potential antimicrobial activity and merit further bioactivity guided fractionation to obtain the bioactive component(s). However, since the crude hexane and DCM extracts could not be screened, their initial fractionation and assay for antimicrobial activity may be of value, with further bioactivity guided fractionation as required.

1.5 Conclusion

The *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities of 52 species of Thai medicinal plants were studied using the tissue source(s) that are used in traditional folk medicine. The crude hexane extract from the vines of *B. strychnifolia* was found to exert a strong *in vitro* cytotoxic activity against human cancer cell lines and certainly merits further enrichment to isolate the potentially promising bioactive component(s). This work provides the basic data base of selected Thai medicinal plants species that can be used to identify potential novel bioactive compounds for pharmacological and phytochemical investigations. However, the other parts of each plant may not be used in folk medicine for reasons other than a lack of bioactive compound(s), such as digestibility, taste or presence of other deleterious components, and so the other tissues of positive plants should be screened. Regardless, the simple approach of using the knowledge of folk medicine as a first guide to select plants for pharmacological screening appears validated, although it should be extended to include not only the other tissue parts of positive plants, but also close phylogenetic relatives and cultivars.

CHAPTER II

UP-REGULATION OF STAT1-HDAC4 CONFERS RESISTANCE TO ETOPOSIDE THROUGH ENHANCED MULTIDRUG RESISTANCE (MDR)1 EXPRESSION IN HUMAN A549 LUNG CANCER CELLS

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Abstract

In spite of many efforts to develop an efficient chemotherapeutic drug for cancer treatment, we have often faced a hurdle to overcome because of cancer cells acquiring the drug resistance. We initiated to investigate how cancer cells acquire resistance to an anti-cancer drug. We used human A549 lung cancer cells, resistant to etoposide that acts as a topoisomerase inhibitor (A549RT-eto) and examined features of the cell compared to A549 parental cells. A549 parental cells showed more sensitive cell death than A549RT-eto cells in dose-dependent manner as expected whereas A549RT-eto cells showed more resistance to etoposide–induced apoptosis compared to A549 cells. Of interest, we found that A549RT-eto cells exhibited higher levels of HDAC4, phospho-STAT1, and P-glycoprotein (P-gp) encoded in *MDR1*, compared to A549 cells. To address whether HDAC4 proteins are involved in etoposide resistance in A549 cells, we administered A549RT-eto cells with trichostatin A, a HDAC inhibitor during etoposide treatment. We then found that the combined treatment more sensitizes etoposide-induced apoptosis and notified down-regulation of HDAC4 and P-gp expression levels, and lower levels of phospho-STAT1. In addition, suppression of STAT1 with siRNA enhanced etoposide-induced apoptosis and reduced expression of HDAC4 and P-gp, suggesting that STAT1 plays a critical role in resistance to etoposide and up-regulation of P-gp. Taken together, we suggest that up-regulation of both STAT1 and HDAC4 determines etoposide resistance through P-gp expression in human A549 lung cancer cells.

Key Words; STAT1; HDAC4; P-glycoprotein; Etoposide

2.1 Introduction

Although STAT1 is well known as a master transcription factor for IFN-related intracellular signaling, leading to anti-viral activity, several lines of evidence shown that STAT1 plays a role as an anti-oncogenesis in part by up-regulation of caspases [120, 121] , cyclin-dependent kinase inhibitor 1A [122], the IFN-regulatory Factor 1 (IRF1)/p53 pathway [123], and down-regulation of the BCL2 family [124] have been accumulated. In contrast, emerging data reveal that in certain cellular contexts, the IFN/STAT1 pathway may facilitate tumor cell growth. One study has reported that resistance to ionization radiation and IFNs is associated with constitutive overexpression of the IFN/STAT1 pathway in radio-resistant tumor cells [125]. Recent reports have also demonstrated that constitutive overexpression of STAT1 are positively correlated with protection of tumor cells from genotoxic stress such as treatment with doxorubicin [60], or cisplatin [126]. Furthermore, since overexpression of the IFN/STAT1 pathway is associated with poor prognosis in different types of cancer, the IFN-related genes are suggested as predictive markers for breast cancer patients resistant to the adjuvant chemotherapy [127].

Histone deacetylases (HDACs) play important roles in the maintenance and function of chromatin by regulating the acetylation state of histones [128]. Recent data suggest that HDACs regulate the acetylation state of many non-histone targets [64, 129, 130]. In particular, HDAC4, belonging to a class IIa family of HDAC, has been highlighted because of the involvement in various biological responses [131]. One study shows that HDAC4 binds to HIF-1α and HDAC4 suppression with siRNA augments HIF-1α acetylation, leading to destabilization of HIF-1α and down-regulation of HIF-1α-targeted gene transcription [129]. Other study shows that HDAC4 directly interacts with FOXO1 and decreases FOXO1 acetylation, leading to up-regulation of FOXO1 transcriptional activity [130]. In addition, recent studies highlight that HDAC inhibition or suppression can activate Stat3 activation through acetylation [132, 133]. However, in ovarian cancer cells resistant to cisplatin, HDAC4 emerges as an activator of STAT1 [64].

Cancer cells often acquire anticancer drug resistance during administration. To explore an efficacious therapeutic recipe for cancer cells acquiring resistance to anti-cancer drugs, we investigated features of human A549 lung cancer cells resistant to etoposide. In this study, we found that STAT1 and HDAC4 are up-regulated and involved in etoposide resistance in A549 cells through P-glycoprotein (P-gp), encoded in multiple drug resistance (*MDR*) 1 gene. Based on the result, we propose that STAT1 and HDAC4 are potential therapeutic targets for treatment of chemotherapeutic resistant lung cancer cells over-expressing P-gp.

2.2 Materials and Methods

2.2.1 Cell cultures and Selection of cells resistant to etoposide

A549 cells or A549 resistant to etoposide (A549RT-eto) cells which was developed and kindly provided by Laboratory of Biochemistry, Chulabhorn Research Institute, Thailand as described elsewhere [93] were cultured in RPMI-1640 medium (*GenDEPOT* TX., USA*)* supplemented with 10% FBS and 1% penicillin, and streptomycin which were obtained from Gibco Ltd. (N.Y., USA) in the incubator at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2.2 Reagents and Antibodies

For immunoblotting, antibodies against phospho-STAT1, STAT1, HDAC4, poly-ADP-ribose polymerase (PARP) and caspase 9 were acquired from Cell Signaling Biotechnology (MA, USA). Anti-P-gp (Calbiochem CA., USA) and Anti-β-actin antibodies (Santa Cruz Biotechnology, CA., USA) were used. Trichostatin A was purchased from Sigma-Aldrich (St. Louis, MO., USA).

2.2.3 Immunoblotting

Cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl [pH 7.5]) containing 0.1 mM Na_2VO_3, 1 mM NaF and protease inhibitors (Sigma). For immunoblotting, proteins from whole cell lysates were resolved by 10%

or 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes., Primary antibodies were used at 1:1000 or 1:2000 dilutions, and secondary antibodies conjugated with horseradish peroxidase were used at 1:2000 dilutions in 5% nonfat dry milk. After the final washing, nitrocellulose membranes were exposed for an enhanced chemiluminescence assay using the LAS 4000 mini (Fuji, Tokyo, Japan).

2.2.4 Short interference RNA transfection

Cells were trypsinized and incubated overnight to achieve 60-70% confluency before siRNA transfection. STAT1 siRNA (commercially pre-made at Bioneer, Daejeon, Korea; 200 nM; sense, 5'-CUGACUUCCAUGCGGUUGA(dTdT)-3'; antisense, 5'-UCAACCGCAUGGAAGUCAG (dTdT)-3') or negative control siRNA (Bioneer Corporation) were mixed with Lipofectamine 2000 (Invitrogen). The cells were incubated with the transfection mixture for 6 h and then rinsed with RPMI-1640 medium containing 10% fetal bovine serum. The cells were incubated for 48 h before harvest.

2.2.5 Reverse transcriptase-Polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Three micrograms of total RNA were converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and PCR was performed using the specific which human MDR1: 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′, MRP2: 5′-ACAGAGGCTGGTGGCAACC-3′ and 5′-ACCATTACCTTGTCACTGTCC-3′, BCRP: 5'-GATCACAGTCTTCAAGGAGATC-3' and 5'-CAGTCCCAGTACGACTGTGACA-3'were used. The primers for β-Actin were sense primer 5′-ACCAACTGGGACGACATGGAGAAA-3′ and antisense primer 5′-TTAATGTCACGCACGATTTCCCGC-3′. After initial denaturation at 95°C for 5 min, PCR was performed for 35 cycles (30 s at 94°C, 30 s at annealing temperature and 60 s at 72°C) using G-Taq polymerase (TaKaRa, Japan). Reaction products (10 µl per lane) were electrophoresed in 2% agarose, stained with ethidium bromide and photographed.

2.2.6 Cytotoxicity assay

The MTT assay is cell viability assay which was performed as previously described [134]. After continuous exposure to the compound for 72 h, the MTT solution (5 mg/ml) was added to each well. After incubation for 2 h at 37°C., the soluble of formazan crystals was measured at 550 nm using a Biochrom Asys UVM 340 Microplate Reader (Holliston, MA, USA).

2.2.7 Luciferase reporter assay

A549 and A549RT-eto cells were transfected with hMDR1-luciferase vectors. To normalize transfection efficiency, a pGK-βgal vector that expresses β-galactosidase from a phosphoglucokinase promoter was included in the transfection mixture. At 48 h post-transfection, cells were washed with cold PBS and lysed in lysis solution (25 mM Tris [pH7.8], 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton-X100) and then extracted with the cell lysis buffer in a Luciferase Assay Kit (Promega, WI., USA). Luciferase activity was determined using the Berthold Lumat LB 9507 Luminometer (Berthold, Tokyo, Japan). The β-galactosidase activity was assayed using the Mammalian β-Galactosidase Assay Kit (Thermo Fisher Scientific, IL., USA) which was determined using the Promega Luciferase Assay System in a PerkinElmer Victor^3 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, MA., USA) at OD_{405} nm. The luciferase activity was normalized to the β-galactosidase activity in the same sample.

2.2.8 Statistical Analysis

Data are presented as a means± standard deviation. The Student' *t* test was used for statistical analysis, with *p* value <0.05 defined as significance.

2.3 Results

2.3.1 A549RT-eto cells exhibit higher levels of HDAC4, phospho-STAT1 and P-glycoprotein (P-gp) compared to A549 parental cells.

We first examined what concentration of etoposide is able to affect cell viability of A549 and A549RT-eto cells. A549 parental cells were more sensitive to growth inhibition at lower concentration of etoposide (5 μg/ml) while A549RT-eto cells were relatively resistant to growth inhibition (Figure 2.1A). However, most of A549 cells were dead at the concentration of etoposide (50 μg/ml) whereas A549RT-eto cells were relatively survived. A549 cells were more sensitive to apoptosis during etoposide treatment than A549RT-eto cells, which were supported by the detection of cleaved PARP, a substrate of active caspase 3 and 7. Since we observed A549RT-eto cells exhibit resistance to etoposide, we wondered what feature A549RT-eto cells possess in comparison with A549 parental cells. When we compared expression levels of STAT1, HDAC4 and P-gp, encoded by *MDR*1, we interestingly found that levels of HDAC4 and P-gp proteins were significantly enhanced in A549RT-eto cells compared to those in A549 parental cells. STAT1 protein levels in A549RT-eto cells were not significantly different from those in A549 cells, but we easily notified active form of STAT1 in A549RT-eto cells. Based on the result, we suspect that the enhanced levels of phospho-STAT1, HDAC4 and P-gp are involved in resistance to etoposide in A549 cells.

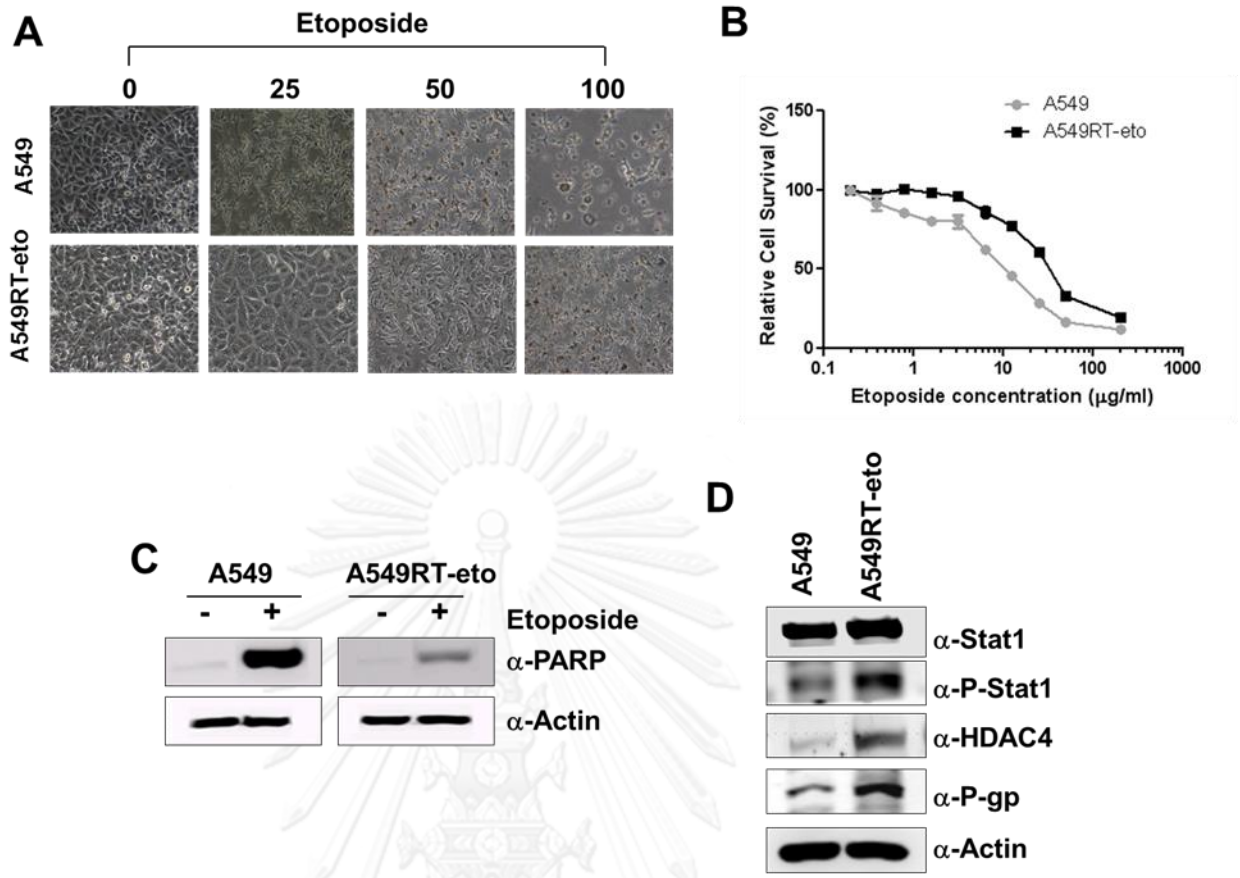

Figure 2.1 Establishment of A549 lung cancer cells resistant to etoposide (A549RT-eto)

(A, B) A549 and A549RT-eto cells were treated with etoposide (0.1, 1, 2, 5, 10, 25, 50 and 100 μg/ml) for 48 h. Thereafter, tetrazolium was added to the cells and the absorbance of the formazan produced by living cells was measured at 570 nm. Results were averaged from triplicate wells and the error bars indicates standard deviation. A549 and A549RT-eto cells were treated with etoposide (25, 50 and 100 μg/ml) and observed at 24 and 48 h after treatment under light microscope. (C, D) Cell lysates from A549 and A549RT-eto cells in the presence or absence of etposide (50 μg/ml) were prepared and separated on a 10% SDS-PAGE gel. The expression of cleaved PARP protein was detected for apoptosis by immunoblotting and protein levels of HDAC4, STAT1, phospho-STAT1, and P-gp were compared by immunoblotting with the corresponding antibodies

2.3.2 HDAC inhibition enhances susceptibility to etoposide in A549RT-eto and A549 cells.

Since we observed enhanced levels of HDAC4, we explored whether HDAC4 are involved in resistance to etoposide in A549 cells. We thus treated A549 and A549RT-eto cells with trichostin A (TSA; 6.25nM), an inhibitor of HDACs and examined cell viability with MTT assay. TSA treatment alone did not influence inhibition of cell growth in both cells. As expected, A549 cells were more sensitive to growth inhibition than A549RT-eto cells. Furthermore, we found that the combined treatment with etoposide and TSA drastically inhibited cell growth approximately 85%, and 65% in A549 and A549RT-eto cells, respectively (Figure 2.2A). Even the combined treatment sensitized A549 and A549RT-eto cells to apoptosis compared to treatment with TSA or etoposide alone because cleaved PARP and caspase 9, an indicator of intrinsic apoptotic cell death, were detected under combined treatment. Moreover, when we examined protein levels of HDAC4, P-gp and phospho-STAT1, we interestingly found that TSA treatment decreased expression levels of HDAC4 and P-gp levels in both cells. However, protein levels of STAT1 were not significantly altered in A549RT-eto cells while A549 cells exhibited a drastic reduction of STAT1 protein levels during TSA treatment. We additionally observed that TSA treatment alone inhibited activation of STAT1 in both cells. Etoposide treatment alone diminished protein levels of HDAC4 in both cells and P-gp protein levels in A549RT-eto cells. We observed that etoposide treatment alone decreased STAT1 protein levels in A549 cells but not in A549RT-eto cells. The combined treatment reduced protein levels of HDAC4, P-gp and phospho-STAT1 in A549Rt-eto cells. Taken together, we suggest that HDAC4 inhibition sensitizes A549RT-eto cell to etoposide-induced apoptosis through decrease of P-gp protein levels (Figure 2.2B).

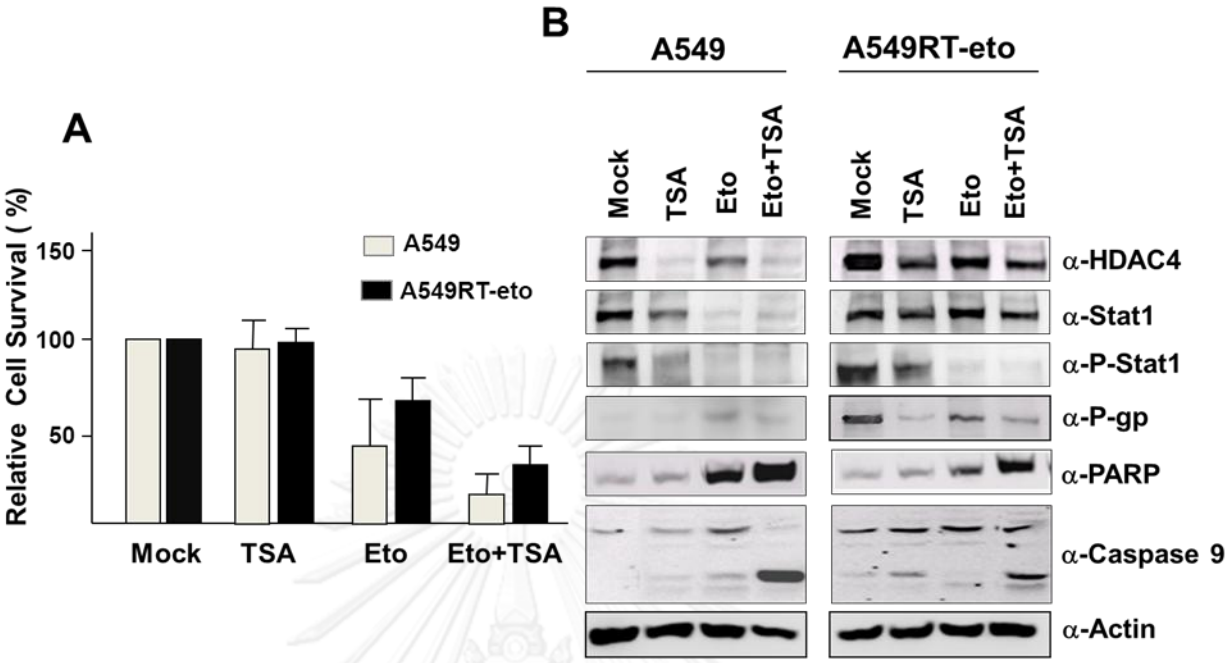

Figure 2.2 TSA treatment suppresses levels of HDAC4, phospho-STAT1 and P-gp proteins, leading to sensitization of etoposide-induced apoptosis.

(A) A549 and A549RT-eto cells were treated with etoposide (50 μg/ml) alone, TSA (6.25 nM) alone, and ectposide plus TSA for 48 h and cell growth was measured by MTT assay. (B) Cell lysates from the treated A549 and A549RT-eto cells were prepared and separated on a 10% SDS-PAGE gel. The expression levels of HDAC4, STAT1, phospho-STAT1, and P-gp were detected by immunoblotting with the corresponding antibodies. Apoptosis was detected by cleaved PARP and caspase 9 using immunoblotting.

2.3.3 Suppression of STAT1 with siRNA enhances susceptibility to etoposide in A549RT-eto cells.

Since we observed that enhanced levels of phospho-STAT1 in A549RT-eto cells, we wondered whether the elevated STAT1 is involved in resistance to etoposide. To answer this question, we introduced STAT1 siRNA to suppress STAT1 levels in A549RT-eto cells. We first optimized STAT1 siRNA concentration to suppress STAT1 expression and used 100 nM of STAT1 siRNA for the later experiment (data not shown). Of interest, we found that suppression of STAT1 itself induced decrease of HDAC4 and P-gp expression levels, indicating that HDAC4 and STAT1 cross-talk each other. Etoposide treatment alone decreased HDAC4, P-gp and phospho-STAT1 protein levels as seen in Fig. 2.3B but is not enough to induce a clear apoptosis in A549RT-eto cells. The combined treatment with STAT1 siRNA and etoposide induced decrease of HDAC4, P-gp and phospho-STAT1 protein levels, resulting in acceleration of apoptosis by activation of caspase 3, 7, and 9 in A549RT-eto cells (Figure 2.3B).

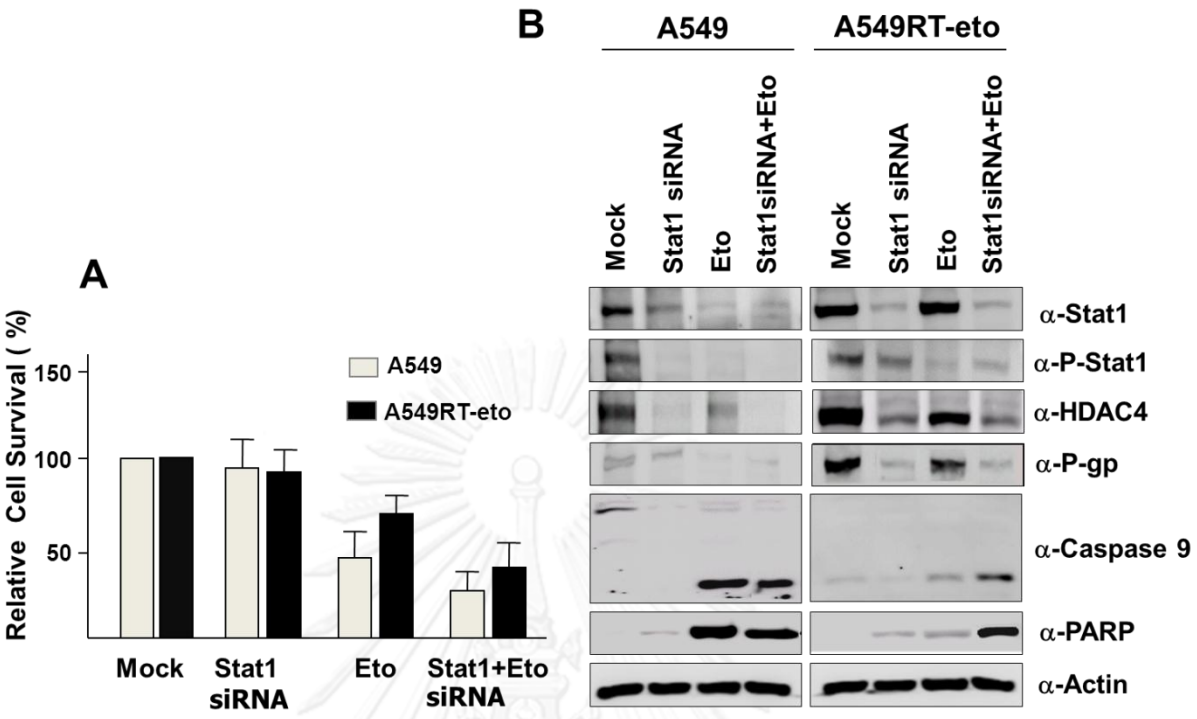

Figure 2.3 STAT1 suppression decreases levels of HDAC4 and P-gp proteins, leading to sensitization of etoposide-induced apoptosis.

(A) A549 and A549RT-eto cells were treated with etoposide (50 μg/ml) alone, STAT1 siRNA (100 nM) alone, and ectposide plus STAT1 siRNA for 48 h and cell growth was measured by MTT assay. (B) Cell lysates from the treated A549 and A549RT-eto cells were prepared and separated on a 10% SDS-PAGE gel. The expression levels of STAT1, HDAC4, phospho-STAT1, and P-gp were detected by immunoblotting with the corresponding antibodies. Apoptosis was detected by cleaved PARP and caspase 9 using immunoblotting.

2.3.4 Etoposide resistance is involved in the enhancement of MDR1 transcript levels and its transcriptional activity in A549RT-eto cells.

Since not only MDR1 gene but also other gene such as MRP2 and BCRP are known to be involved in drug resistance, we thus examined levels of transcript related to multi-drug resistance such as MDR1, MRP2, and BCRP genes in A549 and A549RT-eto cells. We found that A549RT-eto cells exhibited much higher levels of MDR1 transcript whereas A549 cells showed almost no transcript of MDR1 (Figure 2.4A). However, levels of MRP2 and BCRP transcript are very similar in between A549RT-eto and A549 parental cells. The result indicates MDR1 gene is specifically activated for etoposide resistance in A549 cells. Furthermore, we examined MDR1 promoter activity in A549RT-eto and A549 cells. We observed a drastic increase of MDR1 promoter activity in A549RT-eto cells compared to that in A549 parental cells, supporting higher levels of MDR1 transcript in A549RT-eto cells seen in Figure 2.4B. In addition, we examined whether TSA or STAT1 siRNA treatment reduces MDR1, MRP2, and BCRP transcript levels in A549RT-eto cells. TSA treatment alone drastically reduced transcriptional levels of MDR1 but not MRP2 and BCRP while etoposide treatment alone mildly reduced transcriptional levels of MDR1 but not MRP and BCRP. The combined treatment with TSA and etoposide also reduced transcriptional levels of MDR1 (Figure 2.5A). When STAT1 siRNA was treated into A549RT-eto cells, we examined transcriptional levels of MDR1, MRP2, and BCRP gene. We found that STAT1 siRNA treatment alone did not reduce MDR1 transcript levels similar to those treated with control siRNA. The combined treatment with STAT1 siRNA and etoposide reduced MDR1 transcript levels because etoposide treatment suppressed MDR1 transcript levels (Figure 2.5B).

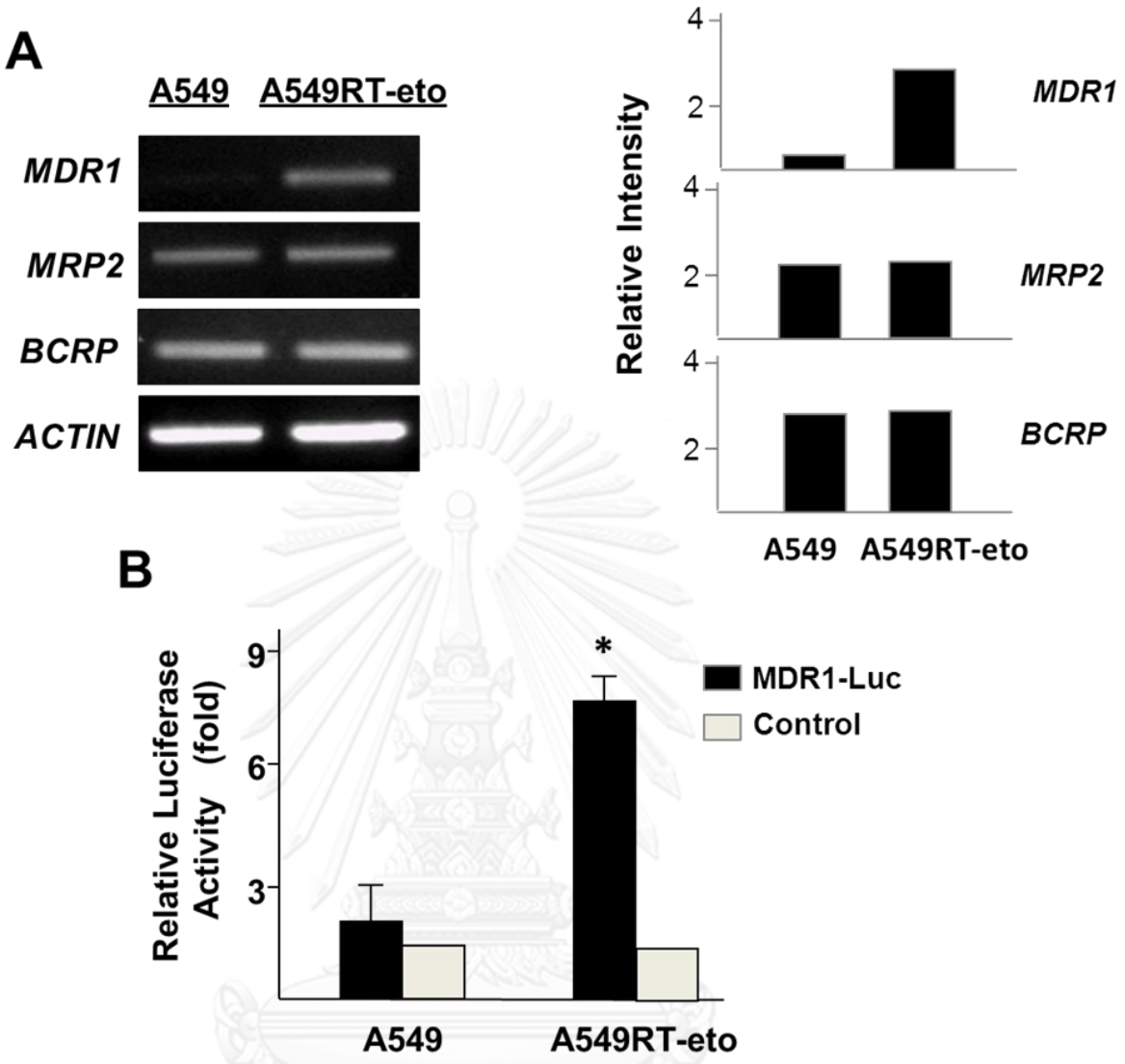

Figure 2.4 Regulation of MDR-related gene transcripts by treatment with TSA and STAT1 siRNA

(A) Total RNAs from A549 and A549RT-eto cells were isolated and subjected to RT-PCR. Transcripts of MDR1, MRP2 and BCRP were examined after optimization of PCR. Relative mRNA ratio of each MDR-related protein was described in comparison with mRNA levels of β-actin after measurement of band intensities using Multi Gauge Vesion 2.1 (Fuji, Tokyo, Japan). (B) *MDR1*-promoter luciferase reporter assay of A549 and A549RT-eto cells.

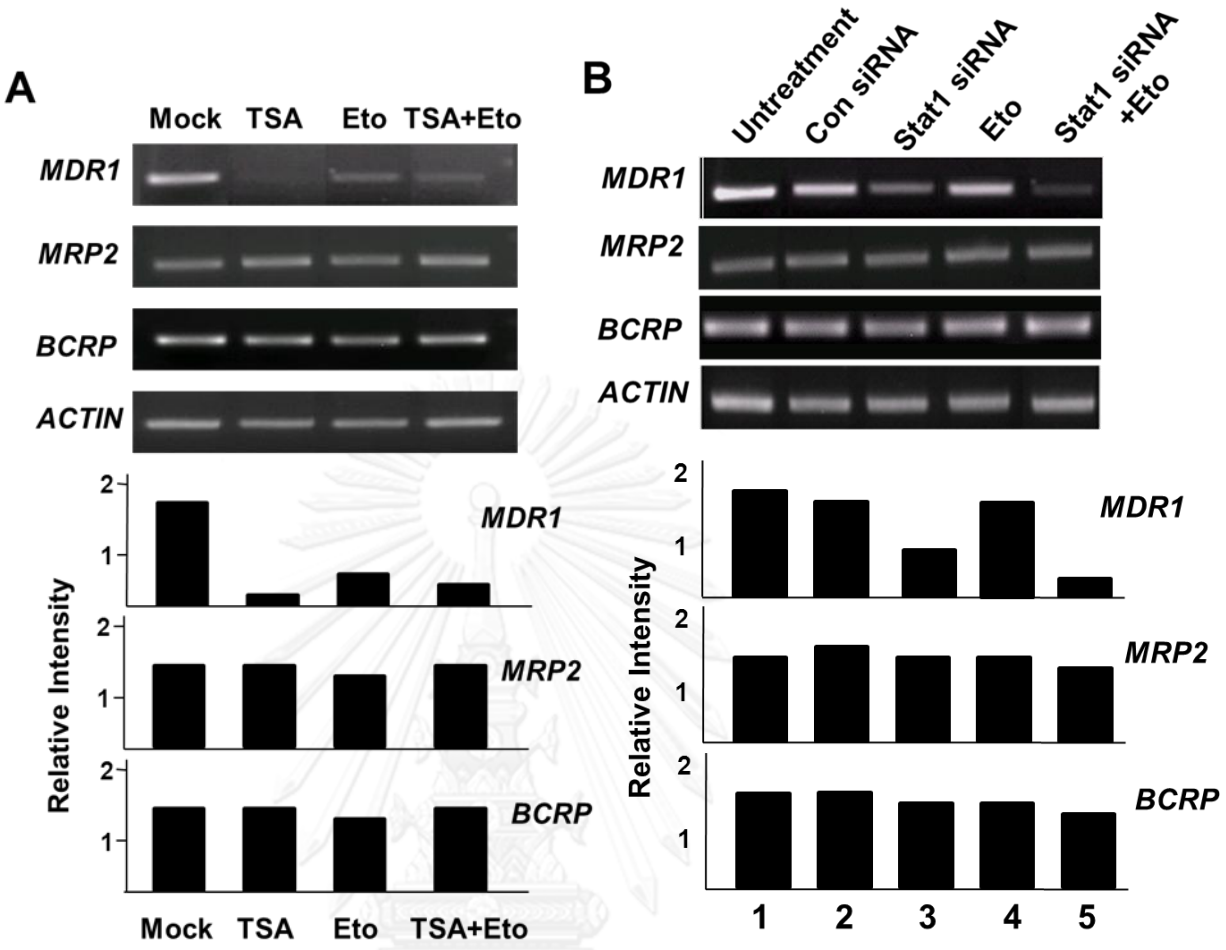

Figure 2.5 Regulation of MDR-related gene transcripts by treatment with TSA and STAT1 siRNA

(A, B) Total RNAs from A549RT-eto cells untreated or treated with TSA alone, etoposide alone, TSA plus etoposide, STAT1 siRNA alone and STAT1 siRNA plus etoposide were isolated and subjected to RT-PCR. Transcripts of MDR1, MRP2 and BCRP were examined after optimization of PCR. Relative mRNA ratio of each MDR-related protein was described in comparison with mRNA levels of β-actin after measurement of band intensities using Multi Gauge Vesion2.1 (Fuji, Tokyo, Japan).

2.4 Discussion

Almost all non-small cell lung cancer (NSCLS) patients display intrinsic chemoresitance, generally limiting the chance of successful chemotherapy [135]. The most frequent form observed in lung cancer patients is multidrug resistance, which has been identified including the overexpression of the ATP-binding cassette (ABC) superfamily of transporters such as P-gp and multidrug resistance-associated proteins (MRP) [136, 137]. In this study, we found that A549 cells with acquired etoposide resistance specifically exhibited up-regulation of P-gp but not other MDR–related genes such as MRP2 and BCRP, which are frequently overeexpressed in many cancer patients with chemotherapeutic resistance. In addition, human H460 lung cancer cells resistant to eptoposide also showed overexpression of lung resistance protein (LRP) [138]. We thus believe that up-regulation of a specific MDR gene is related to the context of cell origin and chemotherapeutic drugs.

Herein, we first reported that expression of P-gp is regulated by both HDAC4 and STAT1. Suppression of HDAC4 activity and STAT1 levels induced decrease of P-gp expression levels, leading to sensitization of apoptosis, which indicates that HDAC4 and STAT1 are responsible for etoposide resistance. Of interest, we also observed that inhibition of HDAC4 activity by TSA reduced STAT1 activity, indicating that HDAC4 may regulates STAT1 activity. Our hypothesis is supported by other study shown that HADC4 interacts with STAT1, resulting in decrease of STAT1 acetylation, which eventually enhances STAT1 phosphorylation in cisplatin-resistant cancer cells [64].

Furthermore, when expression of STAT1 was suppressed with its siRNA, HDAC4 protein levels were also diminished in etoposide-resistant A549 cells, leading to sensitization of etoposide-induced apoptosis through down-regulation of P-gp. Based on these results, we propose that STAT1 and HDAC4 cross-talk each other.

However, when we examined transcript levels of P-gp during STAT1 siRNA treatment, we found that transcript levels of P-gp were not decreased. Considering that P-gp protein levels were decreased during STAT1 siRNA treatment, we hypothesize that suppression of STAT1 may influence P-gp protein levels at post-

transcriptional level or with an indirect way through other proteins. Supporting our hypothesis, a recent study has shown that FBXO15/Fbx15, known as a F-box protein in the ubiquitin E3 ligase complex, regulates P-gp expression levels through the ubiquitin-proteosme pathway [139]. However, it remains unknown how STAT1 suppression is related to up-regulation of FBXO15/Fbx15 until now. Thus, we are now investigating detailed mechanisms by which suppression of STAT1 induces decrease of P-gp protein levels.

CHAPTER III

EXTRACTS OF *Bryophyllum laetivirens* REVERSE ETOPOSIDE RESISTANCE IN HUMAN LUNG A549 CANCER CELLS BY DOWN-REGULATION OF NF-κB

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Abstract

Ethnopharmacological relevance; *Bryophyllum laetivirens* are perennial herbs that grow in the wild and used as a traditional medicinal to treat ailments such as infections, rheumatism, inflammation and cancer.

Aim of the study; To demonstrate that *B. laetivirens* extract (F14) reverses etoposide resistance in human A549 lung cancer cells through down-regulation of NF-**K**B.

Materials and methods; The methanol extracts fraction (F14) were initially tested the cytotoxicity against etoposide resistance in human lung A549 cancer cells. The effect of F14 was evaluated on cell proliferation by MTT assay and protein translational levels of the transcription factor nuclear factor kappa B (NF-**K**B), Sirtuin 1 (SIRT1) and P-glycoprotein (P-gp) by western blotting. Moreover, the effect of F14 on P-gp was studied at the transcriptional by luciferase gene reporter assay and RT-PCR. And using Bay11-7802, an inhibitor of NF-**K**B and SIRT1 siRNA or nicotinamide (NAM), an inhibitor of SIRT1 address whether F14 down-regulation of NF-**K**B and SIRT1 which are involved in resistance to etoposide through P-gp.

Result; The F14 down-regulates expression of NF-**K**B and SIRT1. We then observed that the combined treatment with F14 extract and Bay11-7802 accelerates apoptosis through decrease of P-gp levels, suggesting that NF-**K**B is involved in MDR., sensitizing apoptosis of A549RT-eto cells through down-regulation of P-gp levels, which is encoded by MDR1 gene.

Discussion; We concluded that *B. laetivirens* extract (F14) reverses etoposide resistance, P-gp over-expression, of in human A549 lung cancer cells through down-regulation of NF-**K**B.

Keyword; multidrug resistance; NF-**K**B; SIRT1; *Bryophyllum laetivirens*

3.1 Introduction

Multidrug resistance (MDR) cancer is a major problem in cancer therapy and is often the result of overexpression of the drug efflux protein, P-glycoprotein (P-gp). P-gp is a 170 kDa protein which belongs to the ATP-binding cassette superfamily of membrane transporter proteins [140, 141]. P-gp is an energy-dependent drug efflux pump that maintains intracellular drug concentrations below cytotoxic levels, thereby decreasing the cytotoxic effects of a variety of chemotherapeutic agents, including anthracyclines, vinca alkaloids, and epipodophyllotoxins [140-143]. P-gp also plays a role in inhibition of drug accumulation and caspase activation in the MDR tumor [7, 8]. Of special note, recent lines of evidence have shown that NF-**K**B- or SIRT1-mediated regulation of P-gp plays a critical role in anti-cancer drug resistance [74, 144]. Medicinal plants have been a rich source of therapeutic agents and provided precursors for several synthetic drugs. Despite great development of organic synthesis, currently 75% of prescribed drugs worldwide are derived from plant source [145]. Some species of *Bryophyllum*, which belongs to plant genus Kalanchoe of the *Crassulaceae* family, are perennial herbs that grow in the wild and used as a traditional medicinal plant in tropical Africa, China, Australia, and tropical America [9]. In traditional medicine, some species of *Bryophyllum* have been used to treat ailments such as infections, rheumatism and inflammation [146]. A recent study has also reported that extracts from *Brophyllum pinnata* exhibit anticancer activity against human cervical cancer cells [9]. However, a molecular mechanism by which extracts of the plant show anticancer activity has not been revealed. Since our previous study has shown that human A549 lung cancer cells resistant to etoposide (A549RT-eto) display up-regulation of P-gp, we have screened natural compounds to reverse resistance to etoposide in these cells. In this study, we investigated whether an extract of *B. laetivirens* can efficiently induces cytotoxicity to A549RT-eto cells and further examined molecular mechanisms by which an extract of *B. laetivirens* induce reversal of MDR, leading to apoptosis. We report that an extract of *B. laetivirens* provokes apoptosis of A549RT-eto cells through down-regulation of P-gp, which is mediated by suppression of NF-**K**B expression and activity.

3.2 Materials and methods

3.2.1 Reagents and Antibodies

For immunoblotting, an antibody against cleaved PARP (Asp214) was acquired from Cell Signaling Biotechnology (Beverly, MA, USA). Anti-P-gp (Calbiochem; San Diego, CA, USA) and NF-**K**B p65 (F-6), SIRT1 (H-300), Sp1 (1C6), caspase-9 p35 (H-170), cytochrome c (A-8) and actin (C4) antibodies (Santa Cruz Biotechnology; Santa Cruz, CA, USA) were used. Nicotinamide and BAY11-7082 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

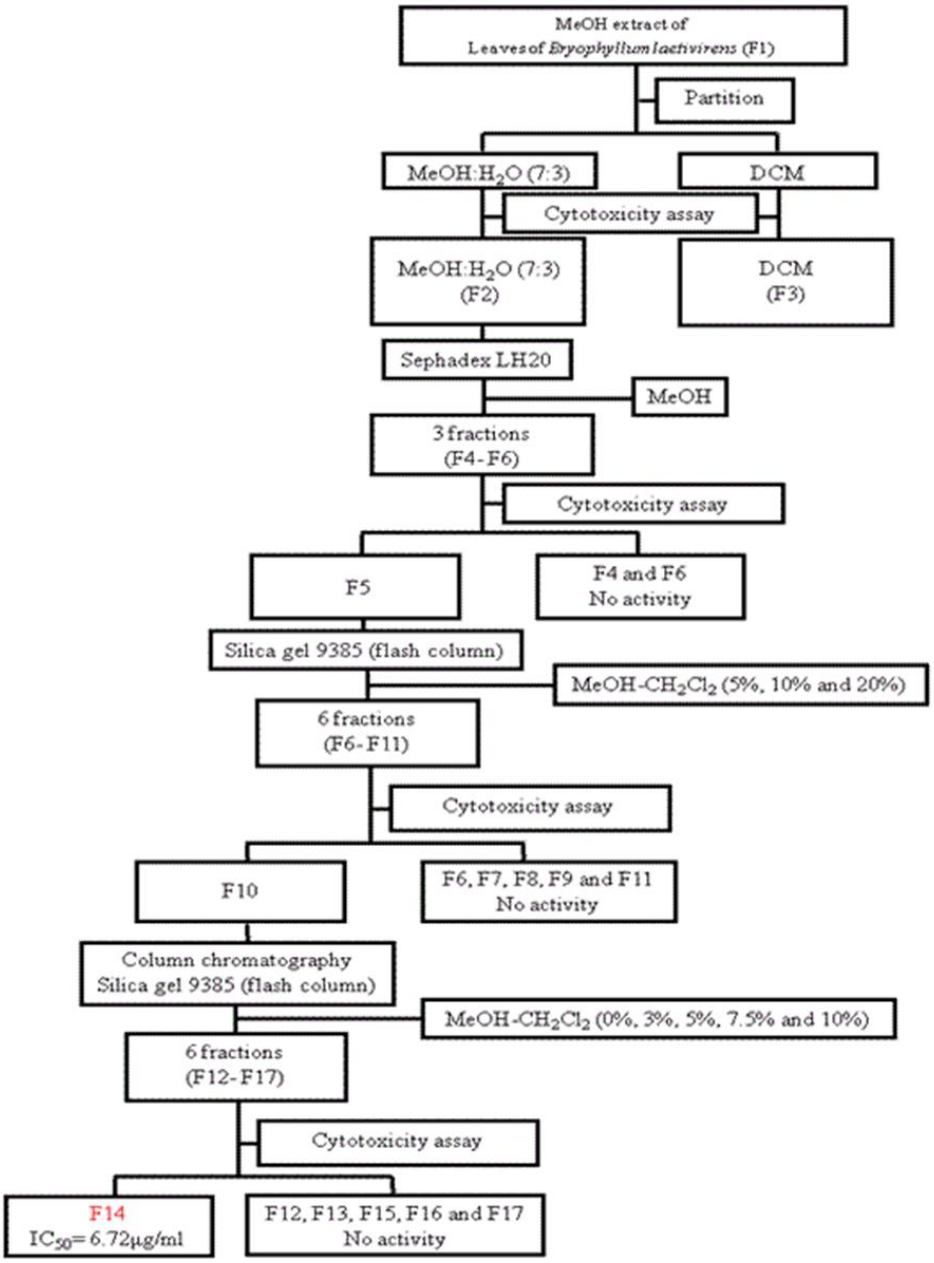
3.2.2 Cell cultures

A549 cells or A549RT-eto cells, which was developed and kindly provided by Laboratory of Biochemistry, Chulabhorn Research Institute, Thailand as described elsewhere [93], were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 1% penicillin, and streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air.

3.2.3 Preparation of extracts from *Bryophyllum laetivirens*

Fresh leaves of *B. laetivirens* (3 kg) were collected during August, 2012 from Inburi, Singburi Province, Thailand. Plant authentication was performed by Ms. Parinyanoot Klinratana and the specimens (A 013630) have been deposited in the Kasin Suvatabandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The leaves were dried at 45°C and ground into powder. The samples were extracted with MeOH. The extracts were partitioned between H_2O and CH_2Cl_2 and then checked for cytotoxicity. A portion of the MeOH extract was chromatographed on Sephadex LH-20 gel (100 g, GE Healthcare, Piscataway, NJ, USA) and the fractions showing cytotoxicity to A549RT-eto cells were collected. The active fractions were then subjected to a silica gel (50 g, 60-230 mesh)

column in hexane and eluted with MeOH:CH_2Cl_2 (5, 10 and 20%). The active fractions were again subjected to this column and eluted with MeOH:CH_2Cl_2 (0, 3, 5, 7.5 and 10%). The fractions showing similar TLC profile were combined and then subjected to evaluate for cytotoxic activity. Isolation of bioactive faction was diagramed in Scheme 1 and the fraction, named as F14 showed one single spot on TLC and the highest cytotoxic activity on A549RT-eto cells.



Scheme 3.1. Flow chart of extraction and column chromatographic separation of *Bryophyllum laetivirens* leaves.

3.2.4 Immunoblotting

Cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 7.5)) containing 0.1 mM Na_2VO_4, 1 mM NaF and protease inhibitors (Sigma). For immunoblotting, proteins from whole cell lysates were resolved by 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes., Primary antibodies were used at 1:1000 or 1:2000 dilutions, and secondary antibodies conjugated with horseradish peroxidase were used at 1:2000 dilutions in 5% nonfat dry milk. After the final washing, nitrocellulose membranes were exposed for an enhanced chemiluminescence assay using the LAS 4000 mini (Fuji, Tokyo, Japan).

3.2.5 Nuclear NF-**K**B pull-down assay

A549 or A549RT-eto cells (1×10^6 cells/ml) were incubated with F14 fraction or DMSO as control for 12 h and nuclear extracts were prepared. Cells were pelleted and resuspended in 0.4 mL hypotonic lysis buffer (20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 0.2% Triton X-100, and 1 mM Na_2VO_4 plus protease inhibitors) and kept on ice for 20 min. After centrifugation at 14000 *g* for 5 min at 4°C, the nuclear pellet was extracted with 0.1 mL hypertonic lysis buffer on ice for a further 20 min. After centrifugation at 14 ,000 *g* for 5 min at 4°C, the supernatants were diluted to 100 mM NaCl and incubated with 25 µL of agarose beads cobnjugated to a consensus NF-**K**B binding oligonucleotide (Santa Cruz Biotechnology) for 1 h at 4°C. After 3 washes, sample buffer was added and boiled for 5 min. The binding NF-**K**B (p65) protein to the oligonucleotide conjugated with agarose was detected by immunoblotting using an anti-p65 NF-**K**B Ab (Santa Cruz Biotechnology).

3.2.6 Short interference RNA transfection

Cells were trypsinized and incubated overnight to achieve 60-70% confluency before siRNA transfection. SIRT1siRNA (commercially pre-made at Bioneer, Daejeon,

Korea; 100 nM; sense, 5′-ACUUUGCUGUAACCCUGUA(dTdT)-3′; antisense, 5′-UACAGGGUUACAGCAAAGU(dTdT)-3′ or negative control siRNA (Bioneer) were mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were incubated with the transfection mixture for 6 h and then rinsed with RPMI-1640 medium containing 10% fetal bovine serum. The cells were incubated for 48 h before harvest.

3.2.7 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Three micrograms of total RNA were converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and PCR was performed using the specific primers: human MDR1: sense 5′-CCC ATC ATT GCA ATA GCA GG-3′ and anti-sense 5′-GTTCAAACTTCTGCTCCTGA-3′, MRP2: sense 5′-ACAGAGGCTGGTGGCAACC-3′ and anti-sense 5′-ACCATTACCTTGTCA CTGTCC-3′, BCRP: sense 5'-GATCACAGTCTTCAAGGAGATC-3' and anti-sense 5'-CAG TCCCAGTACGACTGTGACA-3'were used. The cDNAs of each sample were diluted, and Amplification was carried out for 35 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s. β-actin mRNA was measured as an internal standard. After amplification, the products were subjected to electrophoresis on 1.5 and 2.0% agarose and detected by ethidium bromide staining.

3.2.8 Luciferase reporter assay

A549RT-eto cells were transfected with hMDR1-luciferase or pGL3 empty vector as a control luciferase vector. To normalize transfection efficiency, a pGK-β-gal vector that expresses-galactosidase from a phosphoglucokinase promoter was included in the transfection mixture. F14 extract was added to the transfected cells at 12 h before harvest. At 48 h post-transfection, cells were washed with cold PBS and lysed in lysis solution (25 mM Tris (pH7.8), 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1%

Triton-X100). Luciferase activity was measured with a luminometer by using a luciferase kit (Promega, Madison, WI, USA).

3.2.9 SIRT1 activity assay

SIRT1 activity from A549, A549RT-eto or A549RT-eto cells treated with F14 extract was measured using SIRT1 direct fluorescence kit provided by Cayman (Ann Arbor, MI, USA) as the manufacture's recommended. Briefly, 25 µL of assay buffer was added into the cell supernatant. Subsequently, 15 µL of substrate solution (containing final concentration of 125 µM peptides and 3 mM NAD^+) was added and the sample mixture was incubated for 45 min before addition of stop solution. Fluorescence (an excitation wavelength 360 nm and an emission wavelength 460 nm) was measured using Victor^3 microplate reader (Perkin Elmer; Waltham, MA, USA).

3.2.10 Cytotoxicity assay

MTT assay was performed for the measurement of cell survival as previously described. Dye solution containing tetrazolium was added to the cells in the 96-well plate and incubated for 2 h. The absorbance of the formazan produced by living cells was measured at 570 nm. The relative percentage of cell survival was calculated by the mean absorbance of the treated cell (OD_T) and the mean absorbance of control cell (OD_C) with formula, % Cell survival = (OD_T / OD_C).

3.2.11 Statistical Analysis

Data are presented as a means $\pm$ standard deviation (S.D.). The Student's *t*-test was used for statistical analysis, with p value < 0.05 defined as significance.

3.3 Results

3.3.1 An extract of *Bryophyllum laetivirens* induces apoptosis of human A549 lung cells resistant to etoposide.

We observed that human A549 lung cancer cells resistant to etoposide (A549RT-eto) exhibit up-regulation of STAT1 and HDAC4, leading to the enhancement of P-gp protein levels. Moreover, recent studies have shown that NF-**K**B and SIRT1 are also involved in multidrug resistance (MDR). We thus examined whether NF-**K**B and SIRT1 protein levels are up-regulated in A549RT-eto cells in addition to up-regulation of STAT1 and HDAC4. We found that A549RT-eto cells showed higher levels of NF- **K**B and SIRT1 proteins compared to A549 parental cells (Figure 3.1A). We next wonder whether the elevated protein levels of NF-**K**B in A549RT-eto cells indicates higher activity of NF-**K**B compared to A549 cells. To address this question, we prepared nuclear extract from A549 and A549RT-eto cells. Because the active NF-**K**B protein is translocated into the nucleus and binds its binding site, we examined whether p65 NF-**K**B protein found at higher levels in A549RT-eto cells can bind to NF-**K**B oligonucleotides conjugated with agarose. We found that more active p65 proteins are detected in the oligonucleotide mixtures from A549RT-eto cells than those from A549 cells (Figure 3.1B). With the same reason, we also measured SIRT1 activity because we observed the enhanced protein levels of SIRT1 in A549RT-eto cells compared to those in A549 cells. We found that A549RT-eto cells show higher activity of SIRT1 than A549 cells using a SIRT1 activity kit (Figure 3.1C).

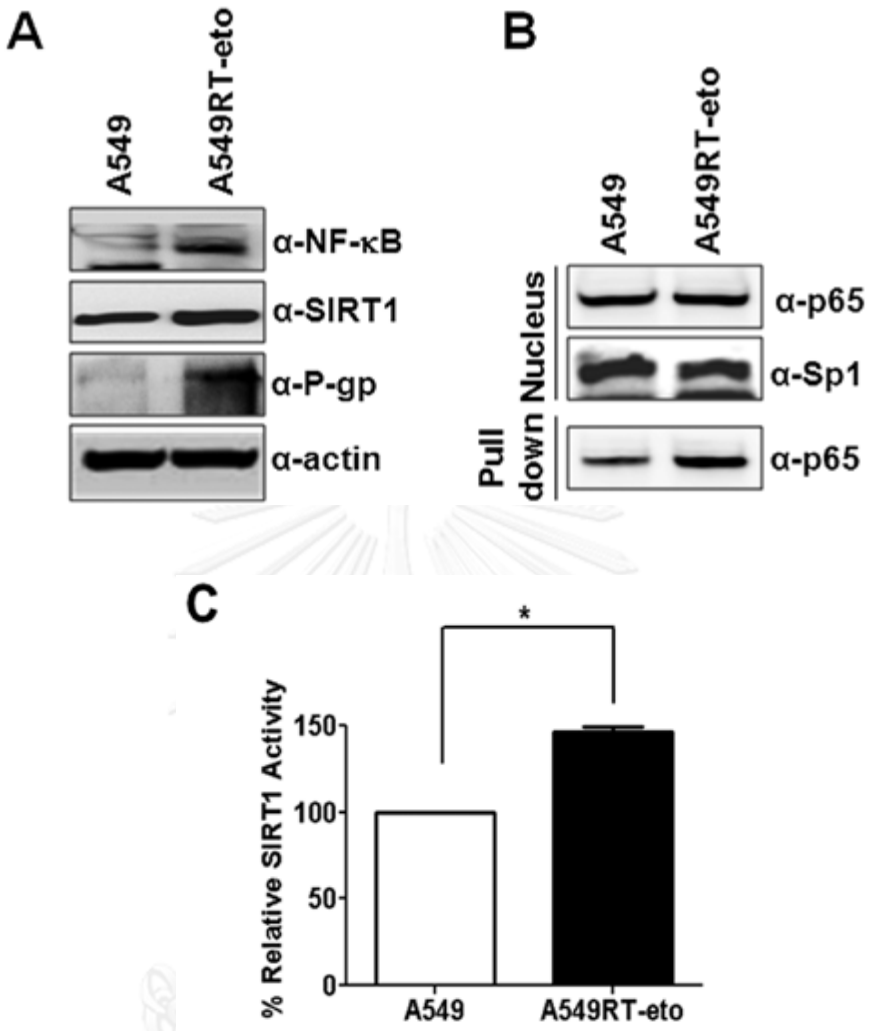


Figure 3.1 A549RT-eto cells exhibits up-regulation of NF-ΚB and SIRT1

(A) The protein expression levels of NF-ΚB, SIRT1, and P-gp from A549 and A549RT-eto cells (B) The binding p65 NF-ΚB to the oligonucleotide of nuclear extracts from A549 and A549RT-eto cells (C) SIRT1 activity of A549 and A549RT-eto cells.

On the basis of our previous study to screen extracts from medicinal plant sources showing the best cytotoxic activity against A549RT-eto cells [134], we chose *B. laetivirens*. We thus purified and collected an active pool from *B. laetivirens*, and then named the active fraction as F14 extract (Scheme 3.1). We first optimized concentration and treatment time of F14 extract, we found that 20 µg/ml of F14 extract significantly induced cell death of A549RT-eto cells at 24 h post-treatment and A549RT-eto cells still survived with naked eyes under microscopy at 12 h post-treatment (Figure 3.1A). However, when we examined protein levels of NF-**K**B, SIRT1 and P-gp in A549RT-eto cells treated with F14 (20 µg/ml) at 12 h post-treatment, we found that F14 treatment drastically reduced expression levels of NF-**K**B, SIRT1 and P-gp (Figure 3.2A and B). In addition, we detected reduced pre-caspase 9 levels, and enhanced cytochrome C levels in the same lysates of A549RT-eto cells, indicating induction of intrinsic apoptosis (Figure 3.2A). Furthermore, when we treated A549RT-eto cells with F14 extract at 0.5 and 5 µg/ml for 12 h, we found that F14 extract at 5 µg/ml is enough to reduce not only expression levels of NF-**K**B, SIRT1 and P-gp but also activities of NF-**K**B and SIRT1 (Figure 3.2C and D).

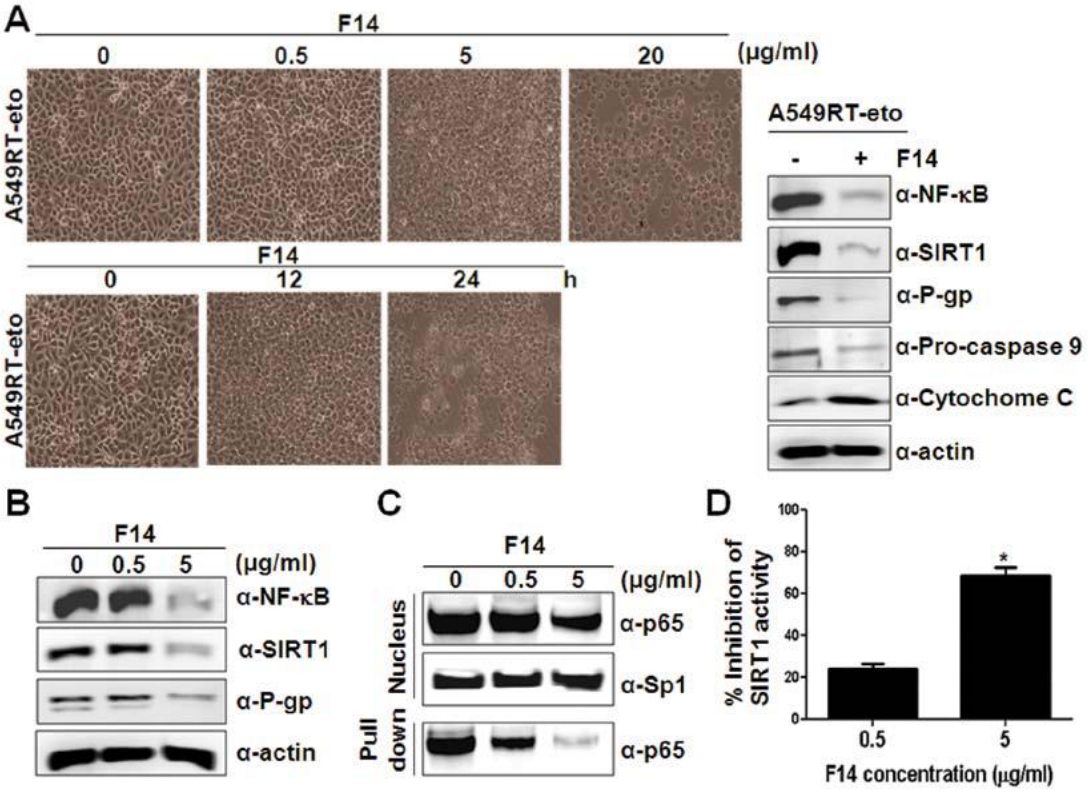


Figure 3.2 F14 fraction suppresses levels of NF-ΚB, SIRT1, and P-gp proteins, leading to F14-induced apoptosis.

(A) The A549RT-eto cells morphology under light microscope and the protein expression levels of A549 and A549RT-eto cells by Western blot assay (B) Western blot assay of treated A549RT-eto cells with F14 at 12 h (C) The binding p65 NF-ΚB to the oligonucleotide of nuclear extracts of treated A549RT-eto cells with F14 by westernblotting assay (D) SIRT1 activity of A549RT-eto cells treated with F14.

3.3.2 F14 fraction reduces MDR1 transcript levels and its transcriptional activity in A549RT-eto cells.

Since not only MDR1 gene but also other genes such as MRP2 and BCRP are known to be involved in drug resistance [147, 148], we thus examined levels of transcript related to multidrug resistance such as MDR1, MRP2, and BCRP genes in A549RT-eto cells during F14 treatment. In addition, we wondered whether decrease of P-gp protein levels is regulated at transcriptional level by F14 treatment. Cells were thus treated with F14 extract (5 μg/ml) for 12 h and total RNAs were isolated. After cDNA synthesis, MDR1, MRP2, and BCRP genes were amplified in A549RT-eto cells treated with F14. We found that A549RT-eto drastically reduced transcriptional levels of MDR1 and slightly reduced of BCRP in a dose-dependent manner but not MRP2 (Figure 3.3A). Furthermore, we examined MDR1 transcriptional activity in the cells using *MDR1*-promoter luciferase reporter vector [74]. As seen in Figure 3.3B, F14 treatment induced a drastic decrease of *MDR1*-mediated luciferase activity in A549RT-eto cells (Figure 3.3B), indicating that F14 extract reduces MDR1 transcriptional activity. On the basis of these results, we suggest that F14 extract inhibited synthesis of MDR1 transcription, resulting in decrease of P-gp protein levels, which eventually sensitizes apoptosis of A549RT-eto cells.

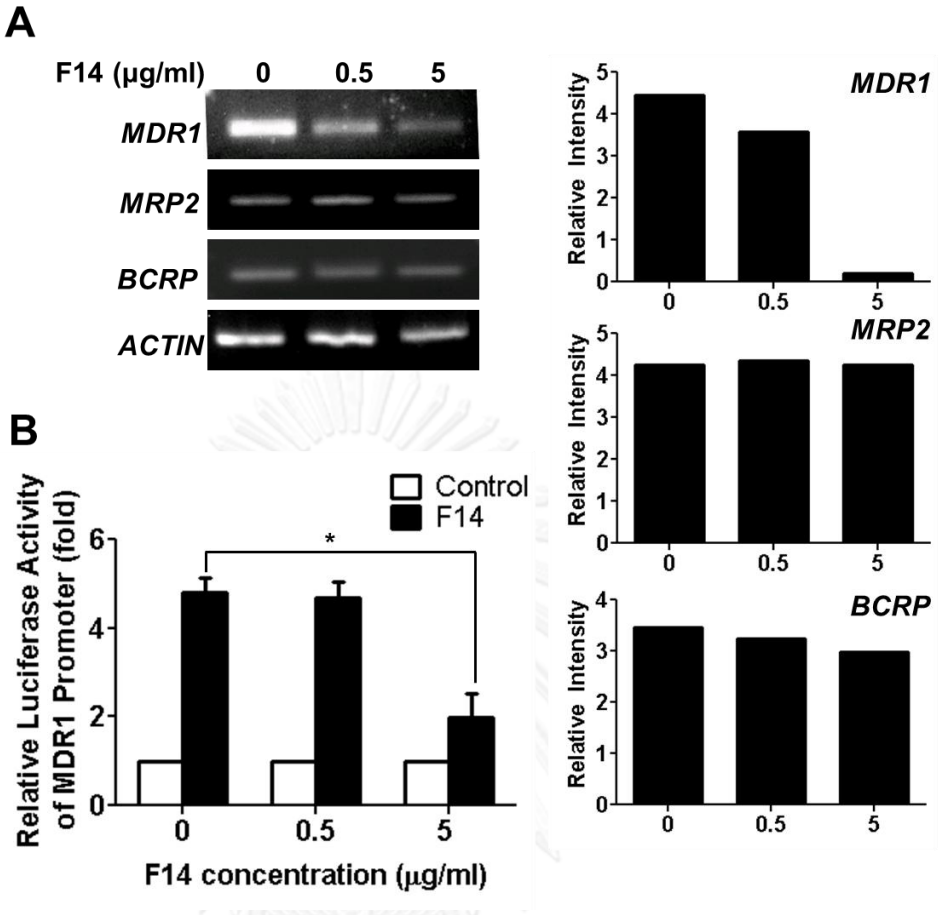


Figure 3.3 F14 fraction reduces MDR1 transcriptional levels and its transcriptional activity in A549RT-eto cells.

(A) Transcripts and relative mRNA ratio of MDR1, MRP2 and BCRP of A549RT-eto cells (B) *MDR1*-promoter luciferase reporter assay of A549RT-eto cells

3.3.3 Inhibition of NF-ΚB sensitized F14-induced apoptosis of A549RT-eto cells through down-regulation of P-gp.

Since we observed enhanced NF-ΚB protein levels and activity in A549RT-eto cells, we explored whether NF-ΚB is involved in resistance to etoposide in A549 cells through up-regulation of P-gp. We thus treated A549RT-eto cells with BAY11-7082 (BAY; 10 μM), an inhibitor of NF-ΚB and examined cell viability with MTT assay. BAY treatment alone did not influence inhibition of cell growth in A549RT-eto cells while F14 extract (1 μg/ml) inhibited approximately 50% cell growth at 24 h post-treatment (Figure 3.4A). Furthermore, we found that the combined treatment with F14 extract and BAY accelerated F14 extract-mediated apoptosis in A549RT-eto cells (Figure 3.4A), which was confirmed by observation of cleaved PARP and pre-caspase 9 (Figure 3.4B). Moreover, when we examined protein levels of NF-ΚB, SIRT1 and P-gp after treatment with Bay alone, F14 extract alone, or Bay plus F14 extract found that BAY treatment alone decreased not only expression levels of NF-ΚB but also P-gp but did not influence protein levels of SIRT1 in the cells (Figure 3.4B). F14 treatment alone drastically diminished protein levels of NF-ΚB, SIRT1 and P-gp in A549RT-eto cells as seen in Figure 3.2A. We also observed that the combined treatment more significantly reduced protein levels of NF-ΚB, SIRT1 and P-gp (Figure 3.4B). These results suggest that NF-ΚB is involved in MDR in A549 cells by up-regulation of P-gp, resulting in resistance to etoposide.

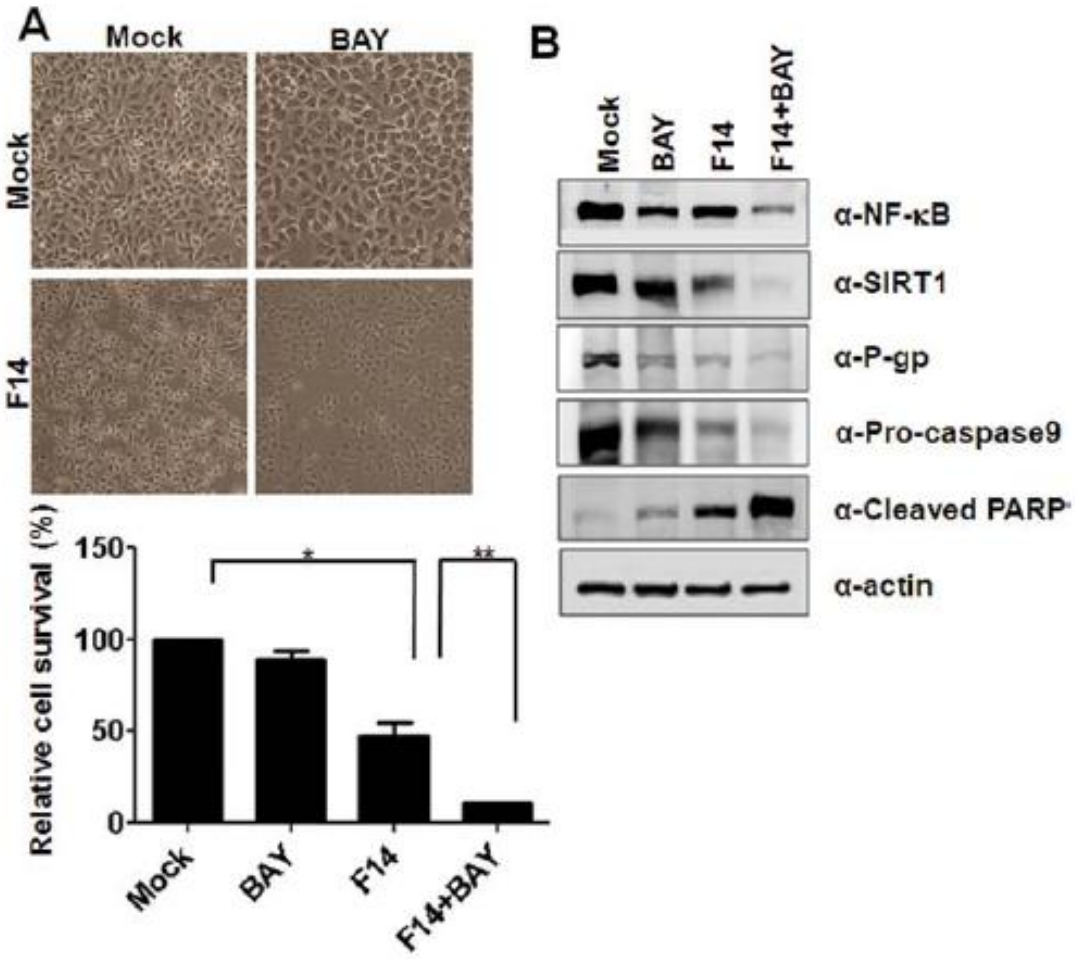


Figure 3.4 BAY 11-7082 treatment suppresses levels of NF-KB, SIRT1, and P-gp proteins, leading to F14-induced apoptosis.

(A) Cell morphology of treated A549RT-eto cells with F14 under light microscope and measuring cell growth by MTT assay (B) Westernblotting assay of treated A549RT-eto cells with F14 and BAY

3.3.4 Suppression of SIRT1 expression does not enhance susceptibility to F14-induced apoptosis of A549RT-eto cells.

We observed that enhanced levels of SIRT1 in A549RT-eto cells (Figure 3.1A and C) and F14 treatment reduced SIRT1 as well as P-gp expression levels (Figure 3.6A and B). We wondered whether down-regulation of SIRT1 protein level is attributed to decrease of P-gp expression, leading to F14 fraction-induced apoptosis. To test this question, we first introduced SIRT1siRNA to suppress SIRT1 levels in A549RT-eto cells. We first optimized SIRT1siRNA concentration (100 nM) (data not shown). We then treated A549RT-eto cells with F14 under suppression of SIRT1 using its siRNA and examined cell viability with MTT assay. Of interest, we found that suppression of SIRT1 did not accelerate F14-induced apoptosis of A549RT-eto cells as seen in combined treatment with Bay and F14 (Figure 3.5A). We also observed that inhibition of SIRT1 activity with nicotinamide (NAM; 500 μM) did not accelerate F14 extract-induced apoptosis of A549RT-eto cells (Figure 3.5B). Furthermore, neither suppression of SIRT1 protein levels itself nor inhibition of SIRT1 activity did significantly affect P-gp expression (Figure 3.5C and D). However, F14 treatment (1 μg/ml) decreased expression of P-gp irrespective of SIRT1 protein levels and activity (Figure 3.5C and D). These results suggest that the elevated levels of SIRT1 are not related to up-regulation of P-gp in A549RT-eto cells.

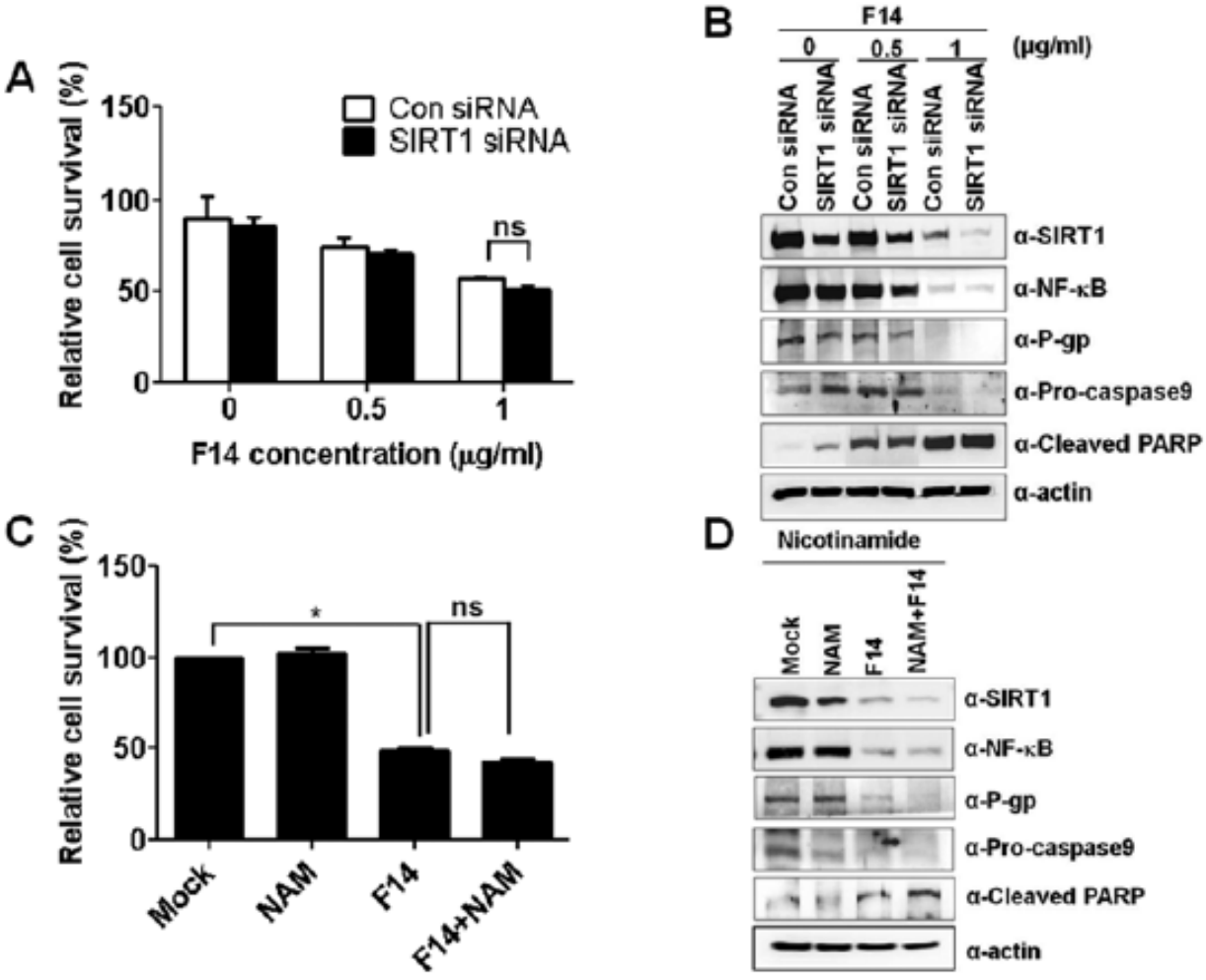


Figure 3.5. Neither SIRT1 suppression nor inhibition does decrease P-gp protein levels and accelerate F14-induced apoptosis.

(A,B) The growth of treated A549RT-eto cells with F14 and SIRT1 siRNA by MTT assay and Western blot assay (C,D) The growth of treated A549RT-eto cells with F14 and NAM by MTT assay and Western blot assay

3.4 Discussion

Medicinal plants are important sources for the potential development of effective anticancer agents [149]. In fact, more than half of the today's anticancer drugs have been originally synthesized from natural products and their derivatives. In this study, we found that a purified fraction (F14) from *Bryophyllum laetivirens* exhibits significant anti-proliferative effects against multidrug resistant A549 cancer cells. Majority of the effective anti-cancer concentrations of plant extracts are greater than 100 μg/ml [149, 150]; however, in this study, cell proliferation was inhibited by treatment with 6.72 μg/ml and the viability was less than 50% after a 12 h exposure (Scheme 3.1). Therefore, the F14 fraction may include a highly effective candidate compound as a future anti-cancer drug. Other study showed that methanol-extracts of *Kalanchoe (Bryophyllum)* hybrid show their cytotoxicity toward MCF-7, NCI-H460, and SF-268 tumor cell lines [151]. Moreover, kalanchosides compounds from *Kalanchoe (Bryophyllum) gracilis* also exhibited significant cytotoxic activity against gastric and nasopharyngeal carcinoma cell lines [152]. However, their studies did not show any detailed mechanisms by which the extracts or compounds from *Bryophyllum* species induce cytotoxicity in various tumor cell lines.

Our study herein demonstrated how F14 fraction from *Bryophyllum laetivirens* sensitizes human lung A549 cells resistant to etoposide to apoptosis. In particular, the F14 reversed A549 cells with multidrug resistance to apoptosis. It was known that several proteins including Ras, Sp1, p53, PKC and NF-**K**B have been involved in the regulation of P-gp expression at the transcriptional level. We herein reported that F14 extract not only diminishes NF-**K**B protein levels but also blocks translocation of NF-**K**B into the nucleus and binding to its binding sites, leading to decrease of MDR1 transcription levels. Furthermore, the combined treatment with F14 fraction and BAY-11-7082 (an irreversible NF-**K**B-specific inhibitor) accelerated apoptosis, suggesting that NF-**K**B is crucial role in MDR and resistance to apoptosis. In addition, since our recent study showed that STAT1 and HDAC4 also play a crucial role in P-gp expression (accepted), we examined whether F14 fraction reduces expression levels of STAT1 and HDAC4 protein. We then found that F14 fraction also decreases protein

levels of STAT1 and HDAC4 (data not shown). Further, we observed that F14 fraction sensitizes etoposide-induced apoptosis in A549RT-eto cells (data not shown). Since we observed that F14 extract targets to multiple proteins including NF-**K**B, STAT1 and HDAC4, we are undertaking search for the detailed mechanism of F14 fraction action.

SIRT1 is a NAD^+-dependent deacetylase which deacetylates histones and nonhistone proteins and has been involved in various biological responses including aging, metabolism and cancer [153, 154]. Since previous studies showed that over-expression of SIRT1 induces the expression of P-gp and leads to resistance to chemotherapy in tumor cells [144, 155], we assumed that an enhanced SIRT1 expression in the A549RT-eto cells might be involved in MDR. We then found that F14 fraction treatment reduces SIRT1 protein levels and its activity. However, we could not know that SIRT1 itself modulates P-gp expression levels because F14 also reduces NF-**K**B expression and its activity, which is involved in regulating transcription of P-gp. To answer this question, we specifically suppressed SIRT1 protein levels by siRNA or inhibited SIRT1 activity with NAM in the absence of F14 extracts. We then found that neither suppression of SIRT1 expression nor its activity did decrease P-gp protein levels, suggesting that SIRT1 itself is not involved in regulation of P-gp expression in A549RT-eto cells at least. Moreover, combined treatment with F14 fraction and SIRT1 siRNA, or NAM did not accelerate apoptosis of A549RT-eto cells compared to F14 fraction alone. When we considered other studies, we might explain that the discrepancy of SIRT1 role in MDR might be attributed to dual functions of SIRT1 on oncogene and tumor suppressor, depending on cellular contexts and subcellular localization of SIRT1 [153, 154]. We still have a question on a role of enhanced SIRT1 protein levels in A549RT-eto cells, which will be a next assignment for the investigation.

3.5 Conclusion

In summary, our study showed that F14, the methanol extract from *B. laetivirens* can reverse etoposide resistance in A549 lung cancer cells though down-regulation of NF-**K**B, leading to decreasing transcription and expression of P-gp, which eventually promotes F14 extract-mediated apoptosis. Since we herein provide an important lead for development of anti-cancer therapeutic drug against MDR cancer, we are definitely under further purification from the *B. laetivirens* leaf extract in order to obtain a single compound as a new and efficacious anti-cancer drug.

CHAPTER IV

FERONIELLIN A-INDUCED AUTOPHAGY CAUSES APOPTOSIS IN MULTIDRUG-RESISTANT HUMAN A549 LUNG CANCER CELLS

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Abstract

During the screening of natural chemicals that can reverse multidrug resistance in human A549 lung cancer cells resistant to etoposide (A549RT-eto), we discovered that Feroniellin A (FERO), a novel furanocoumarin, shows toxicity toward A549RT-eto cells in a dose- and time-dependent manner. FERO reduced the expression of NF-**K**B, leading to downregulation of P-glycoprotein (P-gp), encoded by *MDR1*, which eventually sensitized A549RT-eto cells to apoptosis. FERO specifically diminished transcription and promoter activity of *MDR1* but did not inhibit the expression of other multidrug resistance genes *MRP2* and *BCRP*. Moreover, co-administration of FERO with Bay11-7802, an inhibitor of NF-**K**B, accelerated apoptosis of A549RT-eto cells through decreased expression of P-gp, indicating that NF-**K**B is involved in multidrug resistance. Conversely, addition of Z-VAD, a pan-caspase inhibitor, blocked FERO-induced apoptosis in A549RT-eto cells but did not block downregulation of P-gp, indicating that a decrease in P-gp expression is necessary but not sufficient for FERO-induced apoptosis. Interestingly, we found that FERO also induces autophagy, which is characterized by the conversion of LC3 I to LC3 II, induction of GFP-LC3 puncta, enhanced expression of Beclin-1 and ATG5, and inactivation of mTOR. Furthermore, suppression of Beclin-1 by siRNA reduced FERO-induced apoptosis in A549RT-eto cells and activation of autophagy by rapamycin accelerated FERO-induced apoptosis, suggesting that autophagy plays an active role in FERO-induced apoptosis. Herein, we report that FERO reverses multidrug resistance in A549RT-eto cells and exerts its cytotoxic effect by induction of both autophagy and apoptosis, which suggests that FERO can be a useful anticancer drug for multidrug-resistant lung cancer.

Key words; Feroniellin A, autophagy, apoptosis, multidrug resistance

4.1 Introduction

Medicinal plants have proven to be a rich source of bioactive compounds for therapeutic agents, and currently 75% of prescribed drugs worldwide are derived from plant sources [145]. Feroniellin A (FERO), a novel furanocoumarin is isolated from the roots of *Feroniella lucida*. The chemical structure of furanocoumarins consists of a furan ring fused with coumarin, which is present in many plants. Coumarins possess anticoagulant, antimicrobial, antioxidant, anti-inflammatory, anti-allergic and anticancer properties [156]. Some furanocoumarin derivatives isolated from plants show anticancer activity as topoisomerase I inhibitor [157], an efflux transport inhibitor , or a drug metabolism inhibitor [158, 159]. However, the specific molecular mechanism by which FERO shows anticancer activity has not been revealed.

Multidrug resistance (MDR) is a major problem in cancer therapy and is often the result of overexpression of the drug efflux protein P-glycoprotein (P-gp). P-gp is a 170-kD a protein that belongs to the ATP-binding cassette superfamily of membrane transporter proteins [140, 160]. P-gp is an energy dependent drug efflux pump that maintains intracellular drug concentrations below cytotoxic levels, thereby decreasing the cytotoxic effects of a variety of chemotherapeutic agents [140, 142, 143, 160]. P-gp also plays a role in inhibition of drug accumulation and caspase activation in MDR tumors [7, 8]. Of special note, recent lines of evidence have shown that NF-**K**B- or SIRT1 mediated regulation of P-gp plays a critical role in anticancer drug resistance [74, 144].

Autophagy, an ancient system necessary to maintain homeostasis in eukaryotic cells, degrades long-lived cytoplasmic proteins and organelles and provides nutrients during starvation or stress conditions [80] through programmed processing involving the sequential activity of autophagy related gene (ATG) products. As autophagy is necessary for cellular homeostasis, it is involved in biological processes including development, aging and degeneration [81]. However, aberrant regulation of autophagy is related to many diseases, including cancer and neurodegenerative disorders [82]. As a specific example, the first report connecting

autophagy to cancer showed that allelic loss of the essential autophagy gene *Beclin-1 (BECN1)* is prevalent in human breast, ovarian, and prostate cancers [83], and that *Becn1+/-* mice develop mammary gland hyperplasia and lymphomas as well as lung and liver tumors [84]. Subsequent studies demonstrated that *ATG5-/-* and *ATG7-/-* livers give rise to adenomas [85]. These lines of evidence suggest that autophagy acts as a tumor suppressor in cancer development. Contrary to this, many other reports have shown that autophagy exerts a pro-survival function in tumor cells [86-88]. Additional studies have demonstrated that the inhibition of autophagy by pharmaceutical drugs sensitized cells to apoptotic cell death, and that combination therapies using autophagy inhibitors plus chemotherapy led to faster tumor cell death than did chemotherapy alone [89]. These findings indicate that pro-survival autophagy may represent a major hindrance to successful cancer therapy.

The present study was initiated to screen small molecules derived from plants grown in Thailand in order to reverse MDR in A549RT-eto cells. We have identified that FERO induces autophagy, which is necessary for FERO-induced apoptosis in A549RT-eto cells. Therefore, we propose that FERO represents a powerful candidate for the treatment of multidrug-resistant lung cancer.

4.2 Materials and Methods

4.2.1 Cell cultures

A549RT-eto cells were developed and kindly provided by the Laboratory of Biochemistry, Chulabhorn Research Institute, Thailand and have been described elsewhere [93]. A549RT-eto cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 1% penicillin, and streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air.

4.2.2 Antibodies and Reagents

For immunoblotting, antibodies against Atg5, LC3B (D11), mTOR, Phospho-mTOR (Ser2448), cleaved PARP (Asp214) were acquired from Cell Signaling Biotechnology (Beverly, MA, USA). Anti-P-gp (Calbiochem; San Diego, CA, USA) and actin (C4), BECN1 (H300), BID (FL-195), caspase-9 p35 (H-170), NF-**K**B p65 (F-6), P53 (DO-1), SIRT1 (H-300) and Sp1 (1C6) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rapamycin, Z-VAD and BAY11-7082 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Feroniellins A was isolated from the roots of *Feroniella lucida* and kindly provided by Natural Products Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Thailand as described elsewhere [161].

4.2.3 Immunoblotting

Cells were harvested and lysed with lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 7.5)) containing 0.1 mM Na_2VO_3, 1 mM NaF and protease inhibitors (Sigma). For immunoblotting, proteins from whole cell lysates were resolved by 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes., Primary antibodies were used at 1:1000 or 1:2000 dilutions, and secondary antibodies conjugated with horseradish peroxidase were used at 1:2000 dilutions in 5% nonfat dry milk. After the final washing, nitrocellulose

membranes were exposed for an enhanced chemiluminescence assay using the LAS 4000 mini (Fuji, Tokyo, Japan).

4.2.4 Nuclear NF-**K**B pull-down assay

A549RT-eto cells (1×10^6cells/ml) were incubated with FERO or DMSO as control for 12 h and nuclear extracts were prepared. Cells were pelleted and resuspended in 0.4 mL hypotonic lysis buffer (20 mM HEPES [pH 7.9], 10 mM KCl, 1 mM EDTA, 0.2% Triton X-100, and 1 mM Na_2VO_3 plus protease inhibitors) and kept on ice for 20 min. After centrifugation at 14000 *g* for 5 min at 4°C, the nuclear pellet was extracted with 0.1 mL hypertonic lysis buffer on ice for a further 20 min. After centrifugation at 14000 *g* for 5 min at 4°C, the supernatants were diluted to 100 mM NaCl and incubated with 25 µL of agarose beads conjugated to a consensus NF-**K**B binding oligonucleotide (Santa Cruz Biotech.) for 1 h at 4°C. After 3 washes, sample buffer was added and boiled for 5 min. The binding NF-**K**B (p65) protein to the oligonucleotide conjugated with agarose was detected by immunoblotting using an anti-p65 NF-**K**B Ab (Santa Cruz Biotech.).

4.2.5 Short interference RNA transfection

Cells were trypsinized and incubated overnight to achieve 60-70% confluency before siRNA transfection. Beclin-Human siRNA (commercially pre-made at Bioneer, Daejeon, Korea; 100 nM; sense, 5'-UGGAAUGGAAUGAGAUUAA(dTdT)-3'; antisense, 5'-UUAAUCUCAUUCCAUUCCA(dTdT)-3' or negative control siRNA (Bioneer); 100 nM; sense, 5'-CCUACGCCACCAUUUCGU(dTdT)-3'; antisense,5'-ACGAAAUUGGUGGCGUAGG (dTdT)-3' were mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were incubated with the transfection mixture for 24 h and then rinsed with RPMI-1640 medium containing 10% fetal bovine serum. The cells were incubated for 48 h before harvest.

4.2.6 Reverse transcriptase-Polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Three micrograms of total RNA were converted to cDNA using Superscript II reverse transcriptase (Invitrogen), and PCR was performed using the specific primers: human *MDR1*: sense 5′-CCCATCATTGCAATAGCAGG-3′ and anti-sense 5′-GTTCAAACTTCTGCTCCTGA-3′, *MRP2*: sense 5′-ACAGAGGCTGGTGGCAACC-3′ and anti-sense 5′-ACCATTACCTTGTCACTGTCC-3′, *BCRP*: sense 5′-GATCACAGTCTTCAAGGAGATC-3′ and anti-sense 5′-CAGTCCCAGTACGAC TGTGACA-3′were used. The cDNAs of each sample were diluted, and PCR was run at the optimized cycle number. Beta-actin mRNA was measured as an internal standard. After amplification, the products were subjected to electrophoresis on 2.0% agarose and detected by ethidium bromide staining.

4.2.7 Luciferase reporter assay

A549RT-eto cells were transfected with hMDR1-luciferase or pGL3 empty vector as a control luciferase vector. To normalize transfection efficiency, a pGK-βgal vector that expresses-galactosidase from a phosphoglucokinase promoter was included in the transfection mixture. FERO was added to the transfected cells at 12 h before harvest. At 48 h post-transfection, cells were washed with cold PBS and lysed in lysis solution (25 mM Tris (pH7.8), 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton-X100). Luciferase activity was measured with a luminometer by using a luciferase kit (Promega, Madison, WI, USA).

4.2.8 Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for the measurement of cell survival as previously described [134]. Dye solution containing tetrazolium was added to the cells in the 96-well plate and incubated for 2 h. The absorbance of the formazan produced by living cells was

measured at 570 nm. The relative percentage of cell survival was calculated by the mean absorbance of the treated cell (OD_T) and the mean absorbance of control cell (OD_C) with formula, % Cell survival = (OD_T / OD_C).

4.2.9 Statistical Analysis

Data are presented as a means + standard deviation (S.D.). The Student's *t*-test was used for statistical analysis, with p value < 0.05 defined as significance.

4.3 Results

4.3.1 Feroniellin A induces apoptosis in human A549 lung cells resistant to etoposide.

Previous observations have revealed that human etoposide resistant A549 lung cancer cells (A549RT-eto) exhibit upregulation of STAT1 and HDAC4, leading to elevated levels of P-glycoprotein (P-gp) encoded by multidrug resistance (*MDR*) 1 (unpublished data). Thus, we performed a screen for small molecules derived from medicinal plants in Thailand that reverse MDR. We found that a small compound known as Feroniellin A (FERO) (molecular structure shown in Figure 4.1A) that efficiently induced death in A549RT-eto cells in a dose- and time-dependent manner (Figure 4.1B). We found that parental A549 cells showed more cytotoxicity toward FERO as well (data not shown). Since it has been reported that NF-**K**B and SIRT1 are involved in the regulation of MDR through P-gp protein levels [162]. We sought to examine protein levels of NF-**K**B, SIRT1 and P-gp in A549RT-eto cells treated with 0.05 and 0.5 mM FERO 12 h post-treatment. Our findings show that FERO treatment reduces expression levels of NF-**K**B, SIRT1 and P-gp and enhances expression levels of P53, a representative tumor suppressor protein (Figure 4.1C). In addition, we detected reduced pre-caspase-9 and pre-Bid levels in the same lysates of A549RT-eto cells, indicating induction of intrinsic apoptosis (Figure 4.1C).

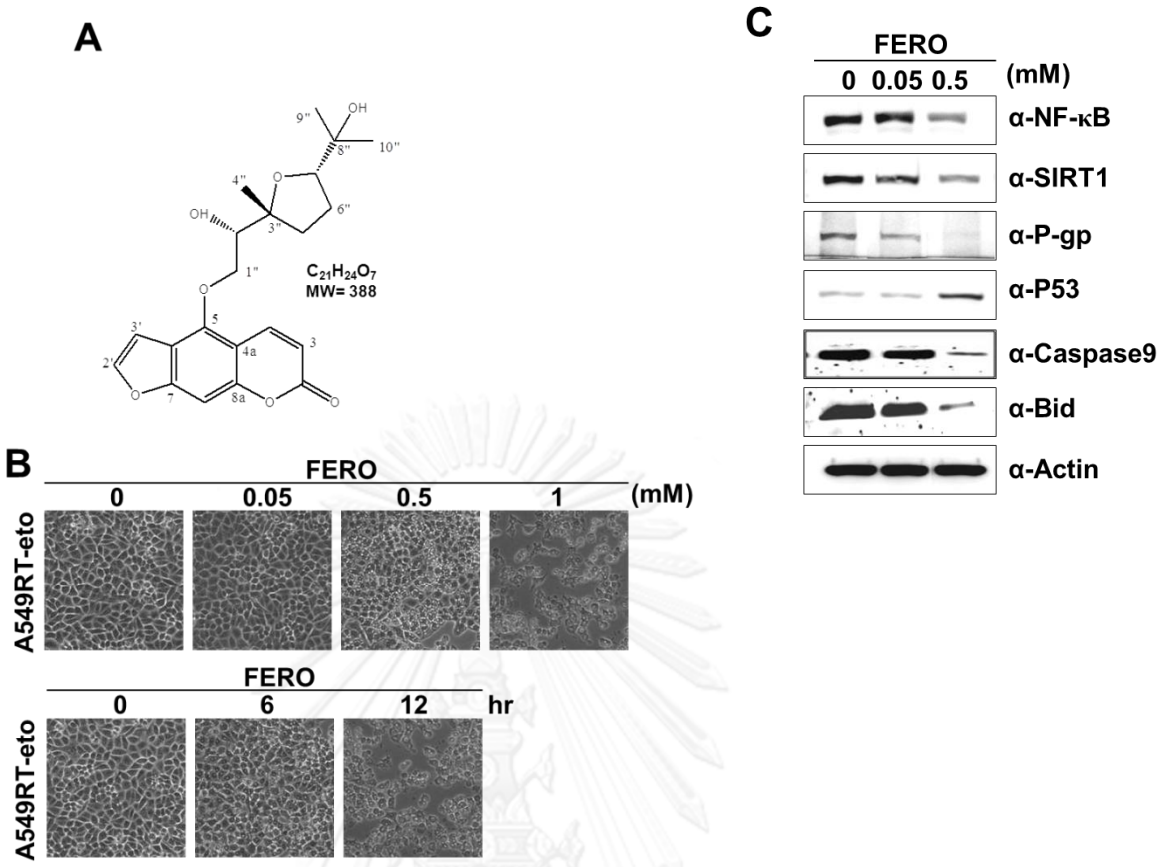

Figure 4.1 Chemical structure of Feroniellin A (FERO) and its effect on cell viability and protein levels in A549RT-eto cells.

(A) The chemical structure and molecular weight of Feroniellin A (FERO). (B) A549RT-eto cells were treated with FERO (0, 0.05, 0.5 and 1 mM) for 12 h and were treated with FERO (1 mM) at 0, 6 and 12 h. After treatment, the morphological changes of cells and cell viability were observed under a light microscope. (C) A549RT-eto cells were treated with FERO (0, 0.05 and 0.5 mM) for 12 h. Cell lysates from A549RT-eto cells treated with FERO were prepared and separated by 12% SDS-PAGE. The expression of pro-Bid and pro-caspase-9 proteins was detected by immunoblotting to confirm apoptosis. Protein levels of NF-ΚB, SIRT1, P53, and P-gp were examined by immunoblotting using the corresponding antibodies.

4.3.2 Feroniellin A reduces MDR1 transcript levels and promoter activity in A549RT-eto cells.

In addition to the *MDR1* gene, other genes such as multidrug resistance-associated protein (*MRP*) *2* and breast cancer resistance protein (*BCRP*) are known to be involved in drug resistance [147, 148], Therefore, we examined transcript levels of *MDR1, MRP2* and *BCRP* in A549RT-eto cells during FERO treatment. RNA was isolated and cDNA generated from A549RT-eto cells treated with 0.05 and 0.5 mM FERO for 12 h and genes were amplified using an optimized number of cycles. We found a significant reduction in *MDR1* transcript levels with slight reductions in *MRP2* levels following FERO treatment in a dose-dependent manner (Figure 4.2A). However, transcript levels of *BCRP* were not reduced by FERO treatment (Figure 4.2B). In addition to transcript levels, we also examined *MDR1* promoter activity in A549RT-eto cells during FERO treatment using an *MDR1*-promoter luciferase reporter vector [74]. FERO treatment induced a dramatic decrease of *MDR1*-mediated luciferase activity in A549RT-eto cells (Figure 4.2B), indicating that FERO reduces *MDR1* promoter activity. Since it has been reported that the transcription factor NF-**K**B is involved in the regulation of P-gp [163, 164], we next examined the nuclear localization of NF-**K**B and its DNA binding activity during FERO treatment. We found that FERO treatment inhibits translocation of NF-**K**B from the cytoplasm to the nucleus, which results in lowered levels of NF-**K**B at its binding site (Figure 4.2C). On the basis of these results, we suggest that FERO inhibits *MDR1* transcription, resulting in a decrease in P-gp protein levels, which leads to sensitization to apoptosis in A549RT-eto cells.

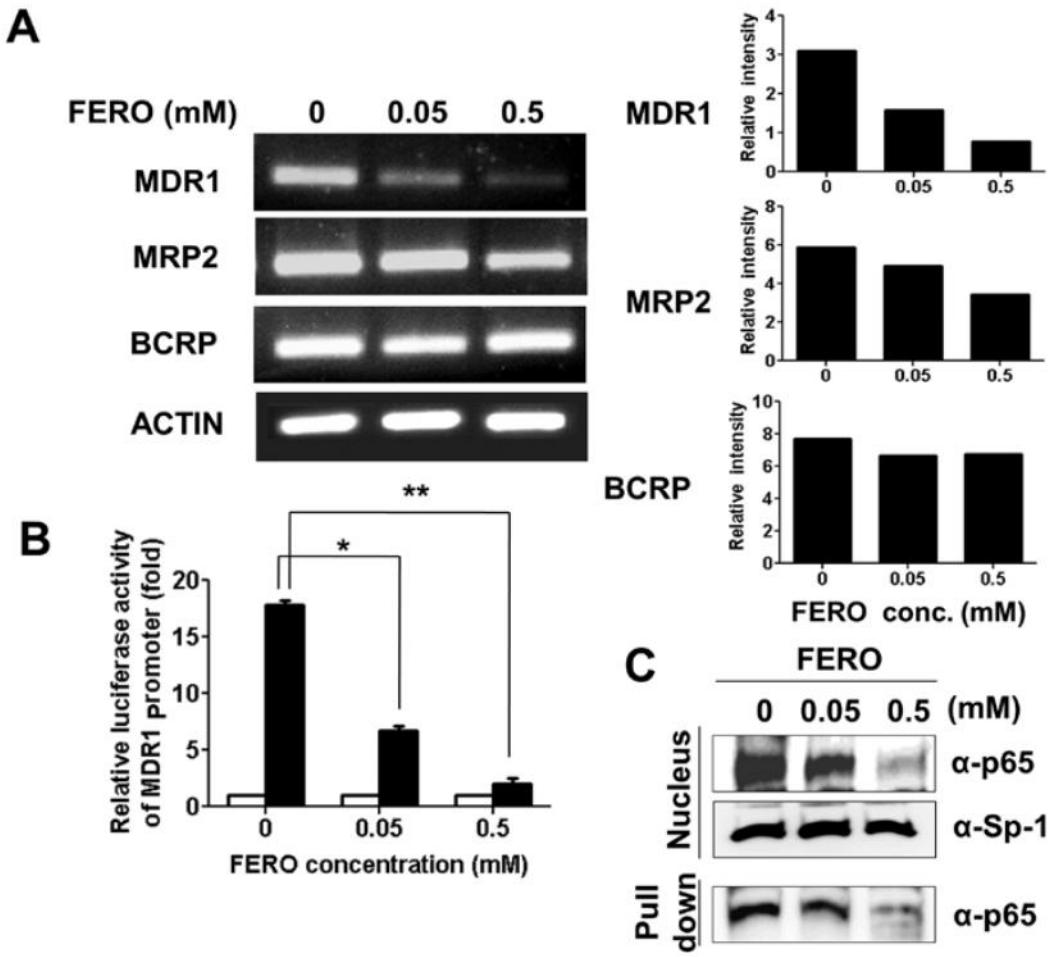

Figure 4.2. FERO suppresses MDR1 transcription and promoter activity and NF-ΚB transcriptional activity.

(A) Transcripts of *MDR1, MRP2* and *BCRP* of A549RT-eto cells following FERO treatment (0, 0.05 and 0.5 mM) for 12 h (B) *MDR1*-luciferase activity of A549RT-eto cells following FERO treatment (0, 0.05 and 0.5 mM) for 12 h (C) NF-ΚB oligonucleotides conjugated to agarose were added to nuclear extracts from A549RT-eto cells treated with FERO (0, 0.05 and 0.5 mM) and the precipitation mixtures were isolated after centrifugation. The binding of NF-ΚB (p65) to the oligonucleotide was detected by immunoblotting using anti-NF-ΚB (p65) antibody.

4.3.3 Inhibition of NF-ΚB accelerates FERO-induced apoptosis of A549RT-eto cells through downregulation of P-gp.

Because we observed enhanced NF-ΚB protein levels and activity in A549RT-eto cells, we next explored whether NF-ΚB is involved in resistance to etoposide in A549 cells through upregulation of P-gp. A549RT-eto cells were treated with 10 BAY11-7082 (BAY), an inhibitor of NF-ΚB, and examined for cellular viability using the MTT assay. BAY treatment alone did not influence inhibition of cell growth in A549RT-eto cells, while 0.05 mM FERO treatment inhibited cell growth by ~30% at 24 h post-treatment compared to DMSO controls (Figure 4.3A). Furthermore, we found that a combined treatment of FERO and BAY accelerated FERO-mediated apoptosis in A549RT-eto cells (Figure 4.3A), which was confirmed by observations of cleaved PARP and pre-Bid (Figure 4.3B). Moreover, when we examined protein levels of NF-ΚB, SIRT1 and P-gp after treatment with BAY alone, FERO alone, or BAY plus FERO, we found that BAY treatment alone decreased not only expression levels of NF-ΚB but also P-gp, but did not influence protein levels of SIRT1 in the cells (Figure 4.3B). FERO treatment alone was shown to drastically diminish protein levels of NF-ΚB, SIRT1 and P-gp in A549RT-eto cells (Figure 4.1C). We also observed that the combined treatment resulted in more significantly reduced protein levels of NF-ΚB, SIRT1 and P-gp (Figure 4.3B). These results suggest that NF-ΚB is involved in MDR in A549 cells by upregulation of P-gp, resulting in resistance to etoposide. To examine whether activation of caspase caused by FERO treatment is involved in apoptotic cell death in A549RT-eto cells, a pan-caspase inhibitor, Z-VAD, was used. FERO treatment alone at 0.5 mM resulted in a significant induction of apoptotic cell death at 24 h post-treatment, while the combined treatment with FERO and Z-VAD blocked FERO-induced apoptotic cell death (Figure 4.4A), which was confirmed by a lack of cleavage of PARP and pre-Bid proteins (Figure 4.4B). These results indicate that activation of caspases is involved in FERO-induced apoptotic cell death in A549RT-eto cells. However, Z-VAD treatment did not block downregulation of P-gp expression induced by FERO, indicating that a decrease of P-gp expression is necessary but not sufficient for apoptotic cell death.

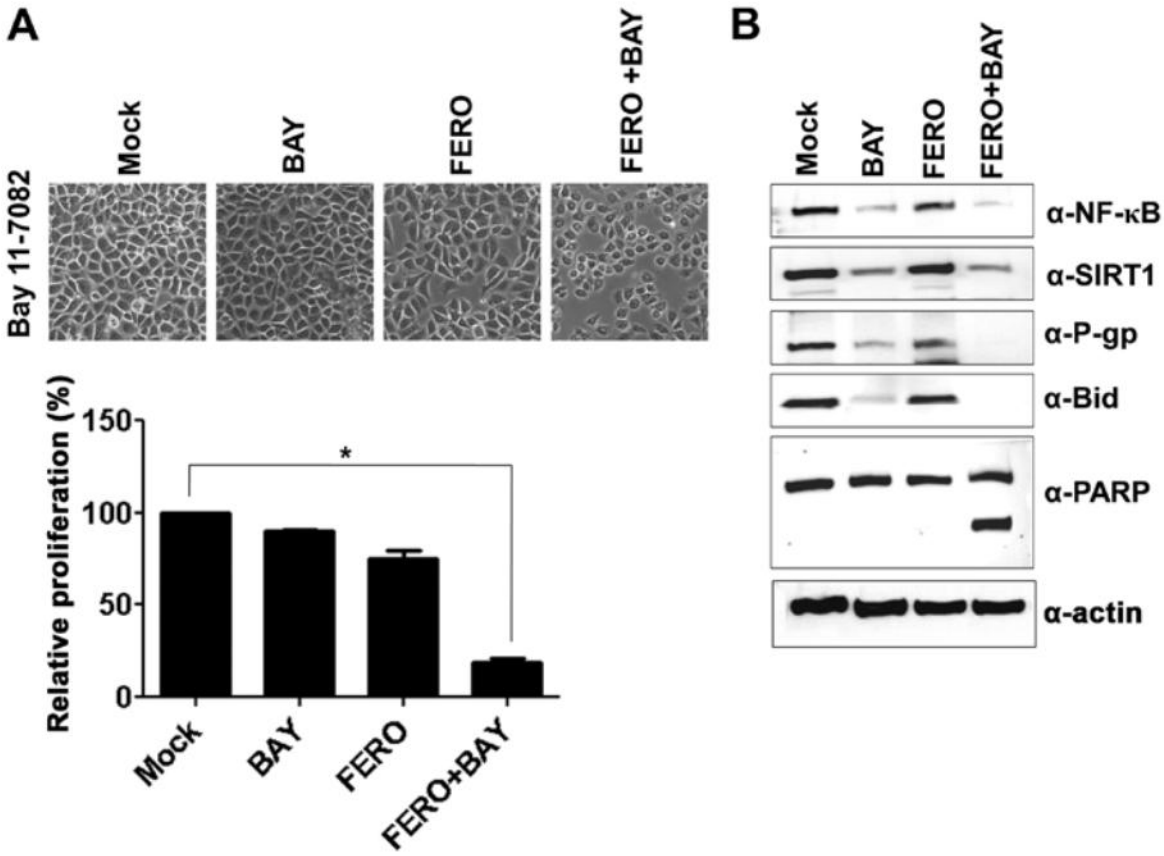

Figure 4.3. NF-ΚB inhibition enhances FERO-induced apoptosis by downregulation of P-gp.

(A) A549RT-eto cells were treated with FERO (0.25 mM) alone, Bay11-7082 (BAY; 2 μM) alone, and FERO plus BAY for 24 h. Changes in cellular morphology were observed under a light microscope and cell growth was measured by MTT assay. Data were calculated as percent of relative cell viability and expressed as the average of three experiments (*p<0.01, Mock vs. FERO+BAY). (B) Cell lysates from the treated A549RT-eto cells were prepared and separated by 12% SDS-PAGE. The expression of pre-Bid and PARP protein was detected for apoptosis by immunoblotting, Protein levels of NF-ΚB, SIRT1 and P-gp were examined by immunoblotting using the corresponding antibodies.

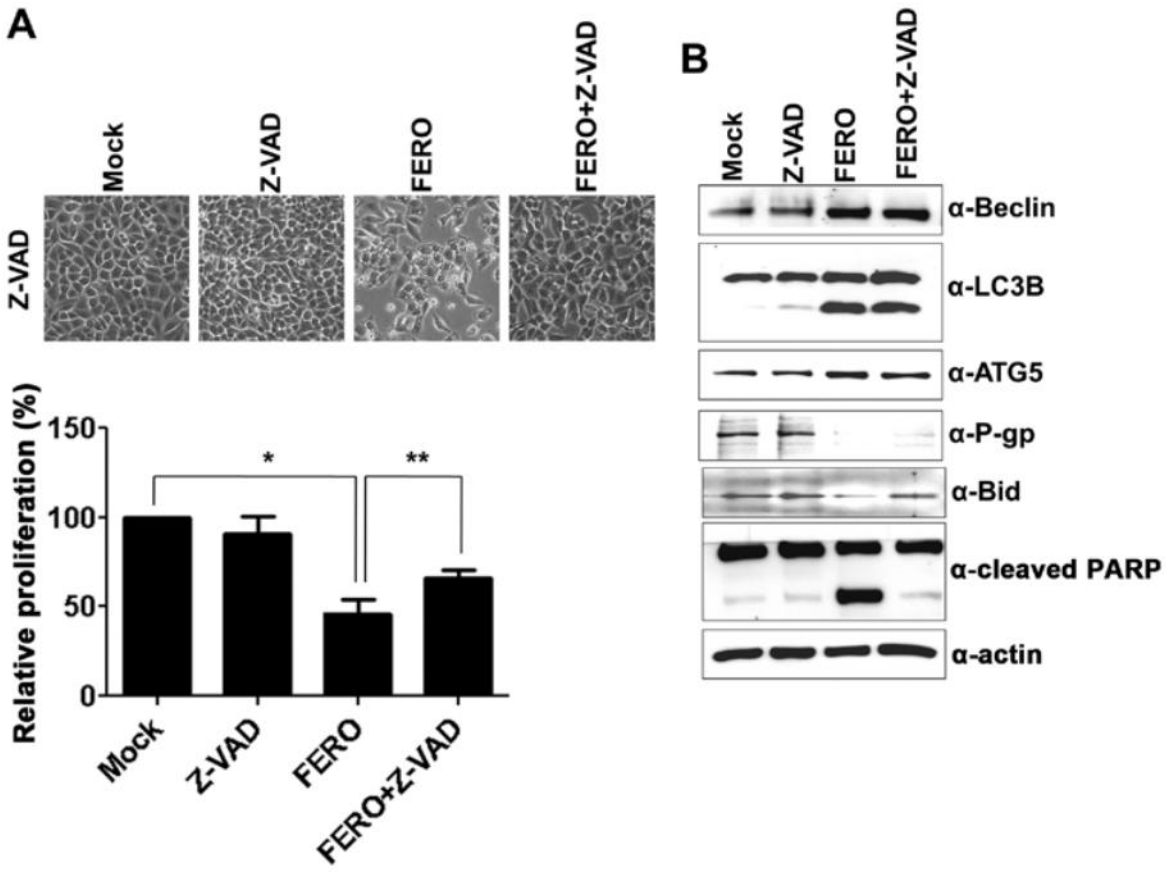

Figure 4.4. Inhibition of caspase activity protects A549RT-eto cells from FERO-induced apoptosis.

(A) A549RT-eto cells were treated with FERO (0.5 mM) alone, Z-VAD (20 μM) alone, or FERO plus Z-VAD for 24 h. Changes in cellular morphology were observed under a light microscope and cell growth was measured by MTT assay. Data were calculated as percent of relative cell viability and expressed as the mean of at least three experiments (*$p<0.05$, Mock vs. FERO; **$p<0.05$, FERO vs. FERO+Z-VAD). (B) Cell lysates from the treated A549RT-eto cells were prepared and separated by 12% SDS-PAGE. Expression of pro-Bid and PARP protein was detected by immunoblotting for apoptosis and protein levels of Beclin-1, LC3B, ATG5, mTOR and P-gp were compared by immunoblotting using the corresponding antibodies.

4.3.4 FERO also induces autophagy in A549RT-eto cells.

Autophagy has a dual role in cancer, as a tumor suppressor and a pro-tumor cell survival mechanism [165, 166]. Therefore, we investigated whether FERO could induce autophagy, which is characterized by the formation of vacuoles, GFP-LC3 puncta, and LC3 I to LC3 II conversion. The presence of vacuoles was apparent in A549RT-eto cells treated with 0.5 mM FERO (Figure 4.5A). There was also a clearly observable accumulation of GFP-LC3 II puncta in A549RT-eto cells treated with FERO, while no GFP-LC3 puncta were present in A549RT-eto cells treated with DMSO (Figure 4.5B). Moreover, we observed LC3 I to LC3 II conversion in FERO-treated A549RT-eto cells in a dose-dependent manner (Figure 4.5C). Lastly, we found induction of Beclin-1, required for the formation of autophagic vesicles [167], and reduction of phospho-mTOR levels, an inhibitor of autophagy, indicating that FERO treatment clearly induced autophagy in A549RT-eto cells (Figure 4.5C).

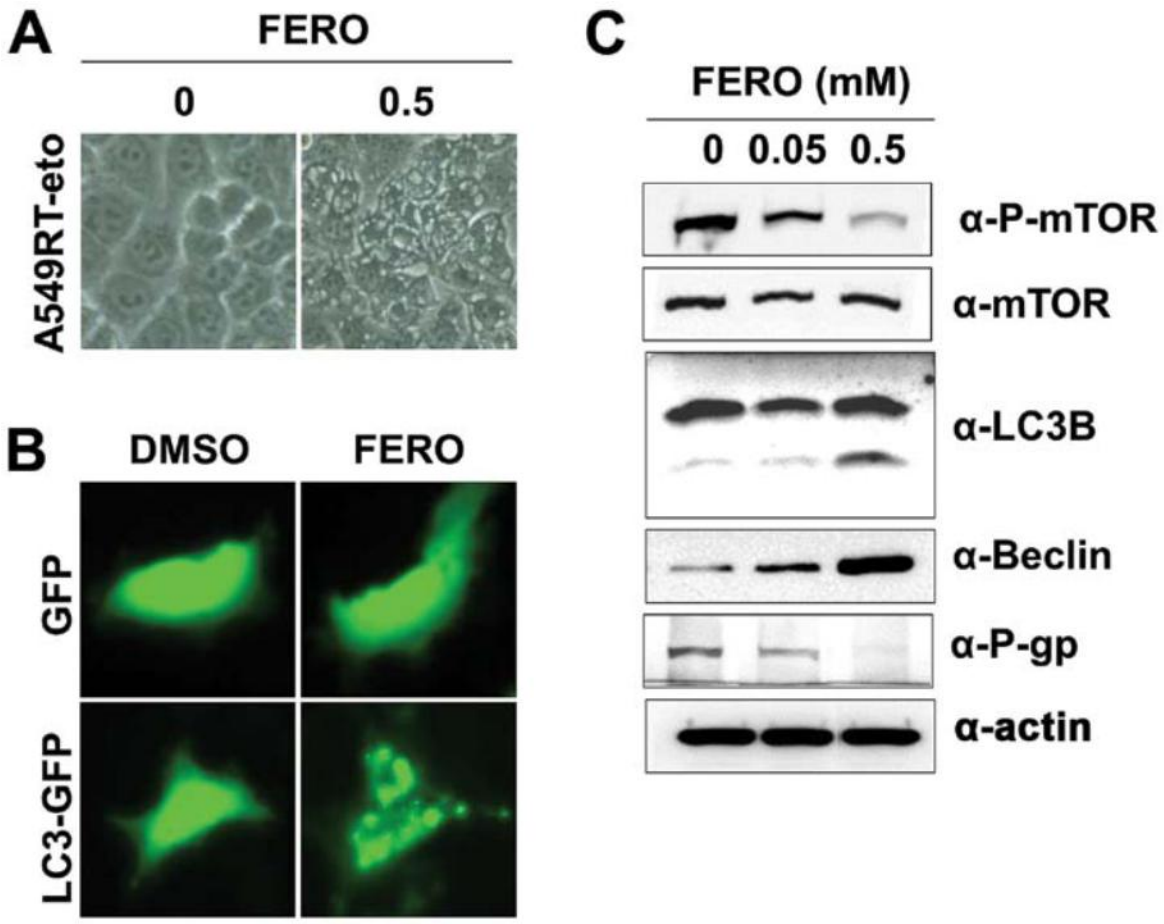

Figure 4.5 Induction of autophagy in A549RT-eto cells by treatment with FERO.

(A) Morphology of A549RT-eto cells was observed under a light microscope at 12 h following treatment with FERO (0.5 mM) (x40 magnification). (B) A549RT-eto cells were transfected with pEGFP-LC3B or pEGFP control vector and then treated with FERO (0.5 mM) or DMSO as a control at 24 h post-transfection. GFP puncta were then analyzed using a fluorescence microscope at 24 h post-treatment. (C) Cell lysates from A549RT-eto cells treated with FERO (0, 0.05 and 0.5 mM) were prepared at 12 h post-treatment and separated by 12% SDS-PAGE. LC3B I cleavage, Beclin-1, P-gp, phospho-mTOR and mTOR proteins were examined by immunoblotting using the corresponding antibodies.

4.3.5 Modulation of autophagy regulates FERO-induced apoptosis in A549RT-eto cells.

Expression of Beclin-1 is known to be essential for double-membrane autophagosome formation required during the initial steps of autophagy [167]. Therefore, we tested whether autophagy impairment via the suppression of Beclin-1 hinders or accelerates FERO-induced apoptosis. We first determined the optimal siRNA concentration for suppression of Beclin-1 expression, which was found to be 100 nM (data not shown). FERO treatment with control siRNA induced apoptosis by activation of caspase-3 or -7 due to cleavage of PARP in a dose-dependent manner (Figure 4.6A). Concomitantly, FERO induced autophagy coincided with an upregulation of Beclin-1 and ATG5, increased LC3 I to LC3 II conversion, and a reduction of phospho-mTOR levels in A549RT-eto cells. However, suppression of *Beclin-1* with siRNA inhibited FERO-induced apoptosis, indicating that autophagy is necessary for FERO-induced apoptosis. Interestingly, we found that suppression of *Beclin-1* by siRNA inhibited the conversion of LC3 I to LC3 II but did not inhibit upregulation of ATG5 expression induced by FERO. These results indicate that conversion of LC3 I to LC3 II occurs later and ATG5 mediated processes take place earlier than Beclin-1 mediated autophagy following FERO treatment (Figure 4.6B). In addition, similar to the observation that Z-VAD suppresses FERO-induced apoptosis but does not block downregulation of P-gp, we also found that suppression of Beclin-1 does not block FERO-mediated decrease of P-gp expression despite the inhibition of FERO-induced apoptosis. This result indicates that downregulation of P-gp expression is necessary but not sufficient for the progression of apoptosis. To verify that autophagy is necessary for FERO-induced apoptosis, we administered rapamycin (RAPA), an inhibitor of mammalian target of rapamycin (mTOR), in order to accelerate A549RT-eto cell autophagy in the presence of FERO [168, 169]. FERO induced apoptosis in A549RT-eto cells as well as dual administration of FERO and RAPA was observed to enhance apoptosis (Figure 4.7A). Co-treatment of FERO and RAPA induced autophagy by upregulating Beclin-1 and ATG5 expression, and converting LC3 I to LC3 II (Figure 4.7B). These results suggest that FERO-induced autophagy promotes apoptotic cell death in A549RT-eto cells.

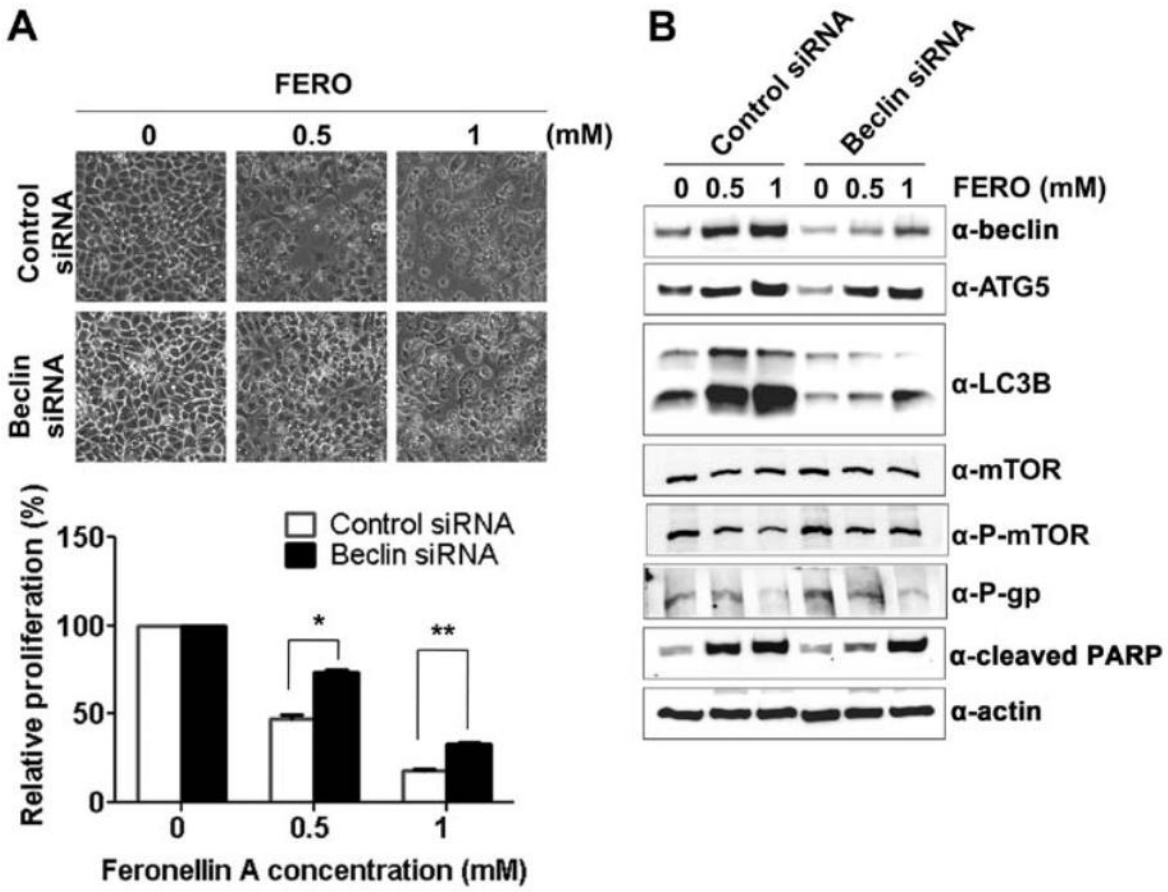

Figure 4.6 Inhibition of autophagy by Beclin-1 siRNA transfection reduces FERO-induced apoptosis in A549RT-eto cells.

(A) A549RT-eto cells were treated with FERO (0, 0.5 and 1 mM) for 24 h following transfection with control or *Beclin-1* siRNAs (100 nM). Cell viability was observed under a light microscope and measured using the MTT assay. Data were calculated as percent of relative cell viability and expressed as the mean of three experiments (*p<0.05 control siRNA vs *Beclin-1* siRNA at FERO 0.5 mM; **p<0.05; control siRNA vs *Beclin-1* siRNA at FERO 1 mM). (B) Cell lysates from the treated A549RT-eto cells were prepared and separated by 12% SDS-PAGE. Cleavage of PARP protein was detected for apoptosis by immunoblotting. Protein levels of Beclin-1, ATG5, mTOR, P-mTOR and P-gp, and cleavage of LC3 I were examined by immunoblotting using the corresponding antibodies.

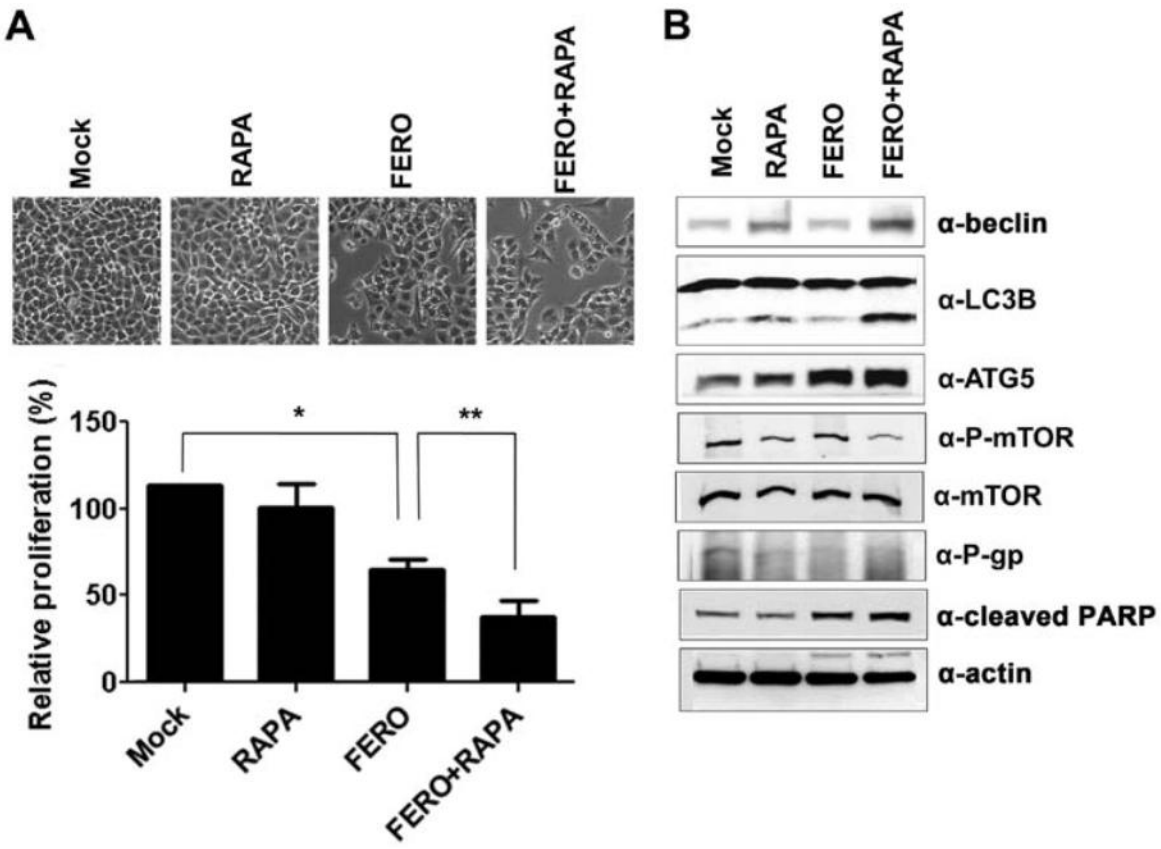

Figure 4.7 Activation of autophagy by rapamycin increases FERO-induced apoptosis in A549RT-eto cells.

(A) A549RT-eto cells were treated with FERO (0.25 mM) alone, rapamycin (RAPA; 10 nM) alone and FERO plus RAPA for 24 h. Cellular morphology was observed under light a microscope and cell growth was measured by MTT assay. Data were calculated as percent of relative cell viability and expressed as the mean of three experiments (*$p<0.01$, Mock vs FERO; **$p<0.05$; FERO vs FERO+RAPA). (B) Cell lysates from the treated A549RT-eto cells were prepared and separated by 12% SDS-PAGE. Expression levels of Beclin-1, ATG5, phospho-mTOR, mTOR and P-gp were examined by immunoblotting using the corresponding antibodies. Cleavage of PARP and LC3 I was detected by immunoblotting using the corresponding antibodies.

4.4. Discussion

Presently, identification of effective chemotherapeutic agents in phytochemicals of great interest to cancer biologists, because of MDR in cancer cells, which are developed following prolonged treatment with anticancer drug. Feroniellin A (FERO) was reported to possess *in vitro* cytotoxicity in human KB carcinoma and HeLa carcinoma cells [161]. However, the molecular mechanism by which this compound exerts cytotoxicity in these cancer cell lines is unknown. For the first time, we have provided a line of evidence showing that FERO reverses MDR1 activity by downregulation of NF-κB, which leads to enhanced apoptotic susceptibility in A549RT-eto cells. Consistent with our results, other studies have shown that metformin, mollugin, and puerarin, which are derived from plants also reverse MDR1 activity effectively through downregulation of NF-κB in human breast cancer cells resistant to adriamycin [163, 170, 171]. Moreover, our study shows that inhibition of apoptosis with Z-VAD does not decrease P-gp expression, indicating that downregulation of P-gp is necessary but not sufficient for apoptosis. As SIRT1, β-catenin and HIF-1α have also been associated with MDR in many cancer models [172-174], we are currently investigating whether FERO affects the expression levels and activity of these proteins, rendering A549RT-eto cells susceptible to apoptosis. Detailed reviews of autophagic progress can be found as described elsewhere [166, 175]. Briefly, autophagy is initiated by activation of the ATG1 complex, which includes ATG1/ATG13/ATG17, among other components. Next, autophagosome nucleation occurs, which requires class III phsophatidylinositol-3-kinase plus Beclin-1/ATG6 in addition to several other factors to recruit proteins and lipids involved in autophagosome formation. Vesicle elongation and completion are mediated by two-ubiquitin-like systems: ATG7 (E1-like) and ATG3 (E2-like), which are necessary for the lipid modification of LC3 (phosphatidylethanolamine; PE), which requires initial cleavage of LC3. Subsequently, the ATG12/ATG5/ATG16 complex mediates LC3-PE binding to the autophagosome membrane. Finally, the completed autophagosome fuses with lysosomes, where the autophagosome contents are degraded. We observed that suppression of Beclin-1 in the presence of FERO results in the inhibition of LC3 I to LC3 II conversion but does not inhibit ATG5 expression.

Considering the sequential progress of autophagy, we assume that LC3 I to LC3 II conversion is very closely linked to Beclin-1 activity but that suppression of Beclin-1 itself does not affect FERO-induced ATG5 expression. Although we have observed that autophagy impairment such as *Beclin-1* suppression with siRNA generated reactive oxygen species (ROS) (data not shown) as reported elsewhere [176, 177], we also found that autophagy impairment with Beclin-1 suppression in fact protects cells from FERO-induced apoptosis. Based on this finding, we speculate that the amount of ROS generated by autophagy impairment does not influence aggravation of A49RT-eto cell survival. In addition, apoptosis inhibition with Z-VAD did not block FERO-induced autophagic progress such as upregulation of Beclin1 and ATG5, and converting LC3 I to LC3 II (Figure 4.4B), indicating that autophagic progress is the upstream event of apoptosis. Recently, autophagy inhibitors such as chloroquine (CQ) or hydroxychloroquine have been used in clinical trials because autophagy is believed to affect tumor survival [178-180]. Therefore, it is possible that conventional chemotherapy with autophagy inhibitors may increase tumor cytotoxicity. In a mouse prostate cancer model, co-treatment with an Src family kinase inhibitor and CQ increased tumor cell numbers *in vitro* but reduced tumor cell growth *in vivo* [181]. Conversely, since it has been suggested that autophagy also plays a role as a tumor suppressor, introduction of autophagy stimulants such as mTOR inhibitors with conventional chemotherapeutic drugs leads to accelerated tumor cell death compared to treatment with anticancer drugs alone [182, 183]. Since there are conflicting reports as to the positive and negative effects of autophagy on tumor cell death [165, 166], the field needs to be cautious when attempting to manipulate autophagy to improve clinical outcomes. In conclusion, our study has demonstrated that FERO-induced autophagy is required for apoptotic progression and suggests that autophagy plays a role as a tumor suppressor in our model.

PART III

THESIS CONCLUSION

3.1 Conclusion

The biological activities, namely *in vitro* cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities of 52 species of Thai medicinal plants were studied. From the results, the four plant extracts, namely *Bauhinia strychnifolia* (Vine), *Coscinum fenestratum* (leaf), *Eurycoma longifolia* (root) and *Bryophyllum pinnata* (leaf) certainly showed merits further to isolate the potentially promising bioactive component(s). For the antioxidant activity, the extracts from only six plant species were found to have an effective DPPH radical scavenging activity, ranging in the order (lowest to highest EC_{50} value) of *Sonneratia caseolaris* leaves, *Coccoloba uvifera* leaves, *Sonneratia alba* leaves, *Lumnitzera littorea* leaves, *Allium sativum* bulbs and *Dolichandrone spathacea* leaves. Nevertheless, the lipase inhibitory and antimicrobial activities from all the 52 extracts were weak comparing to that for their positive control. Therefore, this work focused mainly on *in vitro* cytotoxicity against 5 types of cancer cell lines since four types of plant extracts (mentioned above) were found to efficiently exert a strong activity as well as chemotherapy drugs. Although these potent plants showed cytotoxicity against various types of cancer cell lines, multidrug resistant (MDR) cancer is major obstacle to cancer treatment. In the subsequent work, the mechanism of MDR cancer using the etoposide resistance in human lung A549 cancer cells (A549RT-eto) as a model was therefore studied. From the results, A549RT-eto cells showed more resistance to etoposide–induced apoptosis compared to A549 cells. Of interest, we found that A549RT-eto cells exhibited higher levels of HDAC4, phospho-STAT1 and P-glycoprotein (P-gp) encoded in *MDR1*, than A549 cells.

Based on this result, we concluded that STAT1 and HDAC4 are potential therapeutic targets for treatment of chemotherapeutic resistant lung cancer cells overexpressing P-gp. Furthermore, there have been a number of potent plants reported to overcome MDR cancer problem by down-regulation of the overexpressed P-gp in MDR cancer cells. When the cytotoxicity of 4 selected plants (publication 1)

were tested, the leaf extract from *B. pinnata* showed a strong *in vitro* cytotoxicity against both normal lung (A549) and their MDR (A549RT-eto) cancer cell lines. However, since the antitumor compounds of *B. pinnata* have been isolated and identified already, this study therefore focused on the isolation of the active compounds from *B. laetivirens*. From the result, the obtained isolated methanol extract, F14 also showed a strong cytotoxicity against A549 cancer cells and its MDR cells. Hence, F14 was further applied for the study of the mechanism to overcome MDR cancer. The results showed that F14 could reverse etoposide resistance in A549 lung cancer cells. This mechanism induced apoptosis through down-regulation of NF-**K**B leading to decreasing transcription and expression of P-gp.

From this finding, we further hypothesized whether other types of mechanisms with similar function existed such as autophagy induction. Therefore, 7 purified compounds were later screened to investigate the autophagy induction in human lung A549 cancer cells, leading to apoptosis cell-death. The result showed that the purified compound, Feroneillin A (FERO), exerted cytotoxic activity against MDR cancer, A549RT-eto. However, the treatment with FERO resulted in the differences of the cell morphology compared with F14 (publication 3). From the results, it demonstrated that FERO-induced autophagy was required for apoptotic progression to overcome MDR cancer.

In conclusion, the four plants, *B. strychnifolia*, *C. fenestratum*, *E. longifolia* and *B. pinnata* strong showed merits *in vitro* cytotoxicity against 5 human cancer cell lines. In addition, the other species of the genus *Bryophyllum*, namely *B. laetivirens* exerted revealed potency against A549 cancer cells and their multidrug resistant (MDR) cancer cells, A549RT-eto. Therefore, the extract from *B. laetivirens* can be applied as one of the Thai medicinal plants for further pharmacological and phytochemical investigations in cancer treatment.

3.2 Delimitation and limitation of the Study

- None -

3.3 Suggestion for future work

The obtained bioactive compounds in *B. laetivirens plant*, F14 (publication 3) should be structurally identified, tested in the *in vitro* and then *in vivo* system for the development of anti-cancer therapeutic drug against MDR cancer.

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VITA

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Published publications

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