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MOLECULAR MECHANISMS OF PREVENTIVE AND THERAPEUTIC ACTIVITY OF THAI PLANT EXTRACTS IN SKIN CANCER

Miss Visa Thongrakard

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Clinical Biochemistry and Molecular Medicine
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By	Miss Visa Thongrakard
Field of Study	Clinical Biochemistry and Molecular Medicine
Thesis Advisor	Assistant Professor Tewin Tencomnao, Ph.D.
Thesis Co-Advisor	Professor Ciro Isidoro, M.D., D.Sc.

Accepted by the Faculty of Allied Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Allied Health Sciences

(Associate Professor Prawit Janwantanakul, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Rachana Santiyanont, Ph.D.)

.....Thesis Advisor

(Assistant Professor Tewin Tencomnao, Ph.D.)

.....Thesis Co-Advisor

(Professor Ciro Isidoro, M.D., D.Sc.)

.....Examiner

(Assistant Professor Viroj Boonyaratanakornkit, Ph.D.)

.....Examiner

(Tewarit Sarachana, Ph.D.)

.....External Examiner

(Professor Jenny Emnéus, Ph.D.)

วิสาข์ ทองระกาศ : กลไกระดับโมเลกุลในการป้องกันและรักษาของสารสกัดจากพืชไทยต่อโรคมะเร็งผิวหนัง. (MOLECULAR MECHANISMS OF PREVENTIVE AND THERAPEUTIC ACTIVITY OF THAI PLANT EXTRACTS IN SKIN CANCER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.เทวิน เทนคำเนาว์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.ชิโร อิชิโดโร, 148 หน้า.

ปัจจุบันแพทย์แผนไทยได้ประยุกต์นำสารจากสมุนไพรหลากหลายชนิดสำหรับรักษาผิวหนังและโรคที่เกี่ยวข้องกับการอักเสบของผิวหนัง รังสีอัตราไวโอเลตชนิดบีเป็นปัจจัยสำคัญที่กระตุ้นการทำลายผิวหนัง ซึ่งนำไปสู่มะเร็งผิวหนัง มะเร็งผิวหนังชนิดสะแควมัสเซลล์คาร์ซิโนมาพบมากเป็นลำดับที่สองของมะเร็งผิวหนังทั้งหมด คณะผู้วิจัยศึกษาฤทธิ์ของสารสกัดจากพืชไทยต่อการปกป้องเซลล์ผิวหนังมนุษย์จากการถูกทำลายด้วยรังสีอัลตราไวโอเลตชนิดบีในเซลล์มะเร็งผิวหนังที่ถูกเพาะเลี้ยง (HaCaT cells) พบว่าสารสกัดในส่วนที่มาจากตัวทำละลายไดคลอโรมีเทน และเอทานอล มีสารประกอบฟีนอลรวมและฟลาโวนอยด์ในปริมาณสูง สารสกัดจากขมิ้นในส่วนที่มาจากตัวทำละลายไดคลอโรมีเทนมีความสามารถในการต้านอนุมูลอิสระสูงที่สุด และพบว่าสารสกัดจากขมิ้นในส่วนที่มาจากตัวทำละลายเอทานอล และสารสกัดจากขิงในส่วนที่มาจากตัวทำละลายไดคลอโรมีเทนมีค่าการดูดกลืนรังสีอัลตราไวโอเลตสูงที่สุด ซึ่งสารสกัดจากขมิ้น และขิงในส่วนที่มาจากตัวทำละลายไดคลอโรมีเทนสามารถกระตุ้นการแสดงออกของโปรตีนที่เกี่ยวข้องกับการต้านอนุมูลอิสระคือ Thioredoxin 1 และยังสามารถปกป้องดีเอ็นเอในเซลล์ผิวหนังมนุษย์ที่ถูกเพาะเลี้ยงจากการถูกทำลายด้วยรังสีอัลตราไวโอเลตบี นอกจากนี้คณะผู้วิจัยยังทดสอบฤทธิ์ของสารสกัดจากขมิ้นที่มาจากตัวทำละลายไดคลอโรมีเทน สารบริสุทธิ์เคอร์คูมิน และเรสเวอราทรอล ต่อเซลล์มะเร็งผิวหนังมนุษย์ที่ถูกเพาะเลี้ยง (A431 cells) พบว่าเรสเวอราทรอล เคอร์คูมิน และสารสกัดจากขมิ้น สามารถชะลอการเคลื่อนที่ สามารถยับยั้งวัฏจักรของเซลล์ และสามารถกระตุ้นการตายแบบอะพอพโทซีสในเซลล์มะเร็งผิวหนัง A431 ที่ถูกเพาะเลี้ยง ซึ่งฤทธิ์ของสารเหล่านี้มีความสัมพันธ์กับกระบวนการออโตฟาจี การลดการแสดงออกของยีน BECLIN 1 ซึ่งเกี่ยวข้องกับกระบวนการเกิดออโตฟาจี มีผลต่อการแสดงออกที่เพิ่มขึ้นของโปรตีน p53 และนำไปสู่การยับยั้งวัฏจักรของเซลล์ผ่านโปรตีน p21 เมื่อทดสอบสารสกัดจากขมิ้น หรือเคอร์คูมิน ร่วมกับราพามัยซินซึ่งเป็นสารที่กระตุ้นการเกิดออโตฟาโกโซม สามารถส่งเสริมความเป็นพิษต่อเซลล์ของสารทั้งสองชนิด นอกเหนือจากนี้ยังพบการส่งเสริมฤทธิ์การชะลอการเคลื่อนที่ของเซลล์เมื่อทดสอบเรสเวอราทรอลกับราพามัยซินซึ่งกระตุ้นกระบวนการออโตฟาจีโดยขึ้นอยู่กับ mTOR หรือกับลิเทียมคลอไรด์ซึ่งกระตุ้นกระบวนการออโตฟาจีโดยไม่ขึ้นอยู่กับ mTOR จากผลการศึกษานี้อาจสนับสนุนการใช้ประโยชน์จากสารสกัดจากพืชไทย ในการประยุกต์ใช้เป็นเครื่องสำอาจเพื่อปกป้องผิวหนังจากรังสีอัลตราไวโอเลต นอกเหนือจากนี้สารสกัดจากพืชไทยยังมีฤทธิ์ต้านมะเร็งผิวหนัง โดยยับยั้งการเคลื่อนที่ และยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง ซึ่งฤทธิ์ของสารเหล่านี้เกี่ยวข้องกับกระบวนการออโทฟาจีในแง่ของการส่งเสริมการมีชีวิตรอดของเซลล์

ภาควิชา	เคมีคลินิก	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีคลินิกและอณูทางการแพทย์	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	.....
ปีการศึกษา	2556	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม	.....

5377405037 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE
KEYWORDS: PHYTOCHEMICALS / SKIN CANCER / ULTRAVIOLET B / APOPTOSIS / AUTOPHAGY

VISA THONGRAKARD: MOLECULAR MECHANISMS OF PREVENTIVE AND THERAPEUTIC ACTIVITY OF THAI PLANT EXTRACTS IN SKIN CANCER. ADVISOR: ASST.PROF. TEWIN TENCOMNAO, Ph.D., CO-ADVISOR: PROF. CIRO ISIDORO, M.D., D.Sc., 148 pp.

Thai traditional medicine employs a wide range of indigenous herbs in the forms of tincture or tea for the cure of skin and systemic inflammatory diseases. UVB radiation is the most factors to induce skin damage, including skin cancer. The cutaneous squamous cell carcinoma (SCC) is the second most common form of non-melanoma skin cancer. The protection by Thai plants extracts against UVB DNA damage and cytotoxicity was investigated in human keratinocytes cell line HaCaT. High total phenolic and flavonoid contents were found in the ethanol and dichloromethane fractions. Dichloromethane extract of turmeric was shown to possess the highest antioxidant activity. The maximum UV absorptions were found in the ethanol extract of turmeric and in the dichloromethane extract of ginger. These extracts stimulated the synthesis of Thioredoxin 1, an antioxidant protein, and could protect human HaCaT keratinocytes from UV-induced DNA damage and cytotoxicity. In addition, In addition, the effect of the dichloromethane extract of Turmeric, Curcumin and Resveratrol in skin cancer cell was investigated in epidermoid carcinoma cell line A-431. We found that Resveratrol, Turmeric and Curcumin suppressed cell migration and induced cell cycle arrest and apoptosis in cultured A431 skin SCC cells. These effects were associated with modulation of autophagy. Knock-down of the autophagy protein BECLIN 1 partly rescued p53 stability and led to p21-dependent cell cycle arrest. Rapamycin-mediated inhibition of mTOR increased the production of autophagosomes and greatly enhanced Turmeric and Curcumin toxicity. Moreover, A synergistic inhibitory effect on cell migration was observed in the combination Resveratrol plus rapamycin, as mTOR-dependent autophagy inducer or plus LiCl, as mTOR-independent autophagy inducer. The present data support the utilization of Thai herbs extracts in anti-UV cosmetic pharmaceuticals. Moreover, Thai herbs exert important anticancer effects toward skin carcinoma cells by limiting their cell migration and cell proliferation. These effects depend on the modulation of autophagy, a pro-survival pathway.

Department:	Clinical Chemistry	Student's Signature .....
Field of Study:	Clinical Biochemistry and Molecular Medicine	Advisor's Signature .....
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CHAPTER I

INTRODUCTION

Background and rationale

The skin consists of an outer squamous epithelium, the epidermis, and an inner connective tissue, the dermis. The epidermis undergoes continuous self-renewal due to mitotic activity of the stem cells in the basal layer that provides new keratinocytes (which account for 90–95% of the epidermal layer cells). The latter eventually differentiate into corneocytes, which are then shed from the skin. Keratinocytes are in close contact with the melanocytes, specialized pigment-producing cells derived through the neural crest, forming the so-called epidermal-melanin unit. Imbalance in the growth and differentiation ratios of keratinocytes can result in dysfunctional protection of the epidermal-melanin unit from major environmental insults such as Ultraviolet (UV) radiation, and therefore it predisposes to epidermal carcinogenesis.

There are three major types of epithelial skin cancer: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma. BCC and SCC can be also called non-melanoma skin cancers (NMSCs). In the last few decades, skin cancer has shown a dramatic increase, especially in Caucasians; therefore, it is a serious public health issue [1, 2]. It has been reported that 2 to 3 million of new cases of NMSCs and as many as 130,000 new cases of melanoma are diagnosed each year [3]. Approximately, 80% of all NMSC cases is BCC and the rest is SCC [4]. BCC has a low mortality rate; nevertheless, it can cause substantial morbidity, and cosmetic disfigurement results in health care costs [5]. Usually, BCC does not metastasize; however, it has potential to invade deeper layers of the skin, including muscle, cartilage and bone [6]. Even though BCC has shown the most prevalent, SCC has the potential metastasis leading to higher mortality [7]. The surgery is a major treatment for SCC, but it has limitations. Although combination surgery and chemotherapy is the most preferred treatment, the chemotherapy drug has the side effect [8]. It can be indicated that traditional therapy does not yield satisfactory results for patients. Thus, new agents with high anti-tumor activity to improve satisfaction of patients are required.

Pathogenesis of skin cancer is multifactorial; though, a major factor is ultraviolet (UV) radiation [9]. Several studies have suggested that UVB radiation is the most important factor to induce skin damage, including skin cancer [10]. Generally, exposure of the human skin to UVB radiation induces many adverse effects such as DNA damage, oxidative stress, inflammation, immunosuppression, and gene mutation. All these events are associated with many skin diseases including the development of skin cancer [11-13]. However, the most important effect of UVB radiation is DNA damage and one of hallmark events of UVB exposure to skin is the sunburn cells, which undergo apoptosis [14-16]. Usually, DNA damage can be restored by DNA repair mechanisms; however, if this damage is irreparable, the cells will undergo apoptosis to limit the survival of damaged cells and to escape the risk of developing cancer [17]. For this reason, DNA damage induced by UVB radiation also plays a role in induction of apoptosis. The apoptosis mechanism in response to UVB radiation involves both extrinsic (or death receptor) and intrinsic (or mitochondrial) pathways (reviewed in [18, 19]). Enhancement of DNA repair mechanism might result in a reduced number of apoptotic cells [20]. Generally, skin cells have a variety of mechanism to eliminate UVB-induced cellular damage; nevertheless, excessive UVB exposure can abolish these mechanisms and lead to initiation and development of skin cancer [21].

In the past few decades, macroautophagy, hereafter referred to as autophagy, has gained increase interest not only because of the functional role in different cellular processes (e.g. DNA repair, cell differentiation, cellular stress), but also because its modulation can help the treatment of various diseases including cancer (reviewed in [22]). Autophagy is an intracellular “self-eating” pathway involving the degradation of cytoplasmic substance and the recycling of energy for maintenance of cellular processes during starvation or stress conditions [23]. Autophagy is characterized by sequestration of long-lived proteins and organelles in double-membrane vesicle, called autophagosome, followed by the fusion of the autophagosome with a lysosome to form an autophagolysosome, in which the cellular component are degraded [24]. Basal level of autophagy plays a role in maintaining normal cellular homeostasis by clearance of misfolded proteins and damaged organelles. Under stress condition, autophagy is usually induced to provide energy for promoting cell survival [25, 26]. Hormone therapy, chemotherapy and irradiation frequently induce

autophagy, and in most cases this promotes cell survival and contributes to treatment resistance [26-28]. However, excessive or prolonged activation of autophagy may result in cell death [29]. Therefore, autophagy could be one strategy for cancer therapy.

At present, phytochemicals have gained increased interest for their potential beneficial effects in prevention and therapy of cancer. In this context, the phenolic compounds or extracts rich in polyphenols appear to be the most active. Several studies indicate that polyphenol could have bioactive properties for both prevention and treatment of skin cancer (reviewed in [30]). For example, it has been reported that green tea polyphenols inhibited UV radiation-induced skin carcinogenesis [31], and it has been shown that silymarin (a flavanoid from *Silybum marianum*) protected UV radiation-induced apoptosis and enhanced the DNA repair mechanism [32]. In addition, Singh et al. demonstrated that silymarin suppressed cell proliferation in human squamous carcinoma cell [33]. Further, Jee et al. have shown that curcumin (a polyphenol from *Curcuma longa*) induced apoptotic cell death in human basal carcinoma cell [34]. Therefore, dietary phytochemicals may be considered as a new, safety and efficient strategy for prevention and treatment of skin cancer. However, the protective effects of Thai plant extracts on UVB radiation-induced cellular damage and on inhibition of skin cancer have not been studied at molecular level.

The first aim of the present study was to investigate the potential of individual extracts from fifteen species of Thai plants to protect from UVB-induced cellular damage by enhancing DNA repair mechanism. We focused on the plant extracts that limits apoptosis in keratinocytes subjected to UVB-induced cell death and that do not present DNA mutations. In particular, we determined the potential of these extracts to induce DNA repair damage (removal of Cyclobuthane Pyrimidine Dimer, CPD) in keratinocytes. The immortalized human HaCaT epidermal keratinocytes served as “in vitro” model for UVB-induced cellular damage. A second aim of our study was to investigate the anticancer effects and the molecular mechanisms underlying these effects of selected Thai plant extracts. We tested the anticancer properties only the most active extracts based on antioxidant content and antioxidant activity, in terms of the ability to induce cell death or cell growth arrest. In addition, we also compared the effect of selected extract with the pure substance

that is the major active compound of its selected extract, including Resveratrol, a phytochemical present in the Thai herb (Morus alba Linn.), also possesses anticancer properties. In particular, we analyzed at molecular level the impact of these substances on the regulation of the autophagy and apoptosis pathways, and also assessed the ability of these substances to block cell migration of skin cancer cells. As an “in vitro” cell model, we used the human epidermoid squamous carcinoma A431 cell line (as skin cancer cells). The present study shall contribute to the understanding of the molecular mechanisms of anticancer protective and/or therapeutic effects of Thai plant extracts, which eventually will reflect on the industrial exploitation of Thai plant extracts for general human use for prevention and treatment of skin cancers. In this respect, it is to be noted that we will search for the beneficial effects of the Thai herbs extracts at ‘in vitro’ concentrations that fall into a range pharmacologically relevant ‘in vivo’.

Review of related literature

To support background and rationale of this study, related articles are categorized and review as follows:

The skin and skin cancer

The skin is the body largest organ. Approximately the surface area is around 1.5 – 2.0 m^2 and thickness is around 1 – 4 mm. The skin has many vital functions; for example, it protects the damaging of the internal structures of the body, it also plays a role in detoxication, immunological, metabolic and thermo-regulative. Importantly, the skin is the main organ to protection the body from environmental harmful especially ultraviolet radiation [35]. The skin is composed of three layers as epidermis, dermis and hypodermis [36]. The epidermis is outermost layer of the skin and consists of squamous keratinised epithelium that is around 80% of all the cell is squamous cells or keratinocytes (synthesis the protein celled keratin) and the rest is melanocytes (production of pigment), Langerhans cells (immunological function) and Markel cells (touch receptor). The epidermis is composed of five sublayers from bottom to top layers as stratum corneum, stratum lucidum, stratum granulosum, stratum pinosum, and stratum basale. However, the most important layers are stratum corneum and stratum basale. The stratum basale has the stem cells which divide and mature to

new keratinocytes. The cells move into the higher layers and eventually die. The stratum corneum consists of dead cells called corneocyte that is shed about every 2 weeks [37]. The dermis is beneath the epidermis and contains many specialized cells and structures which have the function to control temperature, fight infection, transmit sensations, be flexible and store water and supply blood and nutrients for the skin. The innermost layer of the skin is the hypodermis that is composed of fat and loose connective tissue. This layer plays a role in protect internal organ from the injury and also helps to regulation of temperature (Figure 1.1) [36].

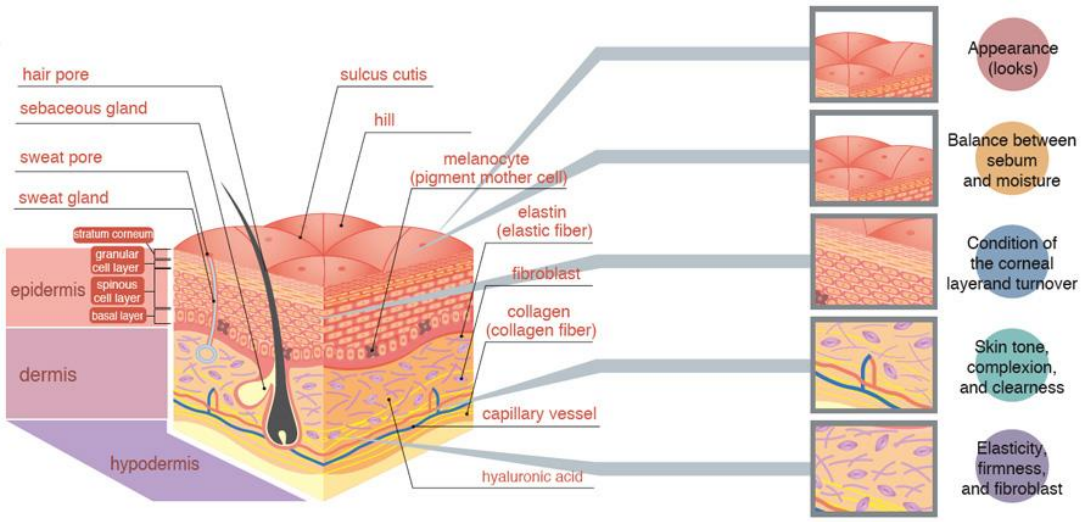


Figure 1.1 The skin is composed of three layers as epidermis, dermis and hypodermis. The epidermis is composed of five sublayers as stratum corneum, stratum lucidum, stratum granulosum, stratum pinosum, and stratum basale. (source: https://www.lifecellaustralia.com/blog/posts/skin-structure)

As mention above, there are three major types of epithelial skin cancer classified by origin of cancer cell: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma. BCC and SCC can be also called non-melanoma skin cancers (NMSCs). BCC arises from basal cells (round cells under the squamous cells) in the lower layer of epidermis called stratum basale (figure 1.2). Mostly, BCC appears on the face, head, neck and hands [38]. SCC arises from squamous cells or keratinocytes (thin and flat cells) in the top layer of epidermis called stratum corneum (figure 1.2). Usually, SCC founds in a sun-exposed area such as face, lips, ears, neck, hand and arms [39]. Melanoma is caused from melanocytes in the lower layer of the epidermis (figure 1.2). It is often found in the upper back or face. The melanoma is the most serious

form of skin cancer and it causes of skin cancer death about 75% of all cases; however, it is less common than NMSCs. It has been reported melanoma affect only 3% of all skin in the U.S. (reviewed in [40]). Approximately, 80% of all NMSC cases are BCC and the rest is SCC. [4]. BCC has a low mortality rate; nevertheless, it can cause substantial morbidity, and cosmetic disfigurement results in health care costs [5]. Usually, BCC does not metastasize; however, it has potential to invade deeper layers of the skin, including muscle, cartilage and bone [6]. Even though BCC has shown the most prevalent, SCC has the potential metastasis leading to higher mortality [7].

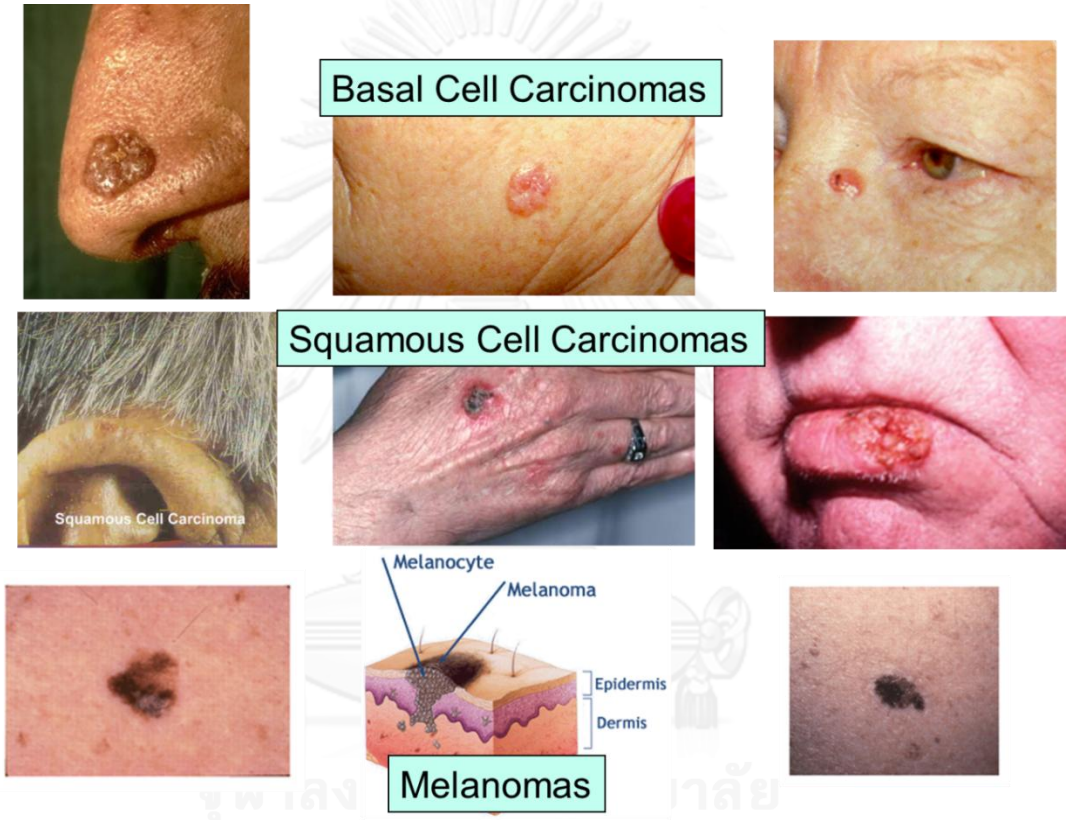


Figure 1.2 The appearance of the three major forms of skin cancer: basal cell carcinomas or BCC (upper), squamous cell carcinomas (middle) and melanomas (lower) [41].

Squamous cell carcinoma (SCC) is the second most common form of non-melanoma skin cancer [4]. It has been reported in 1994 in U.S. that men have the higher risk of SCC than women as 9 - 14% among men and 4 – 9% among woman [42]. The risk factors of SCC were summarized in Table 1.1. However, the most common cause of SCC is exposure to ultraviolet radiation (UV) especially ultraviolet B radiation (UVB).

During the past 50 years the lifestyle has changed, so the incident of exposure to sunlight has increased. Moreover, the people who work outdoor have the higher risk of SCC than the people who work indoor about five times. UV radiation is cause of DNA damage usually in p53 tumor suppressor gene (figure 1.3). If these DNA damage fail to repair, it may lead to tumor formation (reviewed in [43]) (see detail in below). There are many strategies for treatment of SCC such as Mohs' micrographic surgery, excisional surgery, eletrodesiccation and curettage, cryosurgery, radiation therapy and topical chemotherapy (fluorouracil) [44]. The surgery is a major treatment for SCC, but it has limitations. Although combination surgery and chemotherapy is the most preferred treatment, the chemotherapy drug has the side effect [8]. It can be indicated that traditional therapy does not yield satisfactory results for patients. Thus, new agents with high anti-tumor activity to improve satisfaction of patients are required.

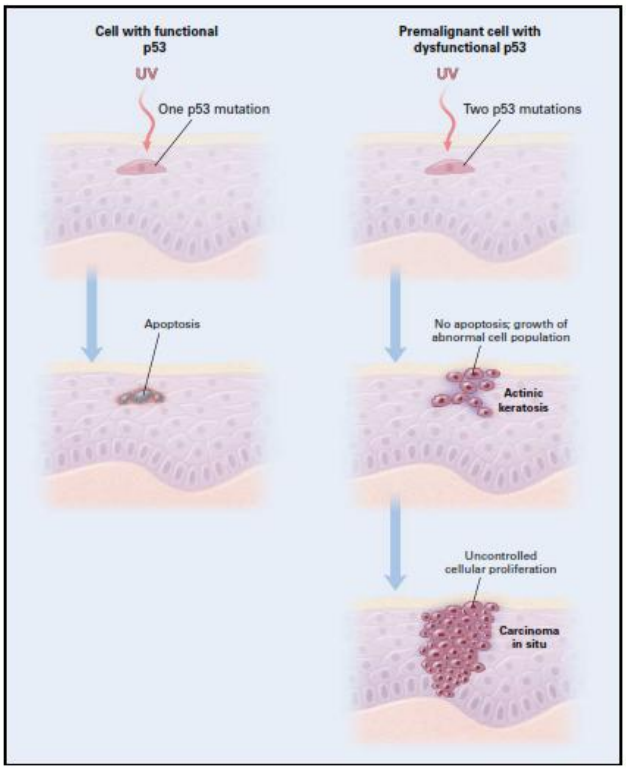


Figure 1.3 UV radiation generates DNA damage in p53 tumor suppressor gene. Left, the one mutation of p53 gene is removed by apoptosis pathway. Right, the two mutations of p53 genes are failed to repair and to remove by apoptosis, it leads to tumor formation [43].

Table 1.1 Risk factors for the development of squamous cell carcinoma (SCC) [43].

Risk factors SCC
Exposure to ultraviolet radiation: Ultraviolet A, Ultraviolet B
Therapy with methoxsalen and ultraviolet A radiation
Exposure to ionizing radiation
Genodermatosis
Oculocutaneous albinism
Xeroderma pigmentosum
Infection with human papillomavirus, especially types 6, 11, 16, and 18
Exposure to chemical carcinogens, arsenic, polycyclic aromatic hydrocarbons
Immunosuppression
Organ transplantation
Leukemia and lymphoma
Immunosuppressive medications
Chronically injured or diseased skin
Ulcers
Sinus tracts
Osteomyelitis
Radiation dermatitis
Certain chronic inflammatory disorders, such as dystrophic epidermolysis bullosa
Precursor lesions
Actinic keratosis
Arsenical keratosis
Radiation-induced keratosis
Bowen’s disease (squamous-cell carcinoma in situ)
Erythroplasia of Queyrat (squamous-cell carcinoma in situ of the penis)

Ultraviolet radiation-induced cellular damage

Pathogenesis of skin cancer is multifactorial such as radiation therapy, chemicals and family history; however, a major factor is ultraviolet (UV) radiation [9]. The spectrum of UV is divided into three types as UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm). UVA is not absorbed by the atmosphere and UVB is not completely absorbed; whereas, UVC is completely absorbed [19]. The remaining of UV radiation incoming to the earth's surface is composed by 10% of the UVB and 90% of the UVA radiation, and is responsible of harming the living systems, including human skin [45]. In the past few decades, the proportion of UVB radiation reaching to the earth's surface is gradually increased as a consequence of depletion of the ozone layer [46]. 80% of UVA penetrates deep into the papillary dermis and around 10% reaches to the hypodermis, whereas almost all of UVB is absorbed by the epidermis. Approximately 70% of UVB is blocked by the stratum corneum [47]. Several studies indicated that UVB radiation could cause skin damages, including skin cancer [10]. For example, it was demonstrated in mouse skin model that UVB radiation act as a strong carcinogen, suggesting that UVB radiation involved in all step of skin carcinogenesis as tumor initiation, promotion and progression [48, 49]. Moreover it has been reported that UVB induce cutaneous carcinogenesis more potential than UVA [50]. Exposure of the skin to UVB induced many responses (figure 1.4) such as inflammatory response, immunosuppression, oxidative stress, gene mutation and DNA damage, all of these involving in skin cancer [11, 12]. UVB-induced inflammatory responses; for example, it stimulates activity of phospholipase subsequently release arachidonic acid and increase the levels of cyclooxygenase-2 (COX-2) and prostaglandin (PG) metabolites. In addition it also enhances blood flow and vascular permeability, leading to edema, erythema and hyperplastic response [13]. It is well know that chronic inflammation plays an important role in all step of tumor development as initiation step, promotion step, and progression step [51]. However, the most important effect of UVB radiation is DNA damage. UVB is directly absorbed by cellular DNA which is the major cellular chromophore, because of its high content in purine and pyrimidine bases that have a maximum absorption of radiation in the 230 and 280 nm wavelength ranges. For this reason, UVB can directly cause DNA damage, typically cyclobuthane pyrimidine dimers (CPD) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4PP), which cross-link adjacent DNA bases (Figure 1.5) [52]. In

addition, when 6-4PP photoproducts exposure to UVB/UVA, they are easily converted into Dewar valence isomers (Figure 1.5) [53].

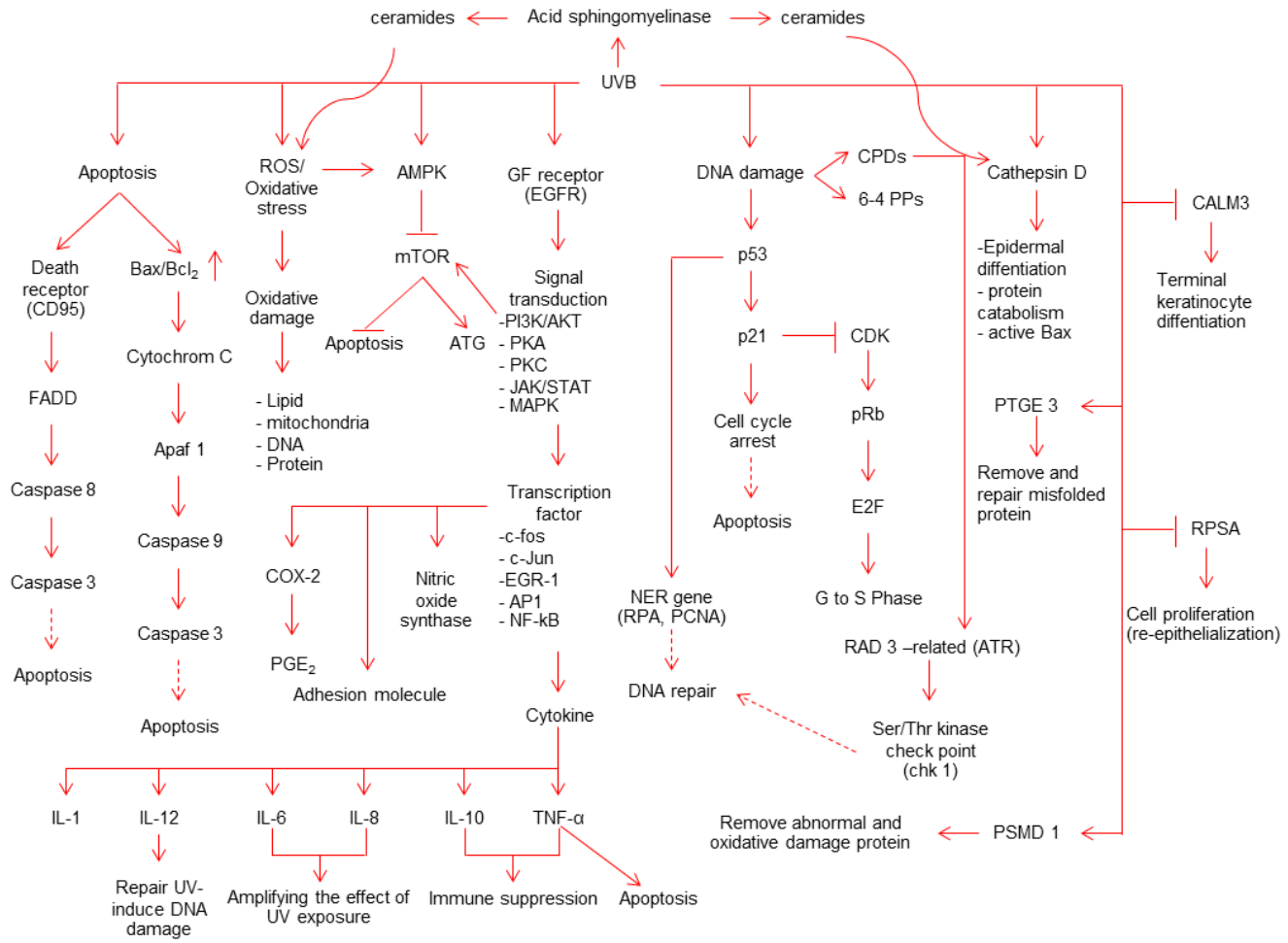


Figure 1.4 UVB induced many signaling pathway result in various responses in human skin such as inflammatory response, immunosuppression, oxidative stress, gene mutation and DNA damage, all of these involving in skin cancer [11, 12]

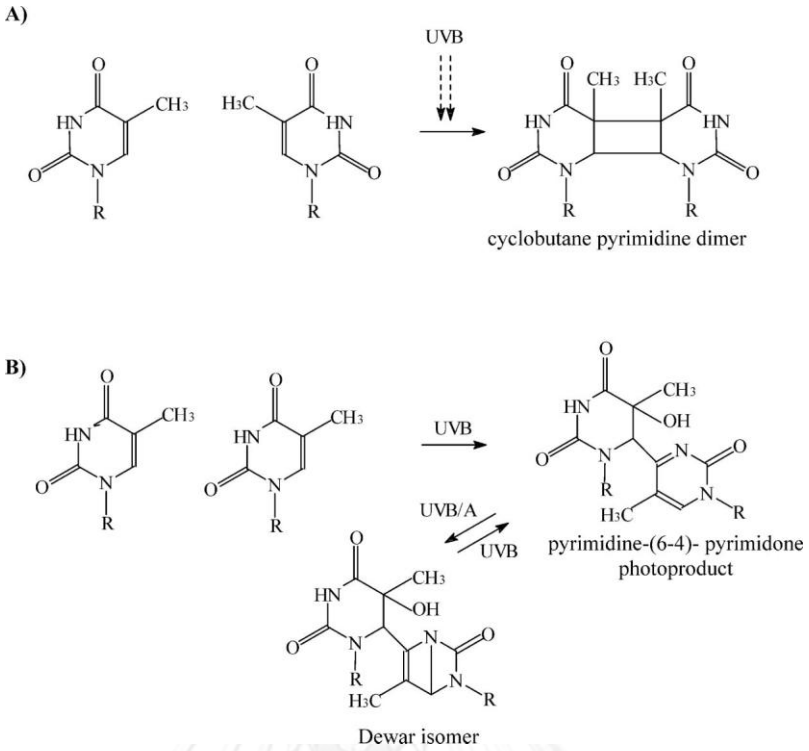


Figure 1.5 Formation of cyclobutane pyrimidine dimer (CPD) from adjacent pyrimidine bases on the same strand (A), pyrimidine-(6–4) pyrimidine photoproduct (6–4PP) and Dewar isomer (B) after absorption of UVA/UVB light energy [21].

In contrast to UVB, UVA is not directly absorbed by cellular DNA. Still, UVA promotes the generation of reactive oxygen species (ROS) that induce oxidizing effects to cellular molecules, including DNA damage such as formation of 8-hydroxyguanine (8-oxoG) [54]. CPD and 6-4PP is the major types of DNA damage induced by UVB radiation, being CPD three times more frequent than 6-4PP [55]. Moreover many observations have been reported that mutagenic and carcinogenic potential of UV is mainly from CPD [56-59]. Thus, the adverse effects of UV radiation on DNA damage are mostly contributed from CPD induced by UVB radiation.

The nucleotide excision repair (NER) is the major DNA repair mechanism against the adverse effects of CPD. There are two modes of NER as global genome NER (GG-NER), repair of lesion over the entire genome, and transcription coupled NER (TC-NER), repair of transcriptionally active genes lesions present in transcribed DNA strands. The difference between GG-NER and TC-NER is the initial steps of DNA damage detection.

Because the TC-NER does not require damage sensing XPC-HR23B complex and DDB proteins (DNA damage binding) factor for damaging detectors, but it detect the damaged DNA when the RNA polymerase II complex encounters a lesion. The rate of GG-NER depends on the type of DNA lesion. For example, CPD is removed much slower from the genome than 6-4PP by GG-NER which is possible that XPC-HR23B complex has a higher affinity for 6-4PP than for CPD. The NER pathway starts with the recognition of the dimer and DNA cut, followed by excision of the dimer, the gap filled by DNA polymerase and, finally, nick sealed by DNA ligase (Figure 1.6) (reviewed in [60, 61]). Genetic defect in the NER pathway, such as it occurs in Xeroderma pigmentosum (XP), leads to accumulation of CPD and is associated with increased risk of skin cancer, indicating a strong causal relationship between CPD and skin carcinogenesis [62].

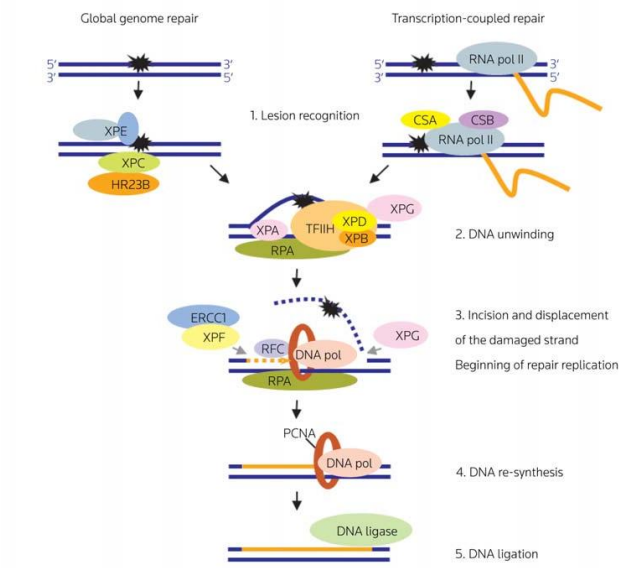


Figure 1.6 Representation of repair of DNA lesions by NER. There are two modes of NER as GG-NER and TC-NER [63]. The difference between GG-NER and TC-NER is the initial steps of DNA damage detection. GG-NER requires damage sensing XPC-HR23B complex and DDB proteins (DNA damage binding) factor for damaging detectors; whereas, TC-NER requires RNA polymerase II complex for damaging detectors. The NER pathway starts with the recognition of the region of damaged DNA and DNA cut, followed by excision of the dimer, the gap filled by DNA polymerase and, finally, nick sealed by DNA ligase.

It has been also evidenced that excessive and irreparable UVB-induced DNA damage leads to apoptosis, as a backup pathway to limit the survival of DNA-mutated cells and thus the risk of developing cancer [17]. The one of hallmark events of UVB exposure to skin is sunburn cells, regarded as a marker for severity of sun damage [14, 16]. Time course studies shows that after UV exposure for 8 h, sunburn cells are maximally expressed after 24-48 h, and after 60-72 h they are no more apparent [64]. On hematoxylin and eosin staining, the sunburn cells show a shrunken eosinophilic cytoplasm with a condensed and pyknotic nucleus [65]. Further characterization of sunburn cells are revealed morphological similarities with apoptotic cell, such as the appearance of cell shrinkage and membrane blebbing in the early stage and then chromatin condensation and DNA fragmentation [15]. According to these morphological features, sunburn cells represent keratinocytes undergoing apoptosis after UV exposure [18]. Indicating that induction of apoptosis of sunburn cells aims to remove the premalignant cells. Therefore, protection of UV radiation-induce adverse effects may result in the reduced formation of sunburn cells or, in other words, result in the reduction of the number of apoptotic cells in the epidermis. The apoptotic pathway of keratinocyte which response to UV radiation can occur both intrinsic/mitochondrial and extrinsic/death receptor – mediated cell – death pathways that are particularly regulated by mitogen – activated protein kinase (MAPK) and p53 tumor suppressor protein (figure 1.7 and 1.8) (reviewed in [19]).

Apoptosis is a cell death process characterized by typical and unique morphological (e.g. membrane shrink, DNA fragmentation, membrane blebbing and formation of apoptotic bodied) and biochemical (e.g. activation of death receptor and caspase activation) events. There are two major apoptosis mechanisms: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (reviewed in [66]). The extrinsic pathway is mediated by activation of death receptors which belong to the tumor necrosis factor family, such as TNF-receptor-1 (TNFR1; also known as DR1), CD95 (also known as DR2, APO-1 and Fas), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1; also known as DR4 and APO-2), TRAILR2 (also known as DR5). Activation of death receptors leads to formation of a death inducing signaling complex (DISC), followed by activation of the cysteine protease caspase-8. Activated

caspase-8 triggers a cascade of signaling events that end with the activation of downstream caspases (e.g. caspase-3) and, lastly, lead the cell to apoptosis [67-69]. The intrinsic pathway is initiated by the permeabilization of the mitochondrial outer membrane and subsequent release of intermembrane proteins, such as the cytochrome C, into the cytoplasm. Cytochrome C then forms a multi-protein complex (called the apoptosome) that includes the Apoptotic Protease Activating Factor-1 (Apaf-1). Subsequently, the apoptosome activates caspase-9, which triggers a cascade of downstream caspase (e.g. caspase-3), and finally leads the cell to apoptosis (figure 1.7) [70, 71].

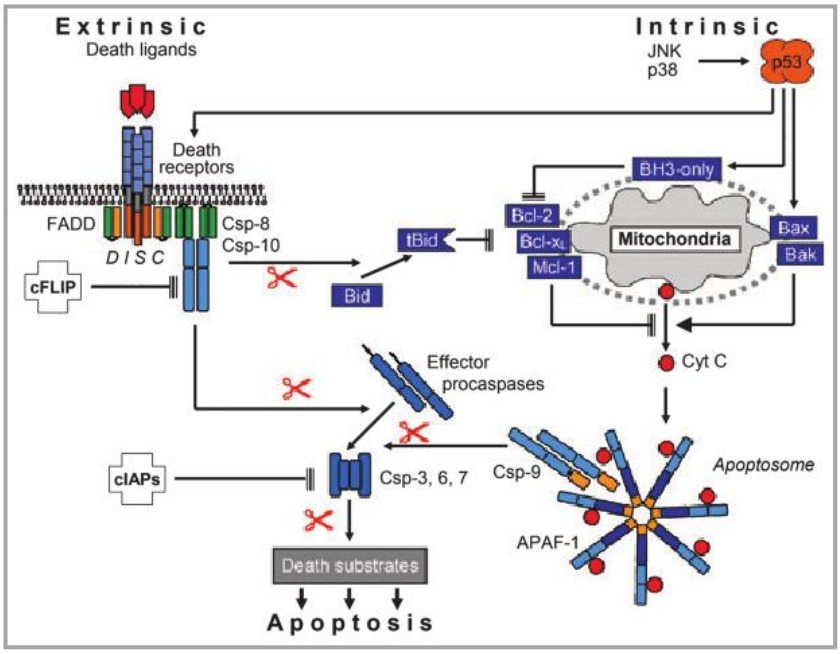


Figure 1.7 Apoptotic pathways. The left side is extrinsic apoptotic pathway start form biding of death ligand, clustering of death receptors, forming of death inducing signaling complex (DISC), followed by activation of initiator caspase-8 or caspase-10, and then activated initiator caspase-8 or caspase-10 triggers a cascade of signaling events that end with the activation of downstream caspases (e.g. caspase-3) result in the cell to apoptosis. The right side is intrinsic pathway which is initiated many signals. For example, activation of p53 by MAPK (JNK and p38) leads to transcription of proapoptotic Bcl-2 proteins (BAX), inhibition of antiapoptotic Bcl-2 proteins (Bcl-2), release of cytochrome *c* from mitochondrial, and then forms a multi-protein

complex (called the apoptosome) that includes the Apoptotic Protease Activating Factor-1 (Apaf-1), after that the apoptosome activates caspase-9, which triggers a cascade of downstream caspase (e.g. caspase-3), and finally leads the cell to apoptosis. Activated caspase-8 can activate Bid protein to proapoptotic, BH3-only Bcl-2 protein tBid (truncated Bid) [19].

There are three sub-families of MAPK family: extra - cellular signal – regulated kinase (ERK; ERK1 and ERK2), c–Jun N–terminal kinase (JNK; JNK1, JNK2, and JNK3), and p38–MAP kinase (α, β, δ, and γ). Usually, ERKs are activated in response to mitogenic stimuli; whereas, JNK and p38 are activated by environmental stresses, especially UV radiation (reviewed in [72]). Non-repaired DNA damage may cause of activation of MAPKs pathway, both JNK and p38, leads to UV-induced apoptosis. JNK and p38 may enhance the mitochondrial apoptosis pathway via activate proapoptotic protein such as Bim and Bmf. However, the most important UV-induced apoptosis is phosphorylation of p53 tumor suppressor protein by JNK and p38. Transcription of many genes such as the genes that encode repair enzymes, the cell cycle inhibitors (p21), death receptors (CD95 and TRAIL receptors), proapoptotic Bcl-2 proteins (BAX, Bid, Noxa, and PUMA) is activated by p53 (figure 1.8). Therefore, activation of p53 leads to activation of DNA repair and cell cycle arrest. However, damaged cells that contain non-repaired DNA damage, p53 triggers activation of apoptosis both intrinsic and extrinsic pathways (reviewed in [19]). Nevertheless, chronic UVB exposure leads to dysregulation of apoptosis and contributes to abnormal proliferation of keratinocytes which contain DNA damage, p53 mutations and alteration in signal transduction pathways, all of which result in skin cancer [73]. Thus, enhancement of NER mechanism to remove CPD might result in decrease in a number of apoptotic cells and also reduce risk of skin carcinogenesis [17, 20].

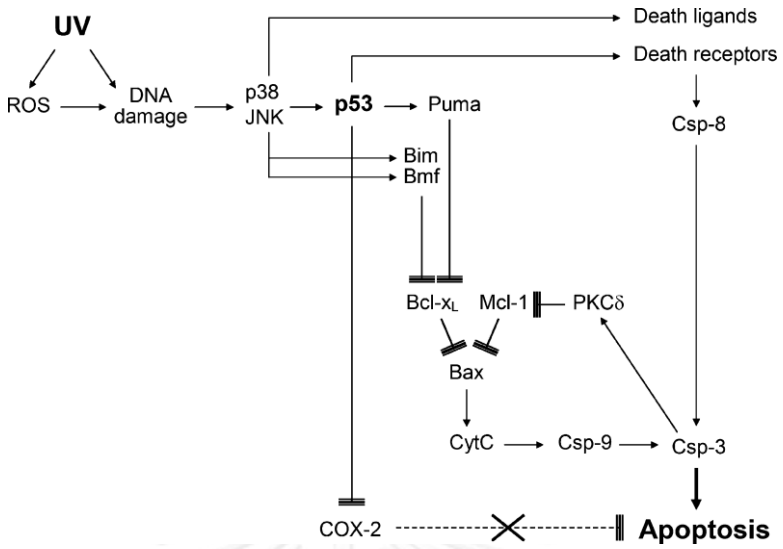


Figure 1.8 UV-induced apoptosis. The apoptotic pathway of keratinocyte which responses to UV radiation can occur both intrinsic and extrinsic death pathways that are particularly regulated by MAPK (p38 and JNK) and p53 tumor suppressor protein [19].

Autophagy

Autophagy is an intracellular "self-eating" pathway involving the degradation of cytoplasmic substance and the recycling of energy and substrates for the maintenance of cellular metabolism during starvation or stress conditions [23]. Dysfunction of autophagy is associated with a number of diseases such as neurodegenerative diseases, cardiomyopathy, diabetes, Crohn's disease, and tumorigenesis [74]. There are three forms of autophagy: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy [24]. Macroautophagy, hereafter called as autophagy, is the mainly pathway. Autophagy is characterized by the sequestration of long-lived proteins and organelles in a double-membrane vesicle, called the autophagosome, followed by the fusion of the autophagosome with a lysosome to form an autophagolysosome, in which the sequestered cellular components are degraded (figure 1.9A) [24]. The CMA involves in selective translocation of the protein in cytoplasm that is tagged with pentapeptide motif related to KFERQ (Lys-Phe-Glu-Arg-Gln) called CMA substrate [75]. This CMA substrate

is recognized by chaperone and heat shock cognate protein of 70 kDa (hsc70) [76], and then delivered to lysosome surface, after that CMA substrate bind to the receptor as lysosome-associated membrane protein type 2A (LAMP-2A) [77]. After binding to LAMP-2A, the CMA substrate undergoes unfolding and then translocated into lysosome which degradation take place by lysosomal protease (figure 1.9C) [78, 79]. The third forms of autophagy is microautophagy, poorly understand in mammalian cells. It is believed that the cytosolic content are directly engulfed by lysosomal membranes (figure 1.9B) [24].

Autophagy is regulated by several highly conserved genes called ATG (*Au*T*opha*G*y* related) gene which had been firstly identified in yeast [80, 81]. A network of other signaling proteins, including the phosphatidyl inositol-3-kinases of class I and class III (the latter is also known as Vps34), Beclin-1 (an oncosuprressor, also is known as Atg6), the dual lipid-protein phosphatase PTEN (which is an ocnosupressor) and microtubule-associated protein 1 light chain 3 (LC3, also known as Atg8) is involved in the regulation and execution of autophagy (reviewed in [82]). The master negatively regulator of the autophagy signaling pathway is the mammalian Target of Rapamycin (mTOR) [83], a kinase that also controls cell growth and protein synthesis, and that is implicated in carcinogenesis. Under nutrient and growth factor available conditions, the class I PI3K/AKT/mTOR axis is activated, resulting in cell growth and cell proliferation and concomitant inhibition of autophagy; whereas, nutrient (e.g., aminoacids, glucose) and growth factor shortage, hypoxia and other stress conditions (energy depletion, oxidative stress, and DNA, protein or organelle damages) induce autophagy and suppress cell growth and cell proliferation [83, 84].

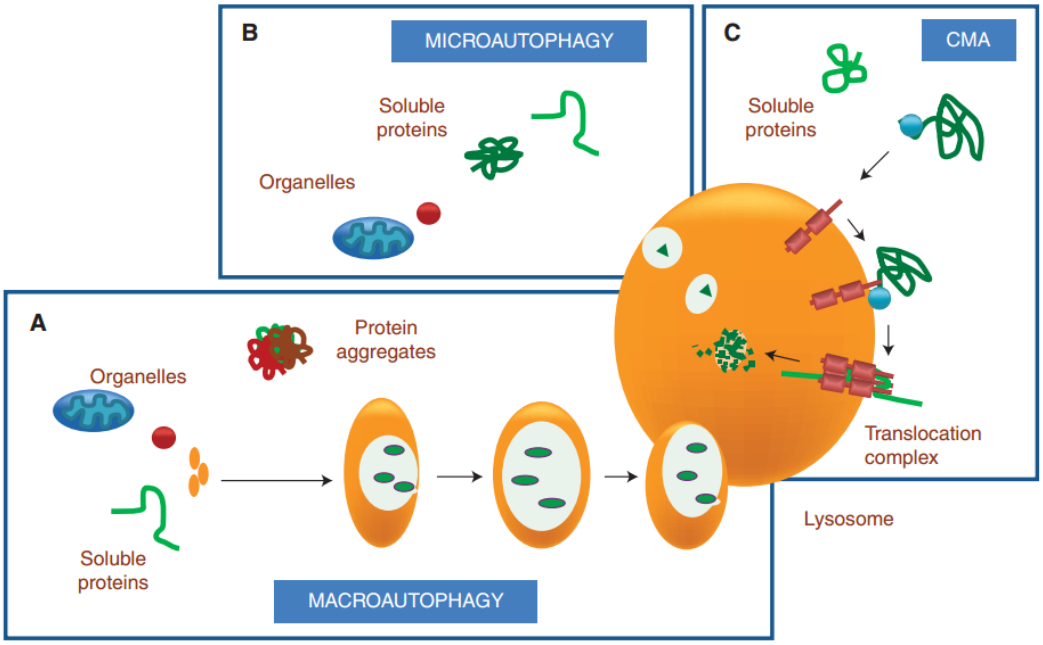


Figure 1.9 Autophagic pathways. (A) Macroautophagy (or called autophagy) is the mainly pathway, is characterized by the sequestration of cytosolic protein in a double-membrane vesicle, called the autophagosome, followed by the fusion these structure with a lysosome to form an autophagolysosome, in which the sequestered cellular components are degraded. (B) microautophagy, the cytosolic contents are directly engulfed by lysosomal membranes as single membrane vesicles. (C) chaperone-mediated autophagy (CMA), the CMA substrate is recognized by chaperone and hsc70, and then delivered to lysosome surface, after that CMA substrate bind to the lysosome receptor. Once binding to lysosome receptor, the CMA substrate undergoes unfolding and then translocated into lysosome which is rapidly degraded by lysosomal protease [85].

The core Atg protein composes of five subgroups: ULK1 protein kinase complex, Vps34-BECLIN1 class III PI3-kinase complex, Atg9-WIPI-1 complex, Atg12 conjugation system, and LC3 (microtubule-associated protein 1 light chain 3) lipidation (Figure 1.10) (reviewed in [86]). 1.) The ULK1 or homolog ULK2 protein kinase complex is consisted of Atg13, FIP200 and Atg101 that is directly control via mTOR kinase by phosphorylates Atg13 to suppress autophagy. Thus, dephosphorylation under nutrient deprivation conditions leads to activation of Atg13 and ULK1/2. The

activating of ULK1/2 phosphorylates FIP200 to induce autophagosome formation. 2.) The Vps34-BECLIN1 class III PI3-kinase complex is classified into three types: Atg14-Vps34-Vps15-BECLIN1 (required for autophagosome formation), UVRAG-Vps34-Vps15-BECLIN1 complexes (positively regulation of autophagosome maturation and endocytic effect), and UVRAG-Vps34-Vps15-BECLIN1 complexes (negatively regulation of autophagosome-lysosome fusion). 3.) The Atg9-WIPI-1 complex is believed that it involving in transport the membrane form trans-Golgi network to formation the autophagosome. In normal condition Atg9 is located to the trans-Golgi network, whereas in starvation condition it is located on the autophagosome. Also, WIPI-1 is located on the autophagosome during autophagic induction. 4.) Atg12 conjugation system is first ubiquitylation-like reaction, is necessary for formation and elongation of the isolation membrane. However, this protein complex will dissociate from fully formed autophagosome. Firstly, Atg12 is activated by Atg7 (E1 ubiquitin activating enzyme-like), followed by transfered to Atg10 (E2 ubiquitin activating enzyme-like). Finally, Atg12 is linked with Atg5 (Atg12-Atg5 conjugate), and then formed with Atg16L1, resulting in Atg12-Atg5-Atg16L1 complex. 5.) LC3 lipidation is also ubiquitylation-like reaction (can be called second ubiquitylation-like reaction, LC3 conjugation system), that is targeted at phosphatidylethanolamine (PE). Thus, this system has been called LC3 lipidation. LC3 is produced as a precursor form and is cleaved at c-terminal to form cytosolic isoform LC3-I by the protease Atg4B. LC3-I is activated by Atg7 (E1-like), and then conjugated to Atg3 (E2-like), lastly conjugated to PE. LC3-II is targeted to elongation of the isolation membrane; however, it is unlikely Atg12-Atg5-Atg16L1 complex. LC3-II remains on fully formed autophagosome until fusion with the lysosome, after that LC3-II on the cytoplasmic side autolysosomes will be delipidated by Atg4B to recycle LC3-I. LC3-II is also located at internal surface of autophagosome and it is degraded in the autolysosome. Due to LC3-I is distributed in the cytosol and LC3-II is located on autophagosome; thus, LC3-II is a promising marker of autophagosomes (reviewed in [24, 86]). It has been reported the cross-talk between the two ubiquitylation-like reaction (figure 1.11). The Atg12-Atg5-Atg16L1 complex can act as E3-like to facilitate LC3-I conjugation to PE [87]. Atg10 can also facilitate LC3-I conjugation to PE by interacting with LC3 [88]. Similarly, it is shown the

coimmuneprecipitation between Atg3 and Atg12. Moreover, overexpression of Atg3 can increase the conjugation of Atg5-Atg12 [89].

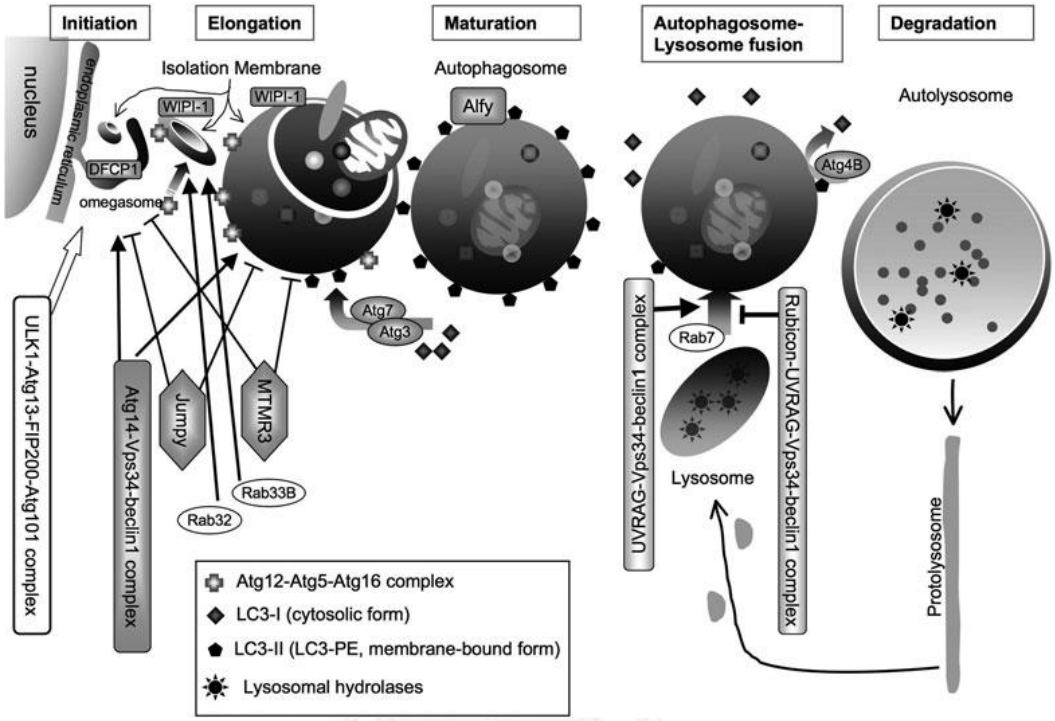


Figure 1.10 The core Atg (*Au*T*opha*G*y*) protein composes of five subgroups: ULK1 protein kinase complex, Vps34-BECLIN1 class III PI3-kinase complex, Atg9-WIPI-1 complex, Atg12 conjugation system, and LC3 lipidation. The ULK1-Atg13-FIP200-Atg101 is negative regulated by mTOR kinase signaling. Under nutrient deprivation, the autophagic induction is activated by ULK1 dephosphorylate, that is the initiation step. After autophagic induction, an omegasome is formed from the endoplasmic reticulum (ER) which associated with DFCP1 (Double FYVE-domain containing protein1). Jumpy and MTMR3 (Two PI(3)P-phosphatase), act as the negatively regulators of omegasome formation. Next step is elongation, the omegasome forms to isolation membrane, which sequestrates the cytosolic protein, including organelle: mitochondria and endoplasmic reticulum, that associated with Atg5-Atg12-Atg16L1 complex and LC3-II. Atg5-Atg12-Atg16L1 complex dissociates from fully formed autophagosome, while LC3-II is still there. Then, maturation step is the enclosed of isolation membrane to form autophagosome. Following the autophagosome

formation is autophagosome-lysosome fusion step, the fusion of lysosome with the autophagosome to form an autolysosome. Finally, the degradation step is degradation of intra-autolysosome component by lysosomal hydrolases [86].

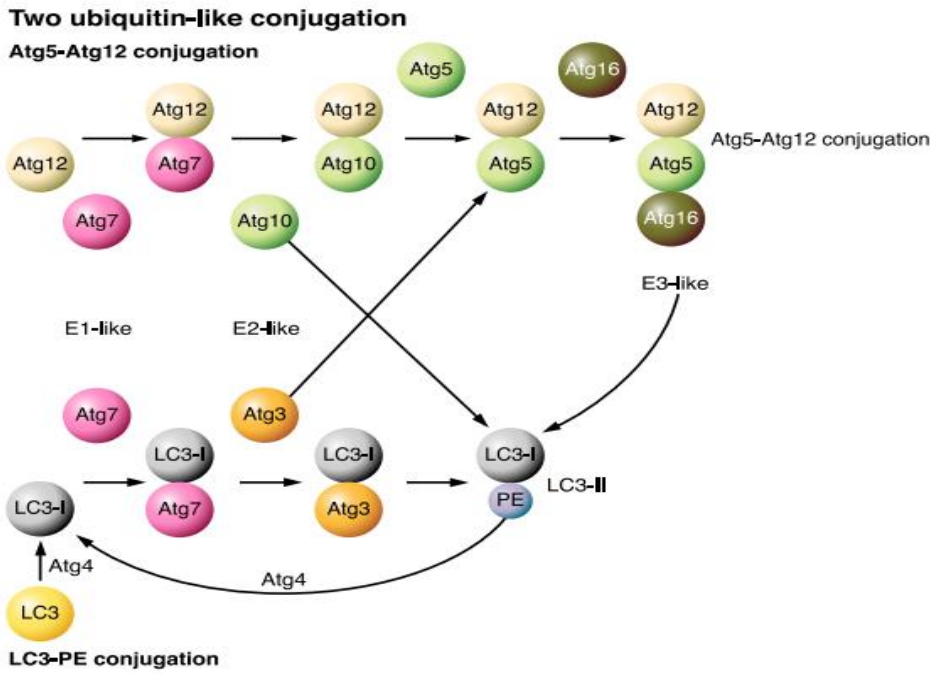


Figure 1.11 Two ubiquitin-like conjugation systems as Atg5-Atg12 conjugation and LC3-PE conjugation are involved in elongation of the isolation membrane. Atg7 (E1-like) and Atg10 (E2-like) are associated with Atg5-Atg12 conjugation; whereas, Atg7 (E1-like) and Atg3 (E2-like) are associated with LC3-PE conjugation. Then Atg5-Atg12 conjugation formed with Atg16L1, resulting in Atg12-Atg5-Atg16L1 complex that also exhibit as E3-like to facilitate LC3-I conjugation to PE. Atg10 can also facilitate LC3-I conjugation to PE by interacting with LC3. Atg3 may involve in increasing of conjugation of Atg5-Atg12 [24].

As mention above the master negatively regulator of autophagy is the mammalian Target of Rapamycin (mTOR) kinase [83]. There are two complex of mTOR: a rapamycin-sensitive mTOR complex1 (mTORC1), which directly regulates autophagy; and mTOR complex2 (mTORC2), which does not directly regulate autophagy [90, 91]. It has been identified that mTORC1 was associated with ULK1/2-Atg13-FIP200 complex. Under nutrient and growth factor available conditions, mTORC1 inhibits

autophagy though phosphorylation of Atg13 and ULK1/2 to suppress their activities. However, under starvation, hypoxia and other stress conditions, mTORC1 disassociate with this complex, resulting in dephosphorylation of Atg13 and ULK1/2 and leading to autophagic induction [92-94]. The PI3K pathway is the major signaling cascade controlling mTORC1 [95]. The class I PI3K is activated by binding of growth factor or insulin to cell surface receptors [96]. The activated class I PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 then activate AKT (protein kinase B) by phosphorylation. Activated AKT phosphorylate and inactivate the tuberous sclerosis complex (TSC) 1/2, resulting in activation of Rheb and subsequently mTORC1. Finally, activation of mTORC1 leads to induction of cell growth and cell proliferation and inhibition of autophagy [97]. Unlikely the class I PI3K, the class III PI3K (or Vps34) stimulates the autophagy by increasing the level of phosphatidylinositol-3-phosphate (PI-3-P), the class III PI3K product [98]. Apart from the class I PI3K/AKT/mTOR axis, several signaling pathways have been reported to regulate autophagy such as LKB1-AMPK-mTOR axis, tumor suppressor p53, BCL-2, and ER stress and Ca^{2+} signaling. Lower energy or metabolic stress condition contributes to reduction of ATP level and increasing of AMP/ATP ratio, resulting in activation of LKB1 (serine/threonine kinase), subsequently AMPK activation, and finally mTORC1 inhibition and autophagic induction [99]. The activation of AMPK can be activated by calcium and calmodulin-dependent protein kinase kinase β (CAMKKβ) [100]. The tumor suppressor p53 shows the complex on regulation of autophagy. Because it has been shown that nuclear p53 was activated by genotoxic stress or oncogenic stress act as the positively regulates autophagy by activating AMPK; whereas, cytoplasmic p53 act as the negatively regulates autophagy (reviewed in [22]). BCL-2 is the one of autophagy inhibition; because it can bind to the BH3 domain of BECLIN1, leading to BECLIN1 inactivation [101]. The accumulation of mis-folded protein in the endoplasmic reticulum (ER) triggers ER stress, which can induce autophagy. The calcium ion release into the cytosol is contributed to ER stress (figure 1.12) (reviewed in [22]).

In principle, autophagy accomplishes a protective role that opposes to cell death inducing injuries, though an exaggerated induction of self-constitutents components

by autophagy may end up with cell death. Recent studies have suggested the crosstalk between autophagy and apoptosis [102]. BCL-2 and BCL-xL are well known for anti-apoptosis; however, it can interact with BECLIN1 to inhibition of autophagy [101]. Atg5 is a one major protein required for autophagosome formation of autophagy pathway, it is also mediated in apoptosis. Yousefi et al. demonstrated that truncated of Atg5 participates in apoptosis by translocating from the cytosol to mitochondria, and then trigger cytochrome *c* release and caspase activation [103]. In some experiment, autophagy plays a role in pro-survival pathway by suppress apoptosis [104]. On the other hand, autophagy can induce cell death both collaboration with apoptosis and serve as back-up the apoptosis mechanism when it defect [105]. However, the connection between autophagy and apoptosis is complex and still requires investigations for a full understanding of the reciprocal regulation of these two processes [106, 107]. Isidoro and collaborators have shown that at least in the case of oxidative stress, autophagy plays an initial protective role that however eventually switches into cell death pathways such as apoptosis and necrosis [108] and that this switch is regulated by the PTEN-AKT-mTOR pathway [109]. Induction of autophagy has also been found associated with anticancer therapy. Huang et al. demonstrated that imiquimod, an immune response modifier, can directly induce autophagy and apoptosis in BCC, and inhibition of imiquimod-induced autophagy enhances cell apoptosis [110]. Similarly, Sun et al. found that epirubicin, a chemotherapy drug, induced both apoptosis and autophagy in breast cancer cell, and inhibition of autophagy restored the sensitivity of breast cancer cell to epirubicin [111]. In these cases, autophagy served as a protection mechanism by preventing the cell to undergo apoptosis under chemotherapy treatment. However, autophagy can also result in cell death. For example, it has been reported that blocking autophagy increased the survival of breast cancer cell after photodynamic therapy [112]. Moreover, α-mangostin, a dietary xanthone, induced autophagic cell death in glioblastoma cells [113]. Furthermore, Trincheri et al. reported that inactivation of autophagy protein in cancer cell may result in resistance to resveratrol-mediated killing [114]. However, very recently it has been questioned about the potential of chemotherapeutic drugs to induce autophagic cell death [115]. Thus, further studies

are needed to clarify the molecular mechanisms of toxicity of chemotherapeutic drugs and the functional role of autophagy in the death-inducing pathway.

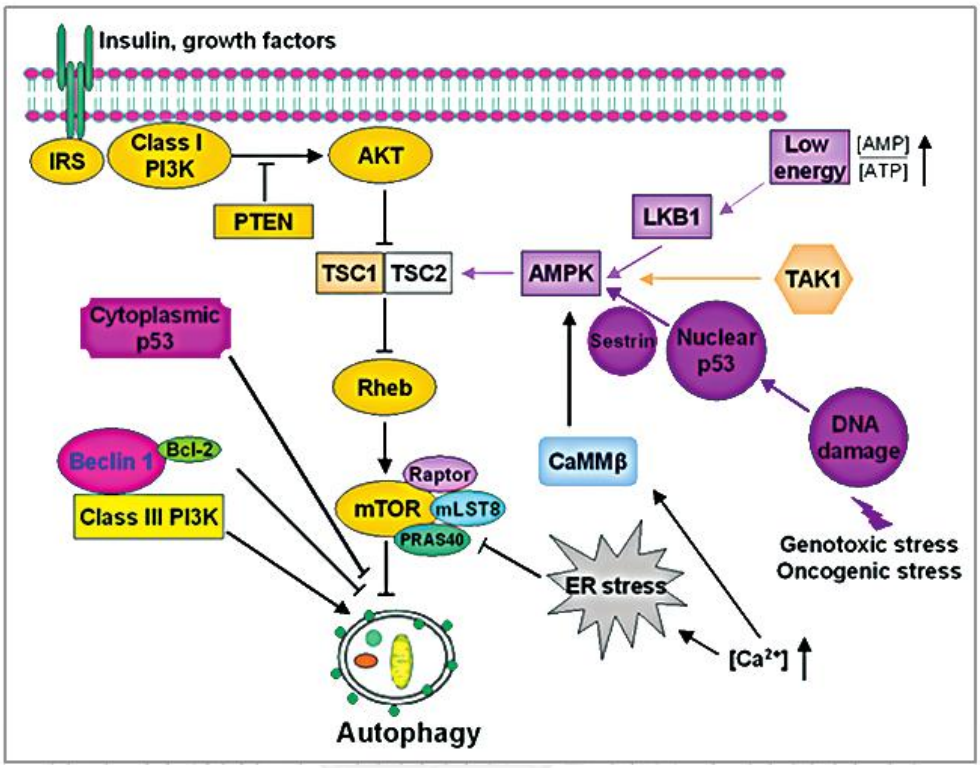


Figure 1.12 Regulation of autophagy. Growth factor and insulin signaling activates the class I PI3K/AKT/mTOR axis leading to autophagy inhibition. On the other hand, the class III PI3K acts as the autophagy induction. The increasing of AMP/ATP ratio form low energy activates the LKB1-AMPK-mTOR axis, consequence induces autophagy. The activation of AMPK can be activated by calcium and calmodulin-dependent protein kinase kinase β (CAMKKβ). The tumor suppressor p53 shows the complex on regulation of autophagy as nuclear p53 activated by genotoxic or oncogenic stress act as the positively regulates autophagy by activating AMPK; whereas, cytoplasmic p53 act as the negatively regulates autophagy. BCL-2 also exhibits autophagy inhibition by binding to BH3 domain of BECLIN1. The calcium ion release into the cytosol is contributed to ER stress, and caused upregulation of autophagy. Moreover, calcium ion can induce autophagy via Ca^{2+}- CAMKKβ-AMPK pathway [22].

Phytochemicals

Skin cancer arises from sun-exposed body sites, thus protection from UVB radiation-induced cellular damage is an effective strategy for the treatment of skin cancer [116]. Many strategies have potential for UV protection such as the use of sunscreens (including cosmetics) or protective clothing; however, due to several causes, these primary prevention methods have shown limitation [117]. Thus, researchers seek new strategies for skin cancer prevention and treatment. Epidemiological studies have revealed that the regular consumption of great quantities of vegetables and fruits is associated with a low risk of cancer [118]. These studies have prompted an increased interest in the potential of natural phytochemicals for the protection and treatment of skin cancer. Indeed, several studies have demonstrated that phytochemicals have various effects for both protection and treatment of skin cancer, such as sunscreen effects, anti-inflammatory effects and anti-oxidant effects (Table 1.2). In this context, the phenolic compounds or extracts rich in polyphenols appear to be the most active (reviewed in [30]). In a pioneristic study, Wang et al. revealed that green tea polyphenols protected against UVB radiation-induced carcinogenesis [119]. More recently, Meeran et al. reported that green tea polyphenols prevented photocarcinogenesis through promotion of DNA repair and inhibition of skin inflammation [31]. Apart from these studies, it has been demonstrated that silymarin (a flavanoid from *Silybum marianum*) and naringenin (a flavonones found in citrus fruits such as lemon and orange) protected UVB radiation-induced damage in keratinocytes through enhancement the DNA repair mechanism and thus preventing apoptosis [32, 120]; further, [6]-gingerol (a phenol from *Zingiber officinale*) reduced the production of ROS, the activation of caspases-3, -8, -9 and Fas expression induced by UVB [121]. In addition, Singh et al. showed that silymarin suppressed cell proliferation in human SCC [33], and Jee et al. revealed that curcumin (a polyphenol from *Curcuma longa*) induced apoptotic cell death in human basal carcinoma cell [34]. Other botanical agents rich in polyphenol also have potential chemopreventive activities such as genistein (from soybean) and resveratrol (largely present in the skin and seed of grape, nuts, and red wine) (reviewed in [30]). Isidoro and collaborators showed that resveratrol could induce apoptosis and autophagy-associated cell death

in colorectal cancer cells [114, 122], and inhibited cell proliferation and cell migration, while promoting cell differentiation, in glioblastoma cells [123]. Therefore, dietary phytochemicals may be considered as a new, safety and efficient strategy for the treatment of skin cancer.

In Thailand, the traditional medicine system has been developed since Ayutthaya period (1350-1767 A.D.) [124]. In the year 1888 during the region of King Rama V, Siriraj hospital was settled, which combination between modern and tradition medicine system. In the year 1913, the tradition medicine system was stopped cause of different doctrines. However, the importance of botany was return; therefore, the Ministry of Public Health, Thailand, was established in 2002. This department is involved in traditional medicine system. In the year 1999, the first list of essential medicines (herbal medicine) was approved. The latest, in 2012, 52 traditional drugs and 21 single herbal drugs was approved. They are classified as modified herbal medicine that is divided into 4 groups: the modified Thai tradition medicine, the modified traditional medicine (e.g. Chinese), the single herbal, and the model herbal drug. Now a day, silymarin, is only one model herbal drug, is licensed cause of safety, efficacy and quality. Most of herbal produce cannot be claimed as model medicine, because it is very difficult to prove the efficacy, adverse effect with the same standard. There are some Thai plants are well known as single herbal produce. 1.) *Andrographis paniculata*, the aerial parts that contain total lactones not less than 6%. It has been reported anti-inflammatory and immunostimulatory effects. 2.) *Curcuma longa*, the rhizome parts contain curcuminoids not less than 5% (as curcumin) and volatile oil not less than 6%. It has been reported curcuminoids or curcumin in rhizome and cineloe in volatile oil can stimulate the bile secretion, which help to digestion and reduce the flatulence. 3.) *Momordica charantia* contain the structure of charantin as a mixture of sitosteryl- and 5, 25 stigmastadiene-3β-ol-D-glucoside. The leaf has been used for relieving fever and the root has been used for remedying liver disease and blood disorder. Interestingly, the fruit is widely studied the anti-diabetic effect (reviewed in [125]). In addition, there are many Thai plants that are widely used in the kitchen, and are well known beneficial on health issue as summarize in table 1.3.

Table 1.2 Phytochemicals for protection and treatment of skin cancer (reviewed in [126]).

Phytochemicals	Source	Mechanism
EGCG	Green tea	• Inhibition of UVB-induced PI3K, c-fos, and activator protein-1 (AP1) and restoration of UVB-induced decrease the level of glutathione (GSH) • Modulation of NF-kB and MAPK pathways • Suppression of tumor incidence, tumor multiplicity and tumor growth • Reduction of matrix metalloproteinases (MMPs) -2, -9
Genistein	Soybean	• Inhibition of UV-induced DNA damage, H_2O_2, and phosphorylation of EGFR • Suppression of PUVA-induced skin thickening, diminished cutaneous erythema, and ulceration
Resveratrol	Grape, red wine	• Inhibition of UVB-induced H_2O_2, COX, NF-kB, surviving, and MAPK pathway • Inhibition of tumor incidence and delay tumorigenesis
Silymarin	Milk thistle	• Inhibition of UVA-induced DNA single strand breaks and caspase-3 activity • Reduction of UVB-induced immunosuppression • Suppression of UVB-induced CPD damage, PCNA, COX, and sunburn cell • Upregulation of p53 and p21 • Decrease in tumor incidence, tumor multiplicity, and tumor volume

Table 1.2 Phytochemicals for both protection and treatment of skin cancer (continued) (reviewed in [126]).

Phytochemicals	Source	Mechanism
Apigenin	Parsley, celery, lettuce	• Inhibition of UV-induced ornithine decarboxylase (ODC) activity • Reduction of cdk2 kinase activity, phosphorylation of Rb, MMP-1 activity, MMP-1 expression, and AP1 activation • Upregulation of p21
[6]-Gingerol	Ginger	• Suppression of UVB-induced ROS level, caspase-3,-8, and -9 activation, FAS expression, COX-2, and NF-kB
Curcumin	Turmeric	• Inhibition of UVB-induced COX-2, JNK, and p38 • Induction of apoptosis by inducing caspase-3,-8, and -9 activation, and cytochrome c release • Inhibition of UVA-induced ODC activity, MMP and protein kinase C
Delphinidin	Pigmented fruits and vegetable such as pomegranate, strawberry	• Inhibition of UVB-induce cell death, apoptosis, DNA damage such as 8-OHdG and CPD
Lycopene	Tomato	• Inhibition of UVB-induce ODC, skin thickness, erythema, sunburn cell • Increasing of pigmentation

Table 1.3 Thai plants widely used for cooking in the kitchen.

Thai plants	Beneficial effect	References
Turmeric (*Curcuma longa* L.)	• Digestive disorder • Arthritis • Anti-cancer (e.g. anti-proliferation, anti-metastasis, angiogenesis, and induct apoptosis) • Skin disease • Delays diabetic cataract • Anti-oxidant properties • Autoimmune disease • Anti-inflammatory effect • Neuroprotective effect	(reviewed in [127])
Asiatic pennywort (*Centella asiatica* (L.) Urb.)	• Anti-tumor • Anti-oxidant properties • Wound healing • Alzheimer's disease	[128-131]
Ginger (*Zingiber officinale* Roscoe)	• Antioxidant • Anti-cancer • Balance the immune system • Cardiovascular protection • Digestive health	[132]
Ivy gourd (*Coccinia grandis* L.)	• Blood sugar lowering effect • Antimicrobial activity • Antidyslipidemic activity • Source of natural antioxidant	[133-136]

Table 1.3 Thai plants widely used for cooking in the kitchen (continued).

Thai plants	Beneficial effect	References
Shallot (*Allium ascalonicum* L.)	• Anti-oxidative and free radical scavenging abilities • against cyclosporine A nephrotoxicity • Against human leukemia cells	[137, 138]
Common basil (*Ocimum basilicum* L.)	• Prevention of cardiovascular disease • Antioxidant properties • Prevention of stroke • Hepatoprotective activity • Anti-inflammatory effects • Antimicrobial activities	[139-144]
Kitchen mint (*Mentha cordifolia* Opiz ex Fresen)	• Itchy skin.	[145]
Leech lime (*Citrus hystrix* DC.)	• Inhibitors of nitric oxide generation • Antifertility effect	[146, 147]
Holy basil (*Ocimum tenuiflorum* L.)	• Anti-inflammatory effects • Antibacterial activities	[148]
Horseradish tree (*Moringa oleifera* Lam.)	• Enhancing of wound healing • Antibacterial activities • Hepatoprotective activity • Anti-cancer • Antioxidant properties • Immunosuppressive activity • Anti-inflammatory effects • Anti-fungal activity • Anti-diabetes mellitus	[149-156]

Table 1.3 Thai plants widely used for cooking in the kitchen (continued).

Thai plants	Beneficial effect	References
Indian mulberry (*Morinda citrifolia* L.)	• Anti-oxidative and anti-inflammatory responses. • Enhancing of wound healing • Induction of apoptosis • Anti-diabetes mellitus • Beneficial immunomodulation effects • UVB-induced transcriptional AP-1 activity	[157-162]
Common cucumber (*Cucumis sativus* L.)	• Antioxidant properties • antimicrobial activities	[163, 164]
Aloe (*Aloe vera* (L.) Burm. f.)	• Antioxidant properties	[165]

Objectives of this study

The whole objective of this study was to find the Thai plant from fifteen species of Thai plants that showed protective effect on UVB-induced cellular damage by enhancing DNA repair mechanism and anticancer effects on squamous cell carcinoma.

1. To investigate the biochemical properties of extracts from fifteen species of Thai plants.

2. To investigate the protective effects of fifteen species of Thai plant extracts on UVB-induced cellular damage through removal of CPD.

3. To investigate the inhibitory effects of Thai plant extracts on squamous cell carcinoma proliferation through induction of apoptosis and autophagy.

4. To investigate the effects of Thai plant extracts on cell migration in squamous cell carcinoma.

Research questions

1. Do the chosen Thai plants extracts contain antioxidant compounds, and do they show antioxidant activities?

2. Do one or more than one out of the fifteen species of Thai plant extracts show the property of UVB absorption and protect human keratinocytes from UVB-induced cellular damage?

3. Do any of the fifteen species of Thai plant extracts possess the ability of enhancing the DNA repair mechanism, thus protecting human keratinocytes from UVB-induced apoptosis?

4. Do any of the fifteen species of Thai plant extracts possess the ability of inhibiting cell viability and of inducing cell apoptosis in squamous cell carcinoma?

5. Do the selected Thai plant extracts with anticancer properties (see point 4) also possess the ability to activate autophagy in squamous cell carcinoma? and, in

affirmative case, is this autophagy promoting cell survival or inducing autophagy-dependent cell death ?

6. Do the selected Thai plant extracts with anticancer properties (see point 4) also play a role in cell migration in squamous cell carcinoma?

Hypotheses

1. One or more than one out of the chosen fifteen species of Thai plant extracts contain antioxidant compounds and possess antioxidant properties.

2. Some of the chosen fifteen species of Thai plant extracts possess UVB absorption properties and exert a protective activity toward human keratinocytes against UVB-induced cellular damage.

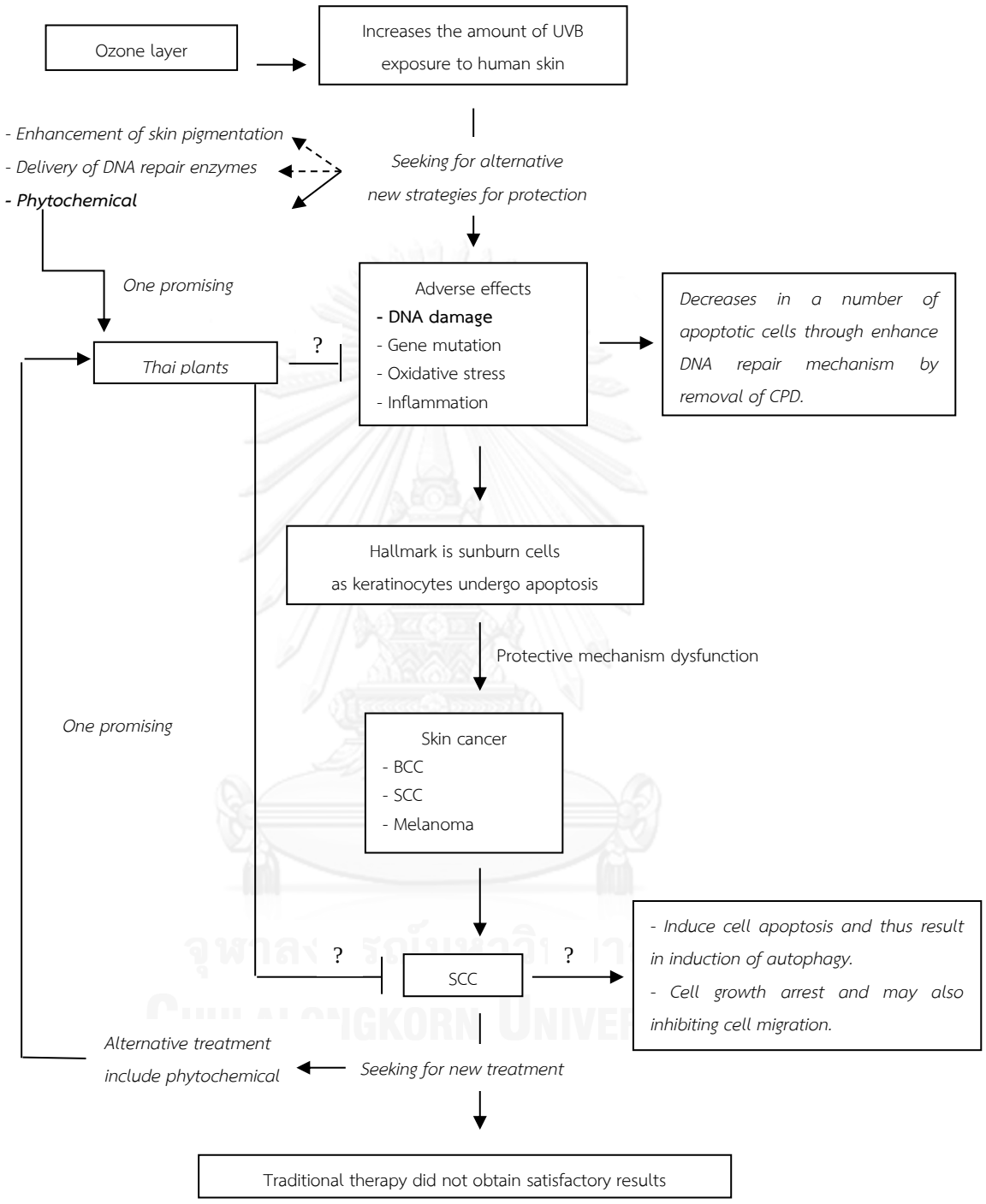
3. The protection mechanism of the Thai plant extracts toward UVB-induced cellular damage in human keratinocytes involves a DNA repair pathway for the removal of CPD.

4. Some of chosen fifteen species of Thai plant extracts (at pharmacologically relevant concentrations) are capable of inhibiting cell viability and of inducing cell apoptosis in squamous cell carcinoma.

5. The (selected) Thai plant extracts showing pro-apoptotic activities toward squamous cell carcinoma (see above point 4) also activate autophagy, which might result in inducing autophagic cell death or autophagy-dependent cell death.

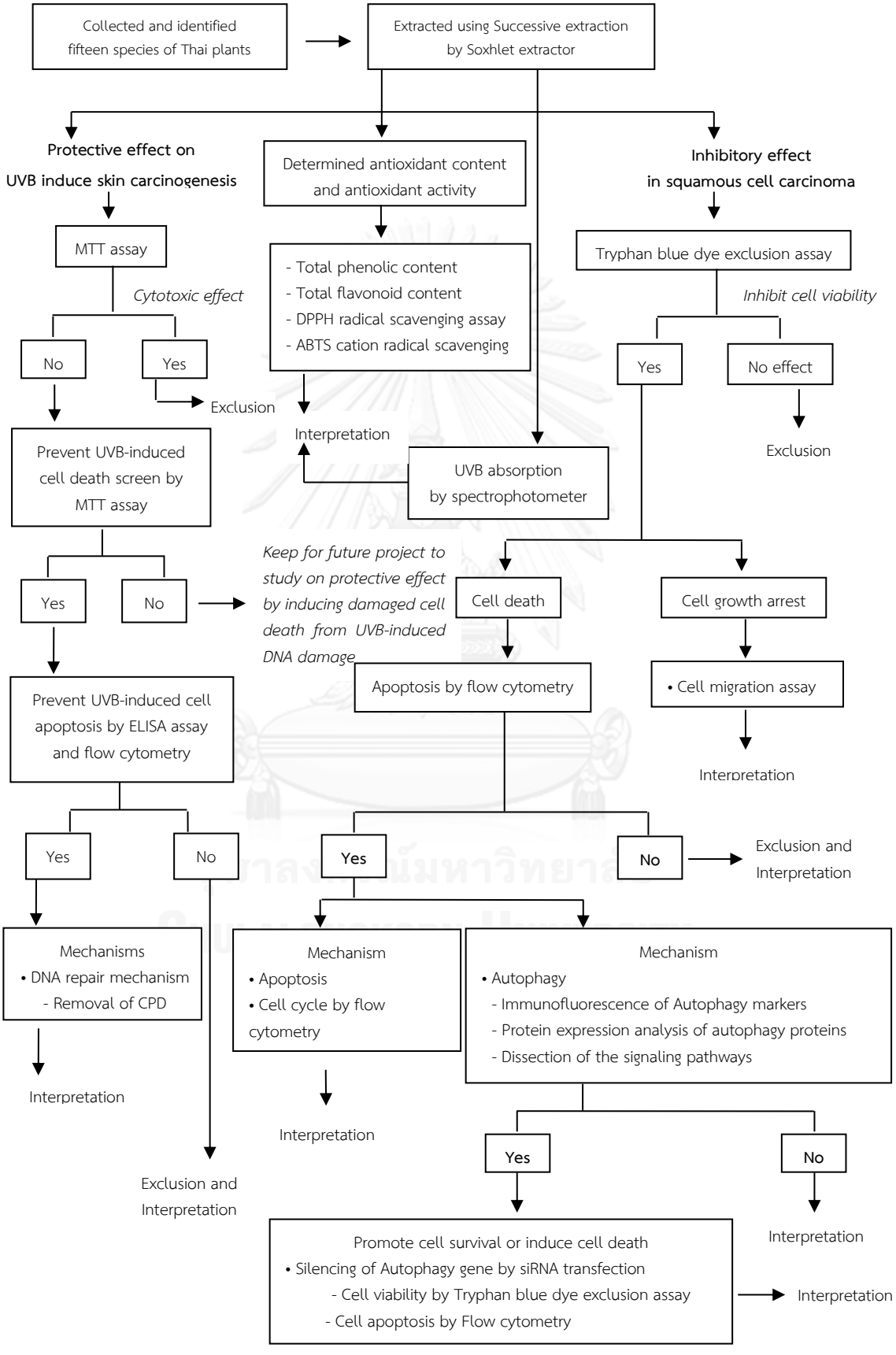
6. The (selected) Thai plant extracts showing pro-apoptotic activities toward squamous cell carcinoma (see above point 4) also play a role in the regulation of cell migration squamous cell carcinoma.

Conceptual framework



BCC, basal cell carcinoma; SCC, squamous cell carcinoma

Experimental design



CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Analytical grade reagents were used in this study.

Chemicals	Companies, Countries
2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)	Sigma-Aldrich, USA
Aluminium chloride ($AlCl_3$)	Merck, Germany
Ascorbic acid (vitamin C)	Merck, Germany
ApoTarget™ caspase-3 protease assay	Invitrogen , *USA*
Chloroquine	Sigma-Aldrich, USA
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Merck, Germany
Dichloromethane	Merck, Germany
Dimethyl sulfoxide (*DMSO*)	Merck, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were	Merck, Germany
Blotto, non-fat dry milk	Santa Cruz Biotechnology, USA
Dulbecco's Modified Eagle Medium/High glucose (DMEM/HG)	Hyclone, UT
Dulbecco's Modified Eagle's Medium	Sigma-Aldrich, USA
Ethanol	Merck, Germany
Fetal bovine serum (FBS)	Sigma-Aldrich, USA
Folin–Ciocalteu's phenol reagent	Sigma-Aldrich, USA
Gallic acid	Merck, Germany
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (170-6515)	Biorad, USA
Goat Anti-Mouse IgG (H + L)-HRP Conjugate (170-6516)	Biorad, USA

IRIS-2 (green fluorescence)–conjugated goat-anti-rabbit IgG (2WS-08) antibody	Cyanine Technology SpA, Italy
IRIS-3 (red fluorescence)– conjugated goat-anti-mouse IgG (3WS-07) antibody	Cyanine Technology SpA, Italy
Immun-blot PVDF membrane and nitrocellulose membrane	Biorad, USA
L-glutamine solution	Sigma-Aldrich, USA
Lipofectamine® 2000 transfection reagent	Invitrogen, USA
Methanol	Merck, Germany
Mouse monoclonal anti-LC3B (L7543) antibody	Sigma-Aldrich, USA
Mouse monoclonal anti-LAMP1 (555801) antibody	BD Pharmingen™, USA
Mouse monoclonal anti-b-tubulin (T 5293) antibody	Sigma-Aldrich, USA
Mouse monoclonal anti-actin (A5316) antibody	Sigma-Aldrich, USA
Mouse monoclonal anti-E-cadherin (610181)	BD Transduction Laboratories™
Monoclonal rabbit anti-S6 ribosomal protein (2217) antibody	Cell Signaling Technology, USA
Monoclonal rabbit anti- phospho-S6 ribosomal protein (Ser235/236) (4856)	Cell Signaling Technology, USA
Mouse anti-Thr167 phospho-BAX antibody	VinciBiochem, Italy
Monoclonal mouse anti-p53 (sc-126)	Santa Cruz Biotechnology, USA
Petroleum ether	Merck, Germany
Potassium persulfate, quercetin	Sigma-Aldrich, USA
Sodium carbonate (Na_2CO_3)	Merck, Germany
Sodium acetate ($NaC_2H_3O_2$)	Merck, Germany
Trypan blue solution	Invitrogen , *USA*
OxiSelect™ Cellular UV-Induced DNA Damage ELISA Kit (CPD)	Cell Biolabs, USA
Opti-MEM® reduced-serum medium	Invitrogen, USA
Propidium iodide	Sigma-Aldrich, USA
Penicillin-streptomycin solution	Hyclone, UT
Penicillin-streptomycin solution	Sigma-Aldrich, USA
Protease Inhibitor Cocktails (P8340)	Sigma-Aldrich, USA

Polyclonol rabbit anti-BAX (2772) antibody	Cell Signaling Technology, USA
Polyclonol rabbit anti-AKT (9272) antibody	Cell Signaling Technology, USA
Polyclonol rabbit anti-Phospho-AKT (Ser473) (9271) antibody	Cell Signaling Technology, USA
Polyclonal rabbit anti-Ser15phospho-p53 (9284)	Cell Signaling Technology, USA
Polyclonal rabbit anti-p21 (C-19) (sc-397).	Santa Cruz Biotechnology, USA
Polyclonal rabbit anti-BECLIN 1 (sc-11427) antibody	Santa Cruz Biotechnology, USA
Polyclonal rabbit anti-thioredoxin 1 (ab26320) antibody	Abcam, UK
Rapamycin	Sigma-Aldrich, USA
ReBlot Plus Strong Antibody Stripping Solution, 10x	Millipore Corporation, USA
Rhodamine123	Alexis Biochemicals, USA
Resveratrol	Sigma-Aldrich, USA
Small interference RNA (siRNA)	MWG Biotech AG, USA
Western lightning™ chemiluminescence reagent plus	PerkinElmer, USA
Z-VAD-fmk	Alexis Biochemicals, USA

2.1.2 Tools and Devices

Instruments	Companies, Countries
-20 °C Freezer	Sanyo, Japan
-80 °C ULT deep freezer	Liofreeze, USA
4 °C refrigerator	Sharp, Japan
6, 12, 96 well cell culture flat bottom with lid	Corning Inc., USA
25, 75 cm^2 cell culture flask	Corning Inc., USA
15, 50 mL centrifuge tube	Corning Inc., USA
2 mL Cryovial tube	Corning Inc., USA
5, 10 mL Disposable serological pipette	Corning Inc., USA

1.5 mL microcentrifuge tube	Eppendorf, Germany
10, 200, 1000 µl	GILSON, France
Analytical balances	Mettler Toledo, Switzerland
Auto pipette	GILSON, France
Block heater	Wealtec Corp., USA
Co_2 incubator	Sheldon Manufacturing, USA
Electrophoresis power supply	Bio-Rad Laboratories, USA
Flow cytometer	Becton Dickinson, USA
Hem cytometer	Hausser Scientific, USA
Incubator	Memmert, Germany
Inverted microscope	Olympus Optical, Japan
Leica DMI600 fluorescence microscope	Leica Microsystems AG, Germany
Larminar flow cabinet	E.S.I Flufrance, France
Light microscope	Olympus Optical, Japan
Liquid nitrogen tank	Taylor-Wharton, USA
Microcentrifuge	Beckman Coulter, USA
Micro refrigerated centrifuge	Vision Scientific, South Korea
Multichannel pipette	GILSON, France
Mini Trans-Blot® Electrophoretic Transfer Cell	Bio-Rad Laboratories, USA
Narrowband TL 20W/01 lamp	Philip, Holland
Rotary evaporator	Heidolph Instruments, Germany
Ultraviolet UV Meter (UVB UV6.2)	Ponpe Instruments, Thailand
UV-Visible spectrophotometer	PerkinElmer, Finland
Vortex mixer	FINEPCR, South Korea
VersaDoc™ Imaging System	Bio-Rad Laboratories, USA

2.2 Thai plants samples

Fifteen species of Thai plants (figure 2.1) in the present study were collected from Bang pra subdistrict, Sriracha district, Chonburi province, Thailand. They were authenticated by Professor Dr. Thaweesakdi Boonkerd and deposited at the Professor Dr. Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The scientific names and parts used for this study are presented in Table 2.1. Curcumin was obtained from Professor Dr. Apichart Suksamrarn, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Thailand, and prepared as previously reported [166].

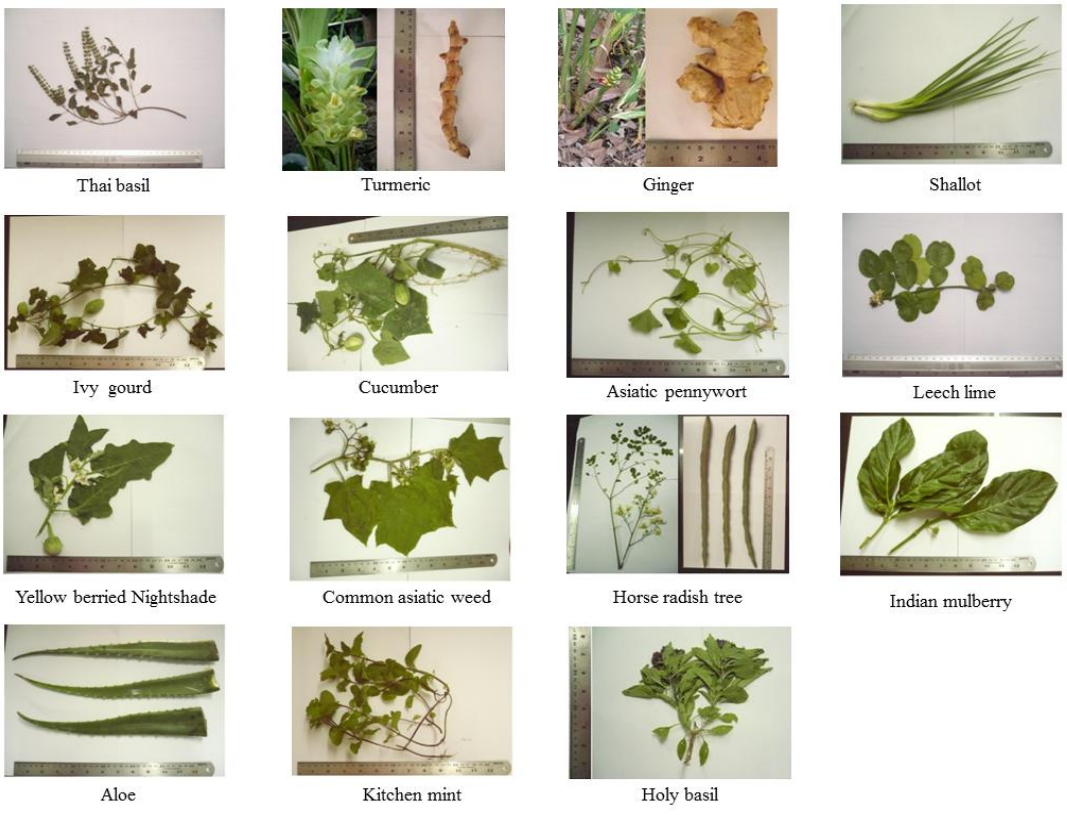


Figure 2.1 Fifteen species of Thai plants.

Table 2.1 Details of common species of Thai plants.

Scientific Name	Family	Common Name	Part used	Herbarium Number
Curcuma longa L.	Zingiberaceae	Turmeric	Rhizome	013396 (BCU)
Centella asiatica (L.) Urb.	Umbelliferae	Asiatic pennywort	Leaf	013400 (BCU)
Zingiber officinale Roscoe	Zingiberaceae	Ginger	Rhizome	013407 (BCU)
Coccinia grandis L.	Cucurbitaceae	Ivy gourd	Rhizome	013439 (BCU)
Allium ascalonicum L.	Alliaceae	Shallot	Whole body	013440 (BCU)
Ocimum basilicum L.	Labiatae (Lamiaceae)	Common basil	Leaf	013441 (BCU)
Moringa oleifera Lam.	Moringaceae	Horse radish tree	Fruit	013442 (BCU)
Morinda citrifolia L.	Rubiaceae	Indian mulberry	Leaf	013443 (BCU)
Mentha cordifolia Opiz ex Fresen	Labiatae (Lamiaceae)	Kitchen mint	Leaf	013445 (BCU)
Solanum torvum Sw.	Solanaceae	Common Asiatic weed	Fruit	013446 (BCU)
Citrus hystrix DC.	Rutaceae	Leech lime	Leaf	013447 (BCU)
Ocimum tenuiflorum L.	Labiatae (Lamiaceae)	Holy basil	Leaf	013448 (BCU)
Cucumis sativus L.	Cucurbitaceae	Common cucumber	Fruit	013449 (BCU)
Solanum aculeatissimum Jacq.	Solanaceae	Yellow berried nightshade	Fruit	013450 (BCU)
Aloe vera (L.) Burm. f.	Asphodelaceae	Aloe	Gel	013451 (BCU)

2.3 Cell culture model

Immortalized human epidermal keratinocyte HaCaT cell line (figure 2.2A), served as "in vitro" model for UVB-induced cellular damage, was purchased from Cell line service (Heidelberg, Germany). The human epidermoid squamous carcinoma A431 cell line (figure 2.2B), served as "in vitro" model for was obtained from University of degli Studi del Piemonte Orientale "Amedeo Avogadro", Novara, Italy. The cells were cultured in DMEM, supplemented with 10 % FBS and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine) at 37°C in a humidified atmosphere at 5% CO_2.

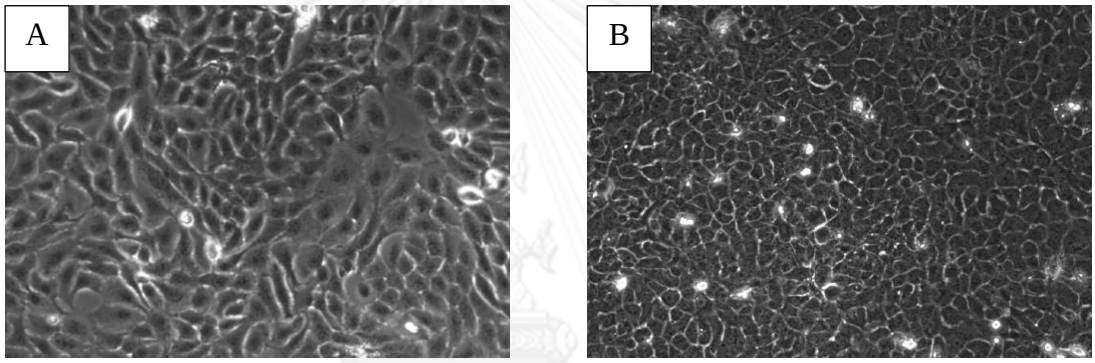


Figure 2.2 Morphology of immortalized human epidermal keratinocyte HaCaT cell line (A) and the human epidermoid squamous carcinoma A431 cell line (B) by phase contrast microscope 40X.

2.4 Experiment procedure

2.4.1 Extract preparation

The scientific names and parts used for this study are presented in Table 2.1 (note that, for simplicity, in the text the common name is used). All Thai plants were successively extracted using a Soxhlet extractor (Figure 2.3). Briefly, 100 g of fresh sample was extracted in organic solvents (1:4, w/v) using series of organic solvents with increasing polarities (petroleum ether, dichloromethane and ethanol) until exhaustion. The plant extracts were concentrated in a vacuum rotary evaporator under reduced pressure and low temperature using the MiVac Quattro concentrator. Finally, the plant extracts were dissolved in DMSO in the concentration of 100

mg/mL as stocks, and stored with protection from light at -20 ° C until further investigation.

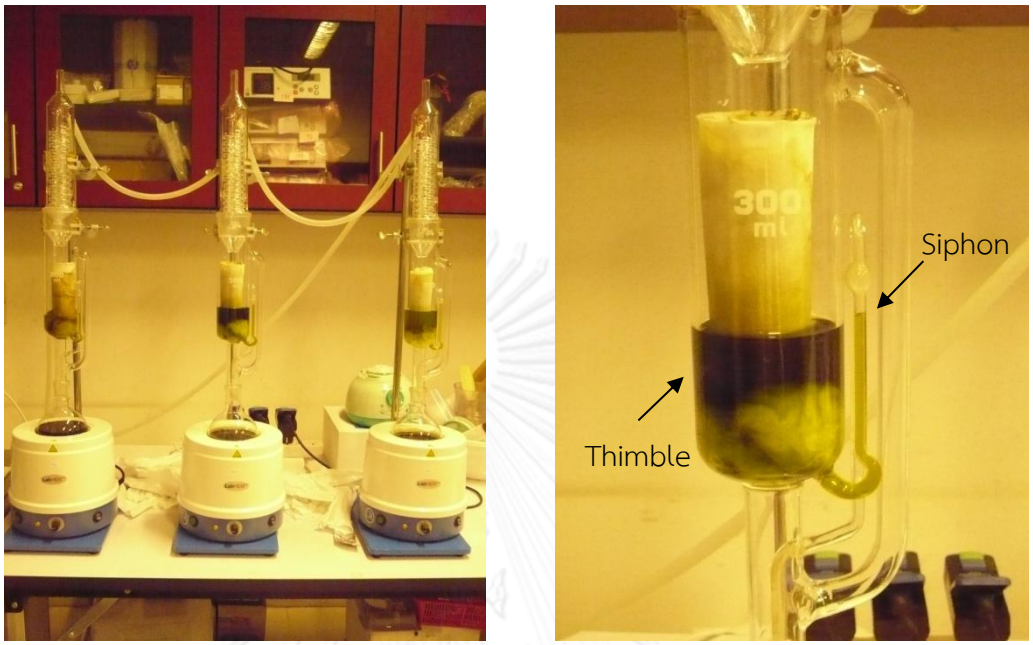


Figure 2.3 Soxhlet extractor, the principal of this technique is the plant material is put into a thimble and the solvent is put into the flask, and then boil the solvent to reach the its boiling point. Vapors of solvent pass through distillation arm, and then condense and drop onto the plant material in the thimble. In this period, the active compounds of the plant are extracted. When the volume of solvent contain the active compound of the plant reaches to the top of siphon tube, the all volume of this solvent will back into the flask. The process is continued until exhaustion.

2.4.2 Determination of antioxidant contents

2.4.2.1 Total phenolic content

The phenolic compound is a mainly type of antioxidant and mainly source is dietary and plants. The polyphenols have shown the antioxidant activity based on hydrogen donation abilities [167]. The total phenolic content of plant extracts was determined by the Folin-Ciocalteu method as described by Singelton *et al*. [168]. This method is based on the oxidation reaction of Folin-Ciocalteu reagent with antioxidant. The antioxidant activity of phenolic compound could reduce yellow color of Folin-Ciocalteu reagent to blue color. Briefly, 50 µL of the extracts (1 mg/mL) or gallic acid

(100 – 6.25 µg/mL) were added to 50 µL of 10% Folin–Ciocalteu's phenol reagent followed by 50 µL of Na_2CO_3 (10% w/v). After incubation at room temperature for 1 h in the dark, the absorbance of the reaction mixture was measured at a wavelength of 760 nm (Table 2.2). Gallic acid was served as a standard. The results are expressed as mg gallic acid equivalent (GAE)/g fresh weight of sample. All samples were analyzed in triplicate.

Table 2.2 The procedure step of Folin-Ciocalteu method.

Reagents	Reagent blank (µl)	Standard (µl)	Sample blank (µl)	Samples (µl)
1. Gallic acid (100 – 6.25 µg/mL)	-	50	-	-
2. Thai plant extracts (1 mg/mL)	-	-	50	50
3. Deionized distilled water	50	-	50	-
4. Folin–Ciocalteu's phenol reagent (10% working reagent)	50	50	-	50
5. Na_2CO_3 (10% w/v)	50	50	50	50
Mixed, incubated at room temperature for 1 h in the dark and then the absorbance of the reaction mixture was measured at a wavelength of 760 nm				

2.4.2.2 Total flavonoid content

The flavonoid is a most common group of polyphenol in dietary and plants [169]. Aluminum chloride colorimetric method was used to determine the total flavonoid content of plant extracts as previously described [170]. This method is based on the the fact that aluminum chloride forms acid stable complexes with the C-3 or C-5 hydroxyl group of flavones and flavonols, and it also forms acid labile complexs with ortho-dihydroxyl groups of flavonoids [171]. Briefly, 50 µL of the extracts (1 mg/mL) or quercetin (200 – 1.56 µg/mL) were mixed with 150 µL of absolute ethanol and 10 µL of 1 M Sodium acetate ($NaC_2H_3O_2$). Then, 10 µL of $AlCl_3$ solution (10% w/v) was added to mixture. The absorbance of the reaction mixture was measured immediately at a wavelength of 415 nm after incubation for 40 minutes in the dark

at room temperature (Table 2.3); quercetin was served as a standard. The results are expressed as mg quercetin equivalent (QE)/g fresh weight of sample.

Table 2.3 The procedure step of Aluminum chloride colorimetric method.

Reagents	Reagent blank (µl)	Standard (µl)	Sample blank (µl)	Sample (µl)
1. Quercetin (200 – 1.56 µg/mL)	-	50	-	-
2. Thai plant extracts (1 mg/mL)	-	-	50	50
3. Absolute ethanol	50	-	-	-
4. Deionized distilled water	-	-	10	-
5. Absolute ethanol	150	150	150	150
6. $AlCl_3$ (10% w/v)	10	10	-	10
7. $NaC_2H_3O_2$ (1 M)	10	10	10	10
Mixed, incubated at room temperature for 40 minutes in the dark and then the absorbance of the reaction mixture was measured at a wavelength of 415 nm				

2.4.3 Determination of antioxidant activities

2.4.3.1 The DPPH (2,2-Diphenyl-1-picrylhydrazyl)assay

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay was performed according to the method of Brand-Williams et al. (1995) [172]. This assay is based on the hydrogen donor of antioxidant which is accepted by DPPH radical. To generate the stable free radical DPPH (DPPH•), DPPH was dissolved in absolute methanol. Fresh DPPH solution was prepared daily, and absorbance of the DPPH solution was adjusted to 0.650 ± 0.020 at 517 nm using absolute methanol. Then, 20 µL of the extracts (1 mg/mL) or ascorbic acid (serving as a standard) (25 – 1.56 µg/mL) were mixed with 180 µL of DPPH solution and incubated for 30 minutes in the dark at room temperature. The absorbance of the reaction mixture was measured at a wavelength of 517 nm (Table 2.4). The results are expressed as mg ascorbic acid equivalent/g fresh weight of

sample. The IC50 was determined in comparison with ascorbic acid. The % scavenging activity (%SC) was calculated using the following formula:

% SC = [[Abs. control – (Abs. sample - Abs. blank sample)] / Abs. control] * 100

Control contained 180 µL of DPPH solution and 20 µL of absolute methanol; whereas, blank sample contained 180 µL of absolute methanol and 20 µL of extracts.

Table 2.4 The procedure step of DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay

Reagents	Control (µl)	Standard (µl)	Sample blank (µl)	Sample (µl)
1. Ascorbic acid (25 – 1.56 µg/mL)	-	20	-	-
2. Thai plant extracts (1 mg/mL)	-	-	20	20
3. Absolute methanol	20	-	180	-
4. DPPH• solution	180	180	-	180
Mixed, incubated at room temperature for 30 minutes in the dark and then the absorbance of the reaction mixture was measured at a wavelength of 517 nm				

2.4.3.2 2,2′-azinobiz(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) assay was performed according to the formerly described method [173]. This assay is based on the reduction of the radical cation of ABTS with antioxidant. The working solution or ABTS•+ solution was prepared by mixing 8 mL of 7.4 mM ABTS•+ and 12 mL of 2.45 mM potassium persulfate solution, and was subsequently incubated for 16-18 h at room temperature in the dark. After that, ABTS•+ solution was diluted by mixing with absolute ethanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. 20 µL of the extracts (1 mg/mL) or ascorbic acid (serving as a standard) (50 – 1.56 µg/mL) were reacted with 180 µL of ABTS•+ solution for 45 minutes in the dark. The absorbance of the reaction mixture was measured spectrophotometrically at 734 nm (Table 2.5). The results are expressed as mg ascorbic acid (vitamin C) equivalent/g fresh weight of

sample. The IC50 was determined in comparison with ascorbic acid. The % SC was calculated using the following formula:

% SC = [[Abs. control – (Abs. sample - Abs. blank sample)] / Abs. control] * 100

Control contained 180 µL of ABTS•+ solution and 20 µL of absolute ethanol; while, blank sample contained 180 µL of absolute ethanol and 20 µL of extracts.

Table 2.5 The procedure step of ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) assay

Reagents	Control (µl)	Standard (µl)	Sample blank (µl)	Sample (µl)
1. Ascorbic acid (25 – 1.56 µg/mL)	-	20	-	-
2. Thai plant extracts (1 mg/mL)	-	-	20	20
4. Absolute ethanol	20	-	180	-
5. ABTS•+ solution	180	180	-	180
Mixed, incubated at room temperature for 45 minutes in the dark and then the absorbance of the reaction mixture was measured at a wavelength of 734 nm				

2.4.4 Determination of UV absorption

To determine the UV absorbing ability of fifteen species of Thai plant extracts. The UV absorption properties of extracts were investigated spectrophotometrically at wavelengths 315-400 nm for UVA and 280-315 nm for UVB. The extracts were measured using a quartz cuvette at a distance of 1 mm. DMSO (0.1% in PBS) was used as a blank solution for this assay. Plant extract samples were measured at the concentration of 100 µg/mL by dissolving the stocks in PBS.

2.4.5 Protective effect on UVB induce skin carcinogenesis: UVB radiation and treatment

A narrowband TL 20W/01 lamp (Philip, Holland), which emits at 305 and 315 nm with a peak emission at 311 nm, was used to irradiate the cells as described previously [174]. A spectro-radiometer (Solarmeter) was used to monitor UVB dosage (mJ/cm^2) at a fixed distance. The cells were seeded and let adhere on cell culture plate for 24 h prior to start the treatments. HaCaT cells were irradiated with UVB by two different experimental protocols. In the first protocol, the cells were pretreated with plant extracts for 30 min, 2, 8, 16 and 24 h, followed by exposition to UVB and further incubated for 24 and 48 h. In the second protocol, the cells were exposed to UVB followed by treatment of plant extracts for 24 h. At the end, cytotoxicity and DNA damage were determined.

2.4.5.1 MTT assay, cell counting and Trypan blue dye exclusion assay

The cytotoxic effect of UVB and of plant extracts treatments was preliminary assessed by MTT assay. This assay is based on measuring the activity of mitochondrial enzymes in living cells that reduce MTT to formazan dyes. Briefly, at indicated time, MTT was added in each well (final concentration 0.5 mg/mL) and the cells were incubate at 37ºC in 5% CO_2 incubator for 4 h, afterword the medium was removed and 200 µl of DMSO was added to each well in order to dissolve formazan. The absorbance was measured at a wavelength of 550 nm. The results were expressed as % cell survival, assuming as 100% the absorbance in control untreated cells. To discriminate viable cells and necrotic cells, the Trypan blue dye exclusion test based on permeability of cell membrane that the live cells have intact the cell membrane that can exclude dye; on the other hand dead cells cannot do so. Apparently, the viable cells have a clear cytoplasm; whereas, the dead cells have a blur cytoplasm. The cells were counted with a hemocytometer.

2.4.5.2 Detection of apoptotic cells

Apoptosis was estimated by photometric enzyme-immunoassay of mono- and oligonucleosomes with the Cell Death Detection ELISA plus kit, following manufacturer's instructions. In brief, the cells were plated in microplate and treated as described in the results section. Necrotic cells in suspension were discharged. Apoptosis in the cell lysate was determined as histone-associated DNA fragments detected by specific monoclonal antibodies labeled with peroxidase and revealed with an appropriate chromogenic substrate. Absorbance was determined with a spectrophotometer. Negative and positive controls were included in the test. Cells unexposed to UVB are referred to as "sham."

2.4.5.3 Caspase-3 activity

Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family [175], plays an importance role in events of cell apoptosis. Pro-caspase-3 is an inactive form that can be activated by upstream proteases such as caspase-8 and caspase-9 [18]. The activity of caspase-3 was determined by the ApoTarget™ caspase-3 protease assay kit. The assay is based on the caspases-3 cleavage of the amino acid sequence DEVD (Asp-Glue-Val-Asp), cleavage site, which is labeled at its C-terminus with para-nitroaniline (pNA), chromophore. Upon cleavage of the substrate by Caspase-3, the absorption of free pNA was quantified using a spectrophotometer. Briefly, after UVB-induced cell apoptosis at indicated time, the adherent cells and suspend cells were collected and centrifuge at 1,200 rmp for 3 minute. The cell pellets were suspended in Cell Lysis Buffer and incubated on ice for 10 minutes, subsequently centrifuged at 13,000 rpm for 1 minute in a microcentrifuge, and then supernatant was transferred (cytosol extract) to a fresh tube and put on ice, after that the protein concentration was measured by Bradford assay. The each protein sample was diluted in 50 μl of Cell Lysis Buffer to a concentration of 50-200 μg of protein, and then 50 μl of 2x Reaction Buffer (containing 10 mM DTT), 5 μL of the 4 mM DEVD-*p*NA substrate (200 μM final concentration) was added to each sample and incubated at 37 °C for 2 h in the dark. The absorbance was measured using a

spectrophotometer at a wavelength of 405 nm. The absorbance of pNA from an apoptotic sample was compared with that of control cells.

2.4.5.4 Detection of Cyclobutane pyrimidine dimers

Cyclobutane Pyrimidine Dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4PP) are the major types of DNA damage induced by UVB radiation, being CPD three times more frequent than 6-4PP [55]. Assay of UVB-induced CPDs was performed by OxiSelect™ Cellular UV-Induced DNA Damage ELISA Kit according to the manufacturer's instructions. Briefly, after UVB irradiation, the cells were fixed and denatured, and the cell containing CPDs was probed with anti-CPD antibody, followed by an HRP conjugated secondary antibody. The unbound secondary antibody was removed, and substrate solution reactive with HRP was added. The absorbance was measured using a spectrophotometer at a wavelength of 450 nm.

2.4.5.5 Western blotting analyses

HaCaT cells were plated in petri dishes and incubated with Thai herbs extracts as indicated, and thereafter cell homogenates were prepared by freeze thawing and ultrasonication in a buffer containing detergents and protease inhibitors (0.2% NaDoc, 1 mM Na_3VO_4, 50 mM NaF, protease inhibitor cocktails) as previously reported [176]. The protein concentration was determined by Bradford assay. A quantity of 30 µg of cell proteins was denatured with Laemmli sample buffer at 95 $^{\circ}$C for 10 minuet, separated by electrophoresis on a 12% SDS containing polyacrylamide gel in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH8.3) and then electroblotted onto PVDF membrane in transfer buffer (25 mM Tris, 192 mM glycine, pH8.3). The PVDF membrane was blocked in 5% non-fat dry milk. The filter was first probed with the rabbit polyclonal antithioredoxin 1 antibody, and after stripping it was reprobed with the mouse monoclonal anti-b-tubulin antibody. b-Tubulin was used as homogenate protein loading control in the lanes. Immunocomplexes were revealed using an appropriate peroxidase-conjugated secondary antibody, and subsequent peroxidase-induced chemiluminescence reaction. Western blotting data were reproduced three

times independently. Intensity of the bands was estimated by densitometry (Quantity one software, Biorad; ImageJ software).

2.4.6 Inhibitory effect in squamous cell carcinoma: cell and treatment

2.4.6.1 Cell cytotoxicity by cell counting and trypan blue dye exclusion assay.

The cells were seeded and let them adhere on cell culture plate for 24 h before starting the treatment. A431 cells were treated with turmeric (10 and 20 µg/mL) or curcumin (20 and 30 µM) or resveratrol (50 and 100 µM) for 24 h, or co-treatment with chloroquine (50 µM) for 24 h, or pre-treatment with Z-VAD-fmk (30 µM) or rapamycin (10 µM) for 1 h and then treatment with turmeric or curcumin or resveratrol for 24 h. The DMSO served as control. To discriminate viable cells, the trypan blue dye exclusion test based on permeability of cell membrane was used (as mention above). The cells were counted with a hemocytometer.

2.4.6.2 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The changing of mitochondrial membrane potential *($\Delta\Psi_m$)* is a marker of mitochondria dysfunction. The loss of $\Delta\Psi_m$ is a one hall mark of apoptosis. Rhodamine 123 (Rho123) was used to evaluate changes in $\Delta\Psi_m$ based on the fluorescence intensity of dye is decreased when the mitochondrial membrane loss of potential. The cells were seeded and let them adhere on coverslip for 24 h before starting the treatment. A431 cells were treated with turmeric at 20 µg/mL or curcumin 30 µM for 16 h. After the indicated times of incubation, the cells were washed with PBS two times, and stained with the Rho123 at 5 µM for 30 min in the dark at 37 ºC. After incubation, the cells were washed with PBS three times. The intensity of Rho123 staining was measured by Fluorescence stained were observed with a Leica DMI600 fluorescence microscope.

2.4.6.3 Flow cytometric analysis of cell cycle

This technique was used to determine the cell cycle progression. The cells were stained by propidium iodide (PI), a fluorescent dye that stains the DNA, and were analyzed using fluorescence-activated cell sorting analysis (FACS) by flow cytometry.

The intensity of fluorescence is proportional to the content of DNA, which changes in the different cell cycle phases. The A431 cells were seeded and let them adhere on cell culture plate for 24 h before starting the treatment. The cells were treated with turmeric 20 µg/mL or curcumin 30 µM or resveratrol 100 µM for 24 h. Or the cells were pre-treated with Z-VAD-fmk 30 µM for 1 h, followed by turmeric 20 µg/mL or curcumin 30 µM or resveratrol 100 µM for 24 h. As indicated time, the cells were trypzined and washed with PBS. After centrifugation at 1,200 rpm for 10 min at 4 ºC, the cell pellets were fixed in 70% ethanol at 4 ºC overnight. Subsequence, the fixed cells were washed with PBS and were incubated with RNase 50 µg/mL for 1 h at 37 ºC. After that the reaction was inactivated by EDTA 5 mM, followed by stained with PI 50 µg/mL in the dark for 15 min at room temperature. The samples were analyzed by flow cytometry [114]. The data were interpreted using the winMDI software. The results were expressed as per cent of cells in each phase of the cell cycle.

2.4.6.4 Propidium iodide (PI) staining method

Propidium iodide (PI) is a red fluorescent nuclear staining and is not permeant to membrane of viable cell. It can be used to discrimination of live cell, apoptotic cell, and necrotic cell. The apoptotic cell is characterized by membrane shrink, DNA fragmentation, membrane blebbing and formation of apoptotic bodied; whereas, the necrotic cell is characterized by cellular swelling, pore in the plasma membrane and intracellular leaking. To determine the necrotic cell death, this method is based on PI cannot penetrate into the membrane of live cell; whereas it can pass into the membrane of necrotic cell death or secondary necrotic cell death. The A431 cells were seeded and let them adhere on coverslip for 24 h before starting the treatment. The cells were pre-treated with rapamycin 10 µM for 1 h and then were treated with turmeric 20 µg/mL or curcumin 30 µM for 8 and 16 h. After the indicated times of incubation, the cells were washed with PBS two times, and stained with the PI at 0.3 µg/mL for 5 min in dark at 37 ºC [108]. After incubation, the cells were washed with PBS three times. The intensity of PI staining was measured by Fluorescence stained were observed with a Leica DMI600 fluorescence microscope.

2.4.6.5 Immunofluorescence

The cells were seeded and let them adhere on cover slip for 24 h before starting the treatment. A431 cells were treated with turmeric 20 µg/mL or curcumin 30 µM or resveratrol 100 µM for 24 h, or co-treatment with chloroquine (50 µM) for 24 h, or pre-treatment with Z-VAD-fmk (30 µM) or rapamycin (10 µM) for 1 h and then treatment with turmeric or curcumin or reveratrol for 16 or 24 h depending on the experiment. After the indicated times of incubation, the cells were washed with PBS two times and fixed with methanol for 20 min, washed two times with PBS for 5 min, permeabilized with 0.2% triton X-100 in PBS for 10 min, and blocked with 10% FBS in PBS for 1 h. And then the cells were incubated with primary antibody of markers of the autophagy-lysosomal system (LC3 and Lamp1) as already reported [108, 114] or primary antibody of markers of apoptosis (BAX) overnight at 4 °C. Appropriately, IRIS-2 – or IRIS-3 – conjugated goat-anti-rabbit IgG or goat-anti-mouse IgG secondary antibodies were applied for 1 h. The localization of proteins was visualized Leica DMI600 fluorescence microscope.

2.4.4.6 Western blotting analyses

The cells were seeded and let them adhere on cell culture plate for 24 h before starting the treatment. A431 cells were treated with turmeric 20 µg/mL or curcumin 30 µM or resveratrol 100 µM for 24 h, or co-treatment with chloroquine (50 µM) for 24 h, or pre-treatment with Z-VAD-fmk (30 µM) or rapamycin (10 µM) for 1 h and then treatment with turmeric or curcumin or reveratrol for 16 or 24 h depending on the experiment. The procedure to perform the western blotting was mention above. The filters were probed with antibodies against autophagy proteins such as anti-LC3B, anti-BECLIN1, and anti-p62, and against signaling pathways involved in the anti-cancer activity such as anti-phospho S6, anti-S6, anti-phospho-AKT, and anti-AKT, and against cell cycle and cell apoptosis such as anti-phospho p53, anti-p53, anti-p21, and anti-BAX, and against cell migration protein such as anti-E-cadherin, after that the filters were stripped to re-probe with anti-b-tubulin or anti-actin antibodies for loading control.

2.4.4.7 Small interfering RNA (siRNA) transfection

To inhibit autophagy, siRNA specifically targeting the autophagy gene BECLIN1 was used for transfection. The sequence and the use of the oligonucleotides used for the silencing of BECLIN 1 mRNA (siRNA-BECLIN) and for sham-transfection as control have been published previously [114]. In brief, adherent A431 cells at about 40 - 50% confluence were transfected with 100 µM siRNA specific for BECLIN1 (5'GGA ACU CAC AGC UCC AUU ACU UAC CAC 3') or control duplex (5'AGG UAG UGU AAU CGC CUU GTT 3') using Lipofectamine® 2000 transfection reagent for 6 h in antibiotic- and serum-free Opti-MEM® reduced-serum medium. After transfection, the cells were cultured in completed medium for 48 h (changing the medium every 24 h). Thereafter, the cells were exposed to Turmeric 20 µg/mL or Curcumin 30 µM for 24 h and then analyzed by western blotting for the protein of interest. The transfected cells homogenates were prepared by freeze thawing and ultrasonication in a buffer containing detergents and protease inhibitors, and then the protein concentration were measured. The efficiency of knocking down was confirmed by western blotting as describe above.

2.4.4.8 Wound healing assay

Wound-healing assay was used to study cell migration *in vitro* according to the procedure described previously [123]. In brief, A431 cells adherent on cell culture plates were cultured till confluence was reached before starting the wound. The wound was made by dragging a sterile blue tip along the center of the cell culture plate. The detached cells were removed by washing twice, after which complete medium supplemented or not with Turmeric 15 µg/mL or Curcumin 25 µM or resveratrol 100 µM was added. The medium was re-newed and the substances re-added every 24 hours, and the experiment lasted 72 h, when the scratch in the control cultures was nearly closed. Photographs were taken at indicated time of incubation by using phase-contrast microscope equipped with a digital camera. The percentage distance of margins of the wound in randomly fields was measured on photographs and with the aid of the free software ImageJ. The rate of cell migration at each time point was calculated on the basis of the area with formula (area of the

wound at time 0 – area of the wound at the given time point)/area of the wound at time 0)

2.5 Statistical analysis

All experiments were performed independently at least three times in triplicate, unless otherwise specified. The siRNA experiment was performed two-times with reproducible results. Data are expressed as the mean ± standard error of the mean (S.E.M). Statistical significance was determined using the Student's t-test (statistical level of significance was set at $p < 0.05$).

CHAPTER III

RESULTS

3.1 Extraction yields and total phenolic and total flavonoid contents

3.1.1 Extraction yields

We took advantage of the successive extraction method to use small quantity of plant materials and solvents and to also reduceorganic wastes. The percentage yield of all Thai plants that were successively extracted with petroleum ether, dichloromethane and ethanol ranged from 0.04% to 3.28% (Table 3.1). In general, the highest percentage yields were obtained from ethanol fractions.

3.1.2 Total phenolic and total flavonoid contents

Total phenolic and total flavonoid contents (shown in Table 3.2) varied depending on the plant type and solvents used. Total phenolic content in extracts ranged from 85.66 ± 2.75 to 3.23 ± 0.09 mg GAE per g fresh weight of sample using the standard curve of GAE (R^2 = 0.9993). In each solvent used for extraction, turmeric was shown to contain the highest level of total phenol, followed by ginger. The lowest content of phenol was found in aloe extract from the petroleum ether fraction. Total flavonoid content ranged from 1092.71 ± 117.49 to 0.75 ± 0.38 mg QE per g fresh weight of sample using the standard curve of quercetin (R^2 = 0.9992). No total flavonoid content was detected in extracts from horseradish tree (petroleum ether and dichloromethane fractions), common cucumber (dichloromethane and ethanol fractions), yellow-berried nightshade (petroleum ether fraction) and aloe (all fractions). The lowest, but detectable, total flavonoid content was found in Common Asiatic weed extract from the petroleum ether fraction. Although the richest total phenolic and total flavonoid contents were found in extracts derived from dichloromethane fractions, extracts from ethanol fractions showed higher total phenolic and total flavonoid contents than dichloromethane fraction extracts in most plants analyzed in this current study. The highest level of total flavonoid content was detected in turmeric extract from dichloromethane fraction, followed by turmeric extract from petroleum ether fraction.

Table 3.1 Extraction yield (%) of common species of Thai plants extracted successively in petroleum ether, dichloromethane and ethanol.

Types of plant	% Yield of Plant Extractions (w/w)		
	Petroleum ether	Dichloromethane	Ethanol
Turmeric	0.37	0.50	1.24
Asiatic pennywort	0.34	0.45	1.65
Ginger	0.80	0.33	0.79
Ivy gourd	0.06	0.16	0.79
Shallot	0.62	0.15	1.22
Common basil	0.35	0.56	1.87
Horse radish tree	0.69	0.84	1.81
Indian mulberry	0.14	0.61	3.28
Kitchen mint	0.12	0.15	0.42
Common Asiatic weed	0.19	0.18	2.04
Leech lime	0.43	0.19	2.75
Holy basil	0.42	0.51	1.07
Common cucumber	0.05	0.08	1.05
Yellow berried nightshade	0.04	0.12	0.70
Aloe	0.34	0.63	0.39

Table 3.2 Total phenolic and flavonoid contents of common species of Thai plant extracts derived from different solvents.

Types of plant	Total phenolic content†			Total flavonoid content‡		
	Petroleum ether	Dichloromethane	Ethanol	Petroleum ether	Dichloromethane	Ethanol
Turmeric	37.19 ± 2.76	85.66 ± 2.75	54.94 ± 4.57	281.50 ± 27.75§	1092.71 ± 117.49§	91.06 ± 12.37§
Asiatic pennywort	4.59 ± 1.11	5.57 ± 1.13	8.28 ± 2.45	6.97 ± 3.28	4.99 ± 1.50	16.38 ± 3.24
Ginger	18.56 ± 2.76	78.49 ± 3.90	27.63 ± 1.59	4.96 ± 0.30	2.67 ± 0.32	2.14 ± 0.84
Ivy gourd	7.86 ± 1.46	10.64 ± 1.68	13.63 ± 0.25	4.27 ± 0.84	17.74 ± 3.14	19.17 ± 2.38
Shallot	6.97 ± 1.21	17.02 ± 1.76	12.10 ± 2.38	3.55 ± 0.72	20.18 ± 1.61	7.81 ± 1.13
Common basil	3.61 ± 0.24	9.05 ± 1.22	21.21 ± 2.93	3.35 ± 0.85	8.03 ± 1.97	18.84 ± 2.10
Horse radish tree	4.86 ± 0.82	5.34 ± 1.23	10.55 ± 1.22	N.A.	N.A.	2.07 ± 0.95
Indian mulberry	8.10 ± 0.69	16.02 ± 0.90	9.80 ± 1.03	13.20 ± 3.09	31.29 ± 2.02	27.32 ± 3.11
Kitchen mint	5.64 ± 0.91	17.20 ± 1.76	25.03 ± 2.52	3.88 ± 1.52	14.01 ± 2.76	41.73 ± 2.60
Common Asiatic weed	5.60 ± 1.70	10.37 ± 0.07	9.60 ± 1.16	0.75 ± 0.38	8.40 ± 1.69	3.83 ± 1.59
Leech lime	5.87 ± 1.61	20.00 ± 1.50	22.73 ± 1.58	8.55 ± 2.51	80.34 ± 3.90	14.61 ± 3.25
Holy basil	6.83 ± 0.18	10.51 ± 1.37	19.67 ± 1.82	3.31 ± 2.20	7.64 ± 1.35	22.94 ± 4.46
Common cucumber	8.51 ± 1.07	6.26 ± 1.48	7.50 ± 0.80	1.32 ± 0.48	N.A.	N.A.
Yellow berried nightshade	8.29 ± 1.95	5.05 ± 0.12	8.37 ± 0.55	N.A.	1.28 ± 0.68	5.90 ± 1.56
Aloe	3.23 ± 0.09	4.55 ± 1.21	4.59 ± 1.07	N.A.	N.A.	N.A.

Values are means ± standard deviation of triplicate independent analyses; † total phenolic content expressed as mg gallic acid equivalent (GAE)/g fresh weight of sample; ‡ total flavonoid content expressed as mg quercetin (QE)/g fresh weight of sample; N.A.: not available; § Curcuminoids might interfere with this method.

3.2 Antioxidant activities

Two different experimental approaches were employed for determination of antioxidant activities. DPPH assay is based on hydrogen donor property of antioxidants and is widely used in natural antioxidant studies because of its simplicity and sensitivity. ABTS assay has also been widely used to evaluate antioxidant activities due to its applicability in both aqueous and lipid phases [177, 178]. Results of the DPPH and ABTS assays are listed in Tables 3.3 and 3.4, respectively. In all antioxidant activity assays, Turmeric extract from dichloromethane fraction had the richest antioxidant activity (85.30 ± 1.02 % SC, IC_{50} = 141.78 µg/mL by the DPPH assay and 93.60 ± 0.25 % SC, IC_{50} = 88.05 µg/mL by the ABTS assay), followed by Ginger extract from dichloromethane fraction (85.21 ± 0.18 % SC, IC_{50} = 165.18 µg/mL by the DPPH assay and 93.44 ± 0.53 % SC, IC_{50} = 100.52 µg/mL by the ABTS assay). The lowest antioxidant activity was found in Asiatic pennywort (2.52 ± 0.58 % SC by the DPPH assay) and Yellow-berried nightshade (6.34 ± 0.94 % SC by the ABTS assay). Of note, the extracts obtained from solvents with higher polarities (dichloromethane and ethanol) showed greater antioxidant activities than those of solvents with lower polarities (petroleum ether).

Correlation analyses were used to evaluate the correlations between the two assays for antioxidant activity (Figure 3.1a) and between the antioxidant contents and antioxidant activities (Figure 3.1 b-f). We found a high correlation between the two techniques employed for evaluating antioxidant activities (R^2 = 0.9102), in agreement with a previous study [179]. The highest correlation was observed between the total phenolic content and antioxidant activity with regard to the DPPH assay (R^2 = 0.8203; Figure 3.1b) and the ABTS assay (R^2 = 0.7213, Figure 3.1c), in agreement with a previous report [180]. On the contrary, total flavonoid content showed low correlation with total phenolic content (R^2 = 0.4593), and antioxidant activity as assayed with DPPH (R^2 = 0.272) and ABTS (R^2 = 0.2027) methods (Figure 3.1, panels d, e, f, respectively). A similar low correlation between total flavonoid content and total phenolic content was already reported [181]. This suggests that phenolic compounds are major contributor to antioxidant activities in these Thai plants. The high

antioxidant activities and high levels of phenolics and flavonoids in Thai plants found in this study support the contention that they may have beneficial health effects.

3.3 UV absorption properties

The spectrum of UV reaching the Earth's surface has wavelengths from 280 to 400 nm: UVA (315-400 nm) and UVB (280-315 nm). UV-absorbing properties of fifteen species of Thai plant extracts are presented in Figure 3.2. The maximum UVA absorption was found in Turmeric extracts from the ethanol and dichloromethane fractions, whereas the maximum UVB absorption was found in Ginger extract from dichloromethane fraction, followed by Turmeric extract from ethanol fraction. In Figure 3.2d the comparison of UVB absorption among the three most active extracts is shown.

Table 3.3 Antioxidant activities of fifteen species of Thai plant extracts derived from different solvents by DPPH assay.

Types of plant	% scavenging activity (%SC)			mg vit C/g fresh weight of sample		
	Petroleum ether	Dichloromethane	Ethanol	Petroleum ether	Dichloromethane	Ethanol
Turmeric	72.56 ± 0.88	85.30 ± 1.02	81.29 ± 0.58	23.00 ± 0.29	27.14 ± 0.33	25.84 ± 0.19
Asiatic pennywort	2.52 ± 0.58	4.13 ± 0.90	7.83 ± 0.71	0.22 ± 0.19	0.74 ± 0.29	1.95 ± 0.23
Ginger	46.33 ± 3.76	85.21 ± 0.18	62.98 ± 0.44	14.47 ± 1.22	27.11 ± 0.06	19.88 ± 0.14
Ivy gourd	N.A.	8.35 ± 0.17	14.20 ± 1.24	N.A.	2.11 ± 0.06	4.02 ± 0.40
Shallot	2.82 ± 1.14	21.32 ± 0.55	10.07 ± 1.56	0.32 ± 0.37	6.33 ± 0.18	2.67 ± 0.51
Common basil	3.52 ± 0.51	10.14 ± 0.31	50.40 ± 4.42	0.54 ± 0.17	2.70 ± 0.10	15.79 ± 1.44
Horse radish tree	4.17 ± 0.24	4.85 ± 1.49	14.21 ± 0.77	0.76 ± 0.08	0.98 ± 0.49	4.02 ± 0.25
Indian mulberry	6.96 ± 0.68	12.2 ± 0.45	16.09 ± 0.88	1.66 ± 0.22	3.38 ± 0.15	4.63 ± 0.29
Kitchen mint	5.51 ± 0.43	30.76 ± 1.19	50.57 ± 2.60	1.19 ± 0.14	9.40 ± 0.39	15.84 ± 0.85
Common Asiatic weed	5.19 ± 0.18	15.67 ± 3.08	12.33 ± 1.68	1.09 ± 0.06	4.49 ± 1.00	3.41 ± 0.55
Leech lime	5.85 ± 1.46	31.66 ± 0.79	27.22 ± 0.42	1.30 ± 0.48	9.70 ± 0.26	8.25 ± 0.14
Holy basil	14.56 ± 0.86	15.58 ± 0.96	41.75 ± 3.08	4.13 ± 0.28	4.46 ± 0.31	12.98 ± 1.00
Common cucumber	N.A.	3.80 ± 0.99	4.95 ± 1.52	N.A.	0.64 ± 0.32	1.01 ± 0.49
Yellow berried nightshade	6.40 ± 0.95	3.44 ± 1.17	16.35 ± 0.94	1.48 ± 0.31	0.52 ± 0.38	4.72 ± 0.31
Aloe	4.84 ± 0.15	2.56 ± 1.02	4.05 ± 1.08	0.97 ± 0.05	0.39 ± 0.28	0.71 ± 0.35

Values are means ± standard deviation of triplicate independent analyses; N.A.: not available

Table 3.4 Antioxidant activities of fifteen species of Thai plant extracts derived from different solvents by ABTS assay.

Types of plant	% Scavenging activity (%SC)			mg vit C/g fresh weight of sample		
	Petroleum ether	Dichloromethane	Ethanol	Petroleum ether	Dichloromethane	Ethanol
Turmeric	78.44 ± 0.70	93.60 ± 0.25	92.68 ± 0.02	44.19 ± 0.42	53.37 ± 0.15	52.81 ± 0.01
Asiatic pennywort	N.A.	12.09 ± 0.93	17.08 ± 0.33	N.A.	4.01 ± 0.56	7.03 ± 0.20
Ginger	60.48 ± 1.66	93.44 ± 0.53	91.28 ± 1.36	33.31 ± 1.00	53.27 ± 0.32	51.96 ± 0.83
Ivy gourd	N.A.	17.36 ± 3.98	32.57 ± 1.22	N.A.	7.20 ± 2.41	16.41 ± 0.74
Shallot	7.23 ± 1.70	41.45 ± 1.56	32.08 ± 2.11	1.07 ± 1.03	21.79 ± 0.94	16.12 ± 1.28
Common basil	7.08 ± 1.16	22.30 ± 2.23	53.29 ± 1.89	0.98 ± 0.70	10.20 ± 1.35	28.96 ± 1.15
Horse radish tree	N.A.	9.02 ± 2.00	31.36 ± 2.71	N.A.	2.15 ± 1.21	15.68 ± 1.64
Indian mulberry	11.57 ± 2.30	31.47 ± 4.27	24.72 ± 1.33	3.69 ± 1.39	15.75 ± 2.58	11.66 ± 0.80
Kitchen mint	12.39 ± 2.25	43.75 ± 2.07	62.39 ± 5.31	4.19 ± 1.36	23.18 ± 1.25	34.47 ± 3.21
Common Asiatic weed	8.25 ± 1.55	38.87 ± 1.61	38.62 ± 2.72	1.68 ± 0.94	20.23 ± 0.97	20.08 ± 1.65
Leech lime	6.74 ± 1.63	52.04 ± 0.77	64.58 ± 2.28	0.77 ± 0.99	28.20 ± 0.47	35.80 ± 1.38
Holy basil	16.10 ± 2.38	27.34 ± 1.30	58.36 ± 1.66	6.44 ± 1.44	13.24 ± 0.79	32.03 ± 1.01
Common cucumber	N.A.	12.00 ± 1.27	14.84 ± 0.60	N.A.	3.96 ± 0.77	5.67 ± 0.36
Yellow berried nightshade	6.34 ± 0.94	7.57 ± 2.19	24.37 ± 1.19	0.53 ± 0.57	1.27 ± 1.33	11.45 ± 0.72
Aloe	6.78 ± 0.39	12.75 ± 3.85	N.A.	0.80 ± 0.24	4.41 ± 2.33	N.A.

Values are means ± standard deviation of triplicate independent analyses; N.A.: not available

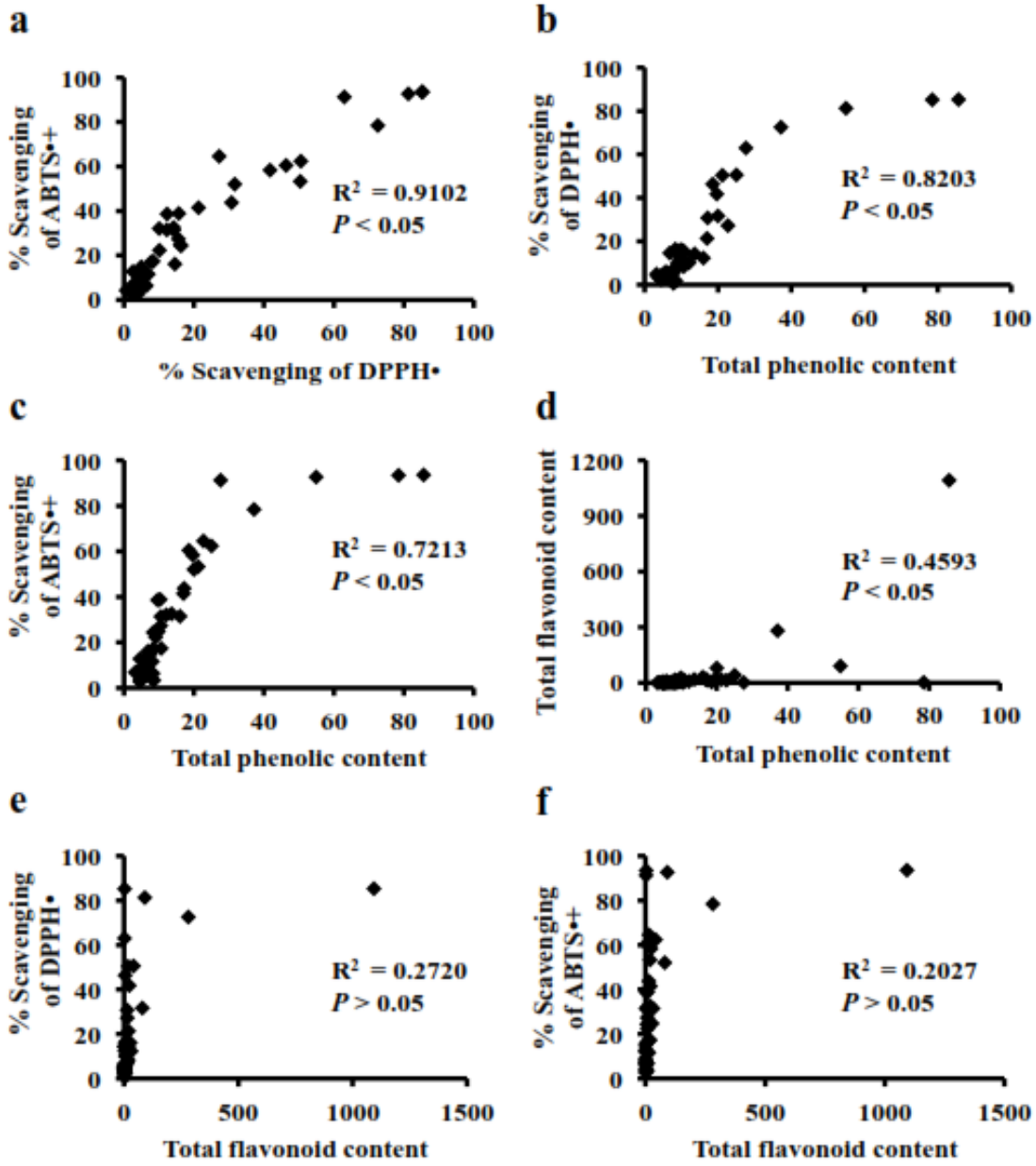


Figure 3.1 Correlation analyses. Correlations (R^2) between different antioxidant activity and total phenolic content (TPC) or total flavonoid content (TFC) of fifteen species of Thai plant extracts. (a) DPPH and ABTS assays (R^2 = 0.9102); (b) TPC and DPPH assay (R^2 = 0.8203). (c) TPC and ABTS assay (R^2 = 0.7213); (d) TPC and TFC (R^2 = 0.4593); (e) TFC and DPPH assay (R^2 = 0.2720) (F) TFC and ABTS assay (R^2 = 0.2027). The statistical significance is indicated.

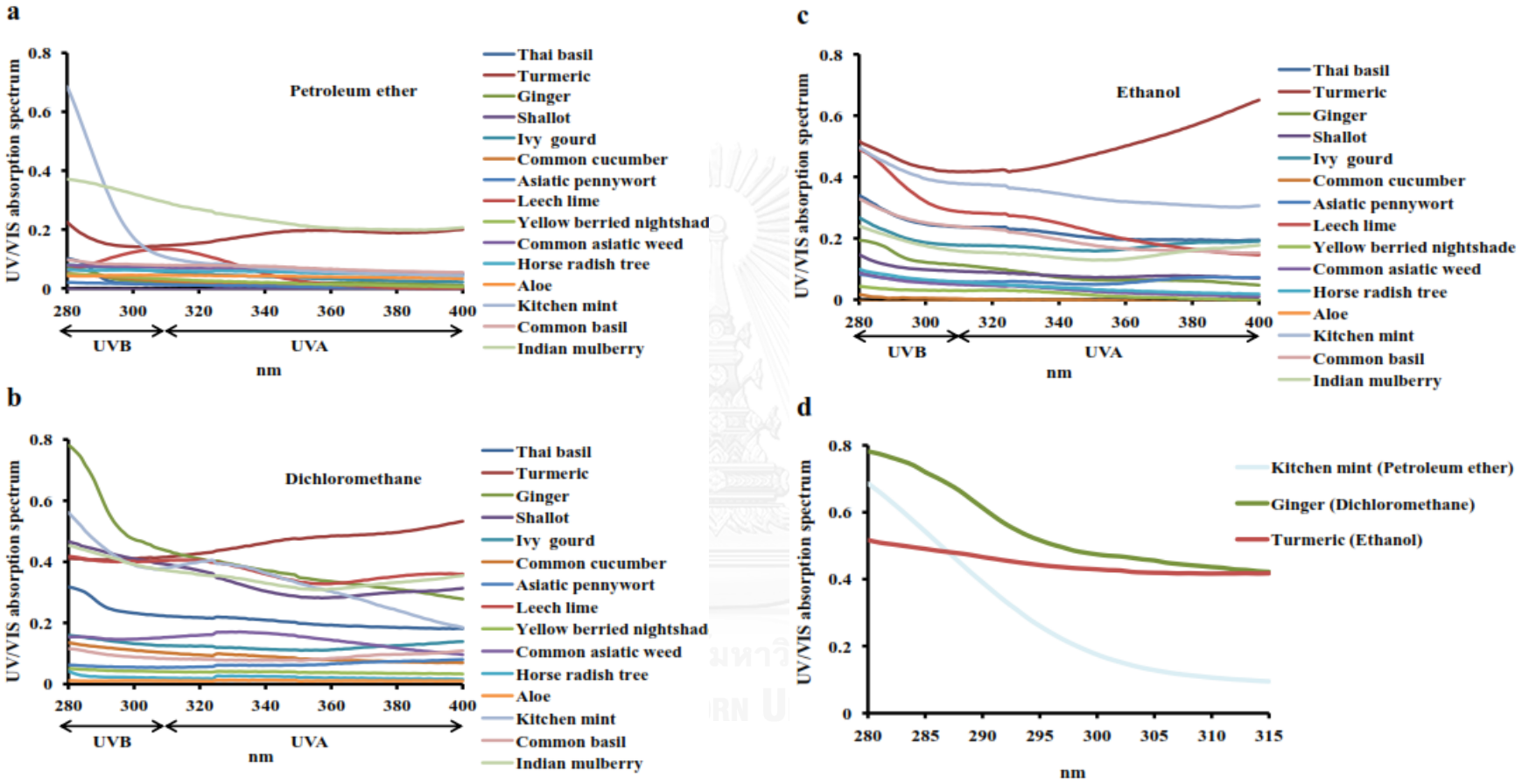


Figure 3.2 UV-absorbing properties of fifteen species of Thai plant extracts. UVA and UVB absorbing properties of the fifteen plant extracts: (a) Petroleum ether fraction; (b) Dichloromethane fraction; (c) Ethanol fraction. (d) Comparison of UVB absorption of the three most active extracts.

3.4 In vitro biological test of protection against UV cell damages by Thai herbs extracts.

The above data showed that certain Thai plant extracts particularly rich in polyphenols have UV-absorbing properties, which could protect against UV-induced cellular damage. We therefore attempted to assess the potential for these extracts to prevent DNA damage and toxicity induced by UV irradiation. Human immortalized HaCaT keratinocyte cells closely resemble normal human keratinocytes and therefore represent a valuable *in vitro* cell model for testing the effects of natural products of cosmetic interest [182]. HaCaT cells are currently widely employed to study the cytotoxic effect of UV irradiation and to evaluate the potential protection by natural compounds [183-187]. In a first set of experiments, we used the MTT assay to assess the toxicity of the Thai herbs in HaCaT cells exposed for 24 h to concentrations of extracts (dissolved in DMSO) ranging from 0.625 to 5 µg/ml. DMSO itself was not toxic at 0.05 %, corresponding to the final content in the incubation medium with the highest concentration of extract (not shown). Most of the 45 extracts were revealed not toxic (data not shown), with the exceptions as detailed below. Dichloromethane extract at all concentrations tested of Common basil, Cucumber and Leech lime consistently improved by 20 % the metabolic activity of HaCaT cells as measured by formazan precipitates (figure 3.3). Strikingly, the petroleum ether extract of Cucumber and of Leech lime were by contrast very toxic at any concentration tested (Figure 3.3). Petroleum ether extract of Common asiatic weed (at all concentrations), of Holy basil (at 2.5-5.0 µg/ml), and the ethanol extract of Indian mulberry (at 5.0 µg/ml) also revealed slightly cytotoxic. We further assessed the toxicity of selected extracts at much higher concentration and for a period of incubation up to 48 h by counting the adherent viable cells. The dichloromethane extract of Aloe and the ethanol extracts of Asiatic pennywort, of Kitchen mint and of Leech lime showed no toxicity at concentrations ranging from 5 to 100 µg/ml for up to 48 h (not shown). Actually, the latter at 5 and 50 µg/ml stimulated HaCaT cell growth by some 50 % by 48 h (Figure 3.4). Dichloromethane extracts of Turmeric at 1 µg/ml and of Ginger at 5 µg/ml were not toxic, while these same extracts at

concentrations of 5-10 µg/ml and 50-100 µg/ml, respectively, were shown very toxic (Figure 3.4).

Next, we tested under several incubation protocols the potential of some extracts to protect HaCaT cells from UVB cytotoxicity. The presence of apoptotic cells in the treated cultures was assayed by determining the amount of mono- and oligonucleosomes in the cytoplasm (see Materials and Methods section). In one set of experiments, a 30 min pre-treatment of the cells with 0.625, 1.25 and 2.5 µg/ml ethanol extracts of Leech lime, of Asiatic pennywort and dichloromethane extracts of Aloe was sufficient to significantly reduce (by approximately 20 %) the extent of apoptosis in cultures exposed to 200 mJ/cm^2 and further incubated for 24 h in normal culture medium (Figure 3.5).

Based on cytotoxic, antioxidant and UVB absorption data, for the next experiments we chose to investigate more in depth the cytoprotective potential of Turmeric (at 1 µg/ml) and of Ginger (at 5 µg/ml) extracts (dichloromethane fraction) toward cell toxicity and DNA damage induced by UVB. In these experiments we adopted a protocol using a dosage of UVB irradiation of 120 mJ/cm^2, which was shown to cause approximately 50 to 60 % cell loss by 24-48 h post-irradiation in HaCaT cultures (Figure 3.6a), and a significant accumulation of CPDs in the cells (Figure 3.6b). Also, 120 mJ/cm^2 UVB irradiation caused a 3.5-fold increase in caspase-3 activity in the cells by 24 h post-irradiation (not shown), and strongly limited the cloning efficiency of treated cells between 24 and 48 h (Figure 3.6a; compare cell density at 24 and 48 h of UV-treated versus control, considering that cell density at day 0 was 25.000 ± 6.000 cells/cm^2). We then tested the protective activity in several protocols of pre-incubation time with the herbs extracts. Optimal protection was observed with a 8 h pre-incubation with 1 µg/ml Turmeric or 5 µg/ml Ginger, followed by UVB exposure and further incubation for 24 or 48 h in serum-free medium supplemented with a halved concentration of extracts. Under this condition, Turmeric and Ginger extracts elicited a significant reduction in the accumulation of CPDs at 24 and 48 h in live but not in dead cells (Figure 3.7a), which associated with reduced activation of caspase-3 activity at 48 h (Figures 3.7b) and some cell protection (Figures 3.7c). A similar

protocol, in which the pre-incubation time was increased to 24 h elicited almost a similar protection, albeit less extensive (not shown).

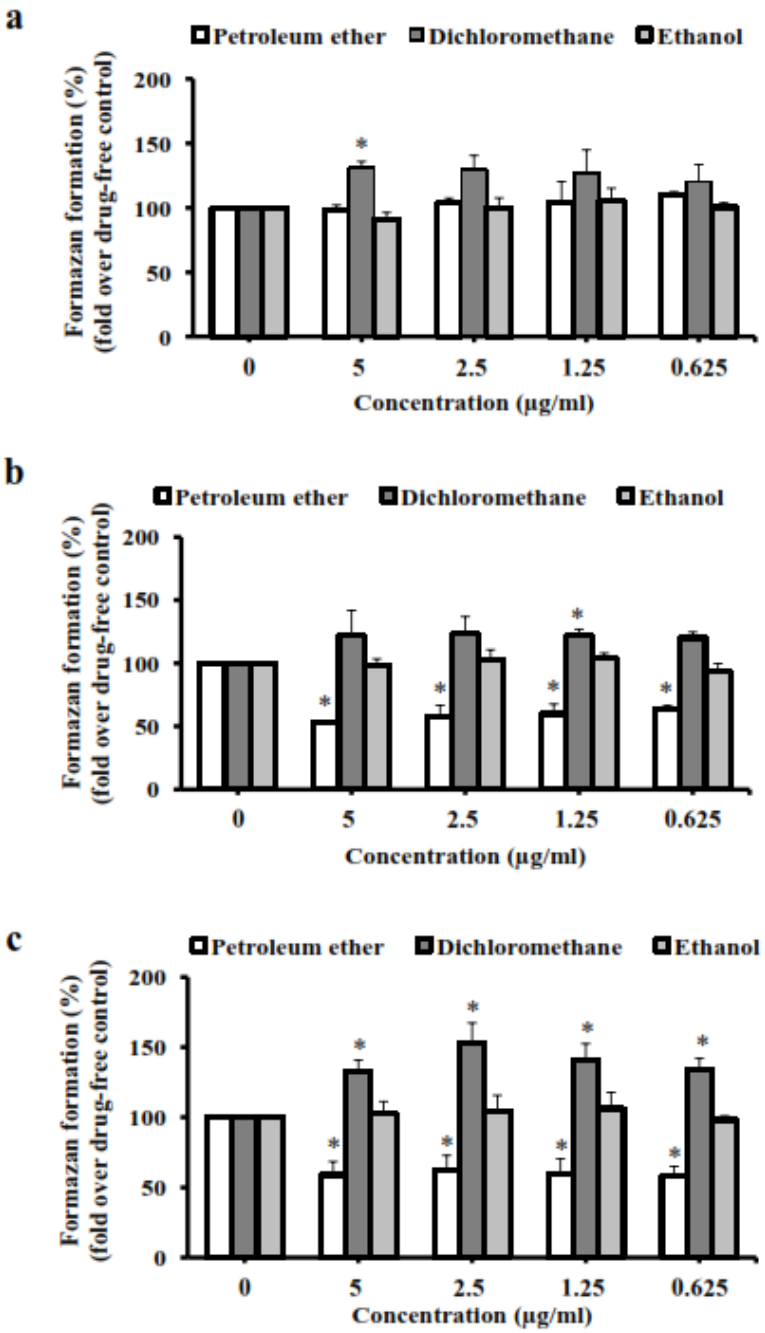


Figure 3.3 Effect of Thai herbs extracts in HaCaT cells as determined by MTT assay. HaCaT cells treated for 24 h with various concentrations of the extracts of (a) Common basil, (b) Cucumber and (c) Leech lime. * $P < 0.05$, significant difference between comparison groups using Student's t test.

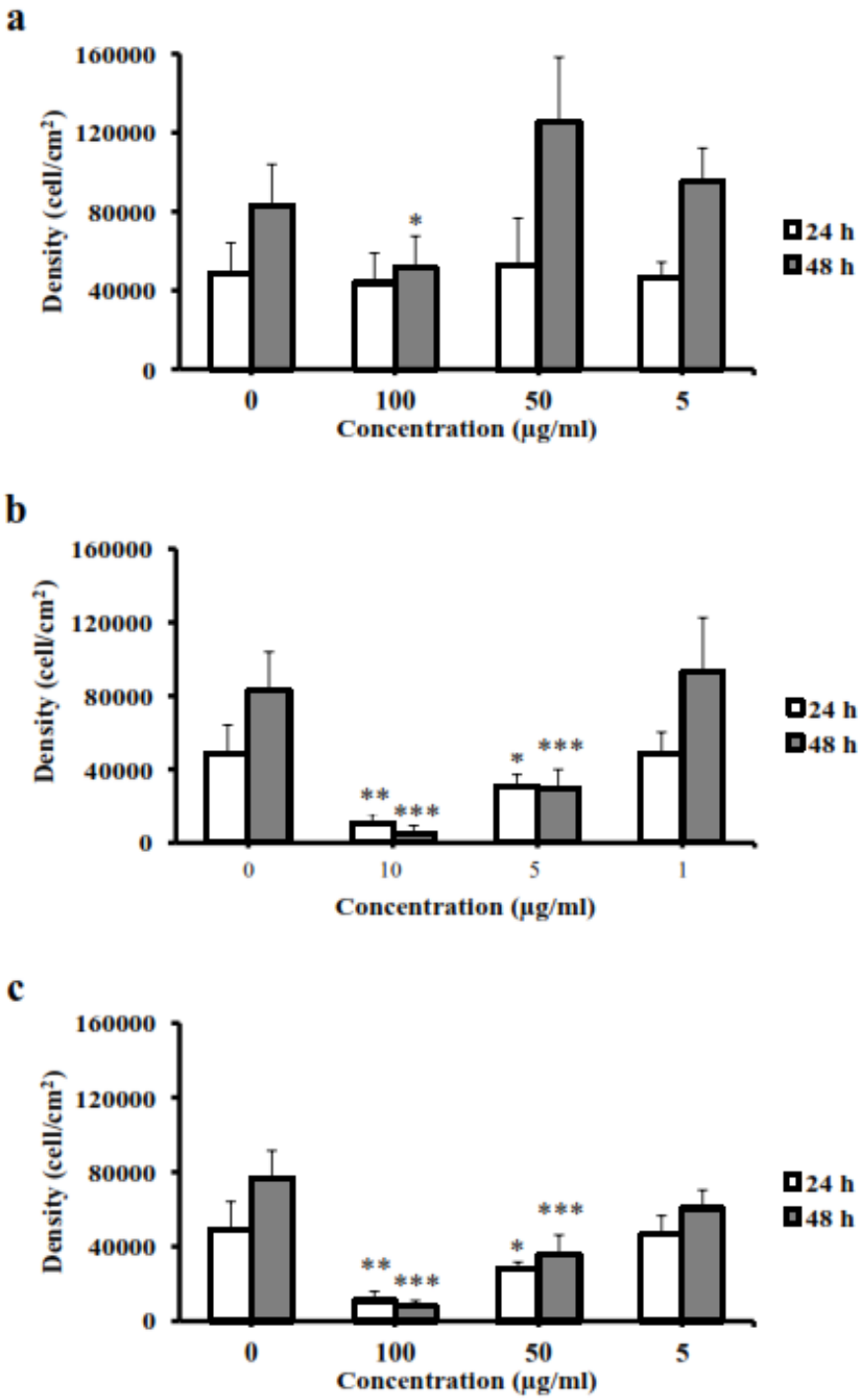


Figure 3.4 The effect of Leech lime, Turmeric and Ginger on cell growth in HaCaT cells. Adherent viable cells were counted after treatment for 24 and 48 h with various concentrations of the ethanol extract of Leech lime, and of dichloromethane extracts of Turmeric and of Ginger. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, significant difference between comparison groups using Student's *t* test.

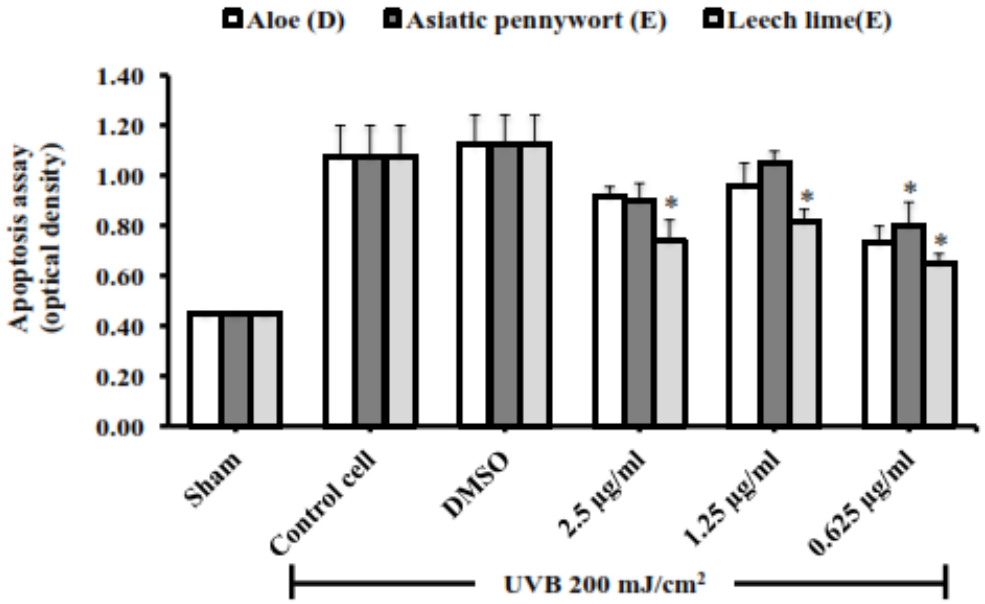


Figure 3.5 Apoptosis assay after UVB exposure in cells pretreated with herb extracts. The cells were pretreated with plant extracts for 30 min followed by exposition to 200 mJ/cm^2 UVB and further incubated for 24 h. At the end, the extent of apoptosis was assessed with the Cell Death Detection ELISA plus kit, which measure the production of mono- and oligo-nucleosomes. The cultures pretreated with the ethanol (E) extracts of Leech lime or of Asiatic pennywort or with dichloromethane extract (D) of Aloe showed a reduced number of apoptotic cells compared to control (i.e., not pretreated with herb extracts) culture (statistical significance * $p < 0.05$). Sham refers to UVB un-exposed cells.

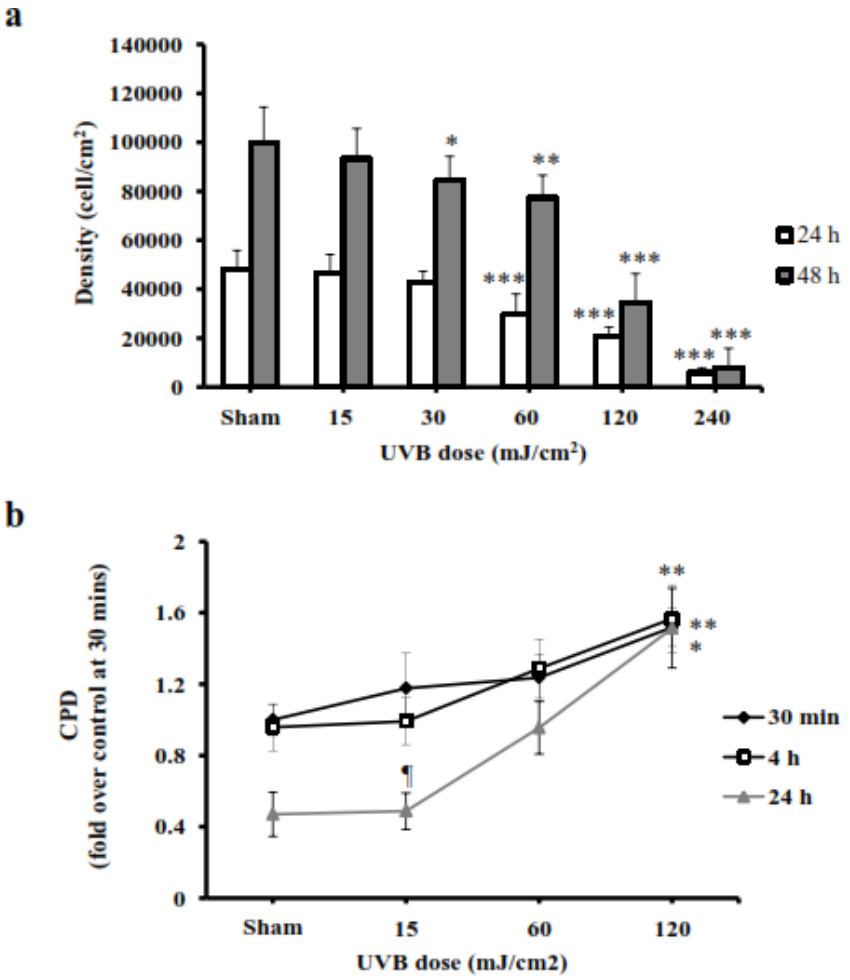


Figure 3.6 Dose-dependent cytotoxic effects of UVB in HaCaT cells. HaCaT cells were plated on petri dishes and let adhere for at least 24 h before starting the treatment. At day 0 cel density was 25.000 ± 6.000 cells/cm^2. (a) in UVB un-exposed cultures (sham), cells duplicated every 24 h. UVB-induced cell loss in a dose-dependent fashion as determined by cell counting of adherent viable cells at 24 and 48 h post-UVB irradiation. Cloning efficiency of irradiated cultures was largely compromised at doses of 120 and 240 mJ/cm^2. (b) UVB-induced DNA damage as assessed by ELISA evaluation of CPDs formation at 30 min, 4 h and 24 h post-UVB (15, 60 or 120 mJ/cm^2) irradiation. Sham indicates UVB un-exposed cells. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significant difference between comparison groups; ¶ $p < 0.05$, significant difference between UVB-treated cells at 30 min and at 24 h post-UVB (15 mJ/cm^2) using Student's t test.

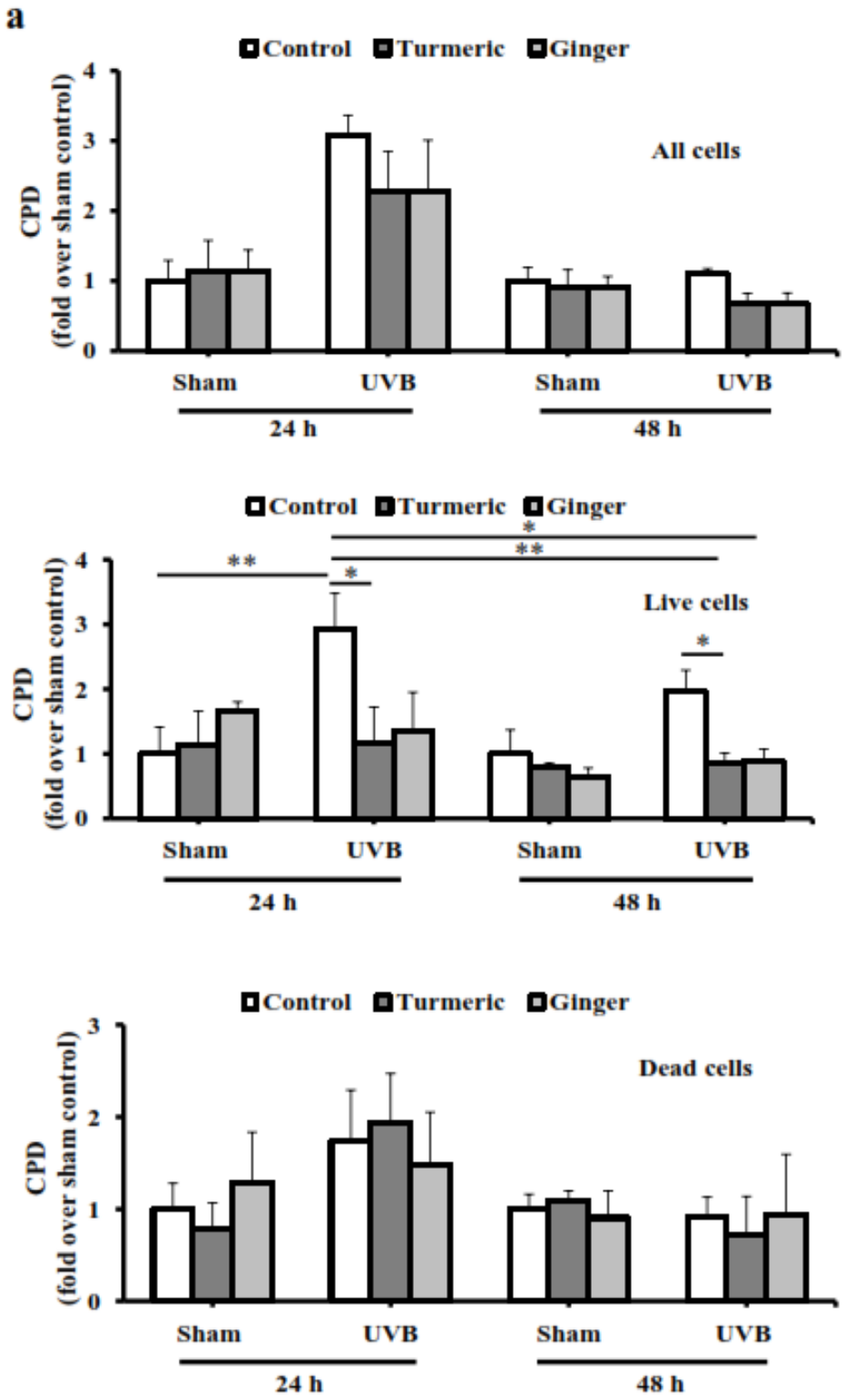


Figure 3.7 Protective activities of Turmeric and Ginger extracts against UVB cytotoxicity. (See detail in below).

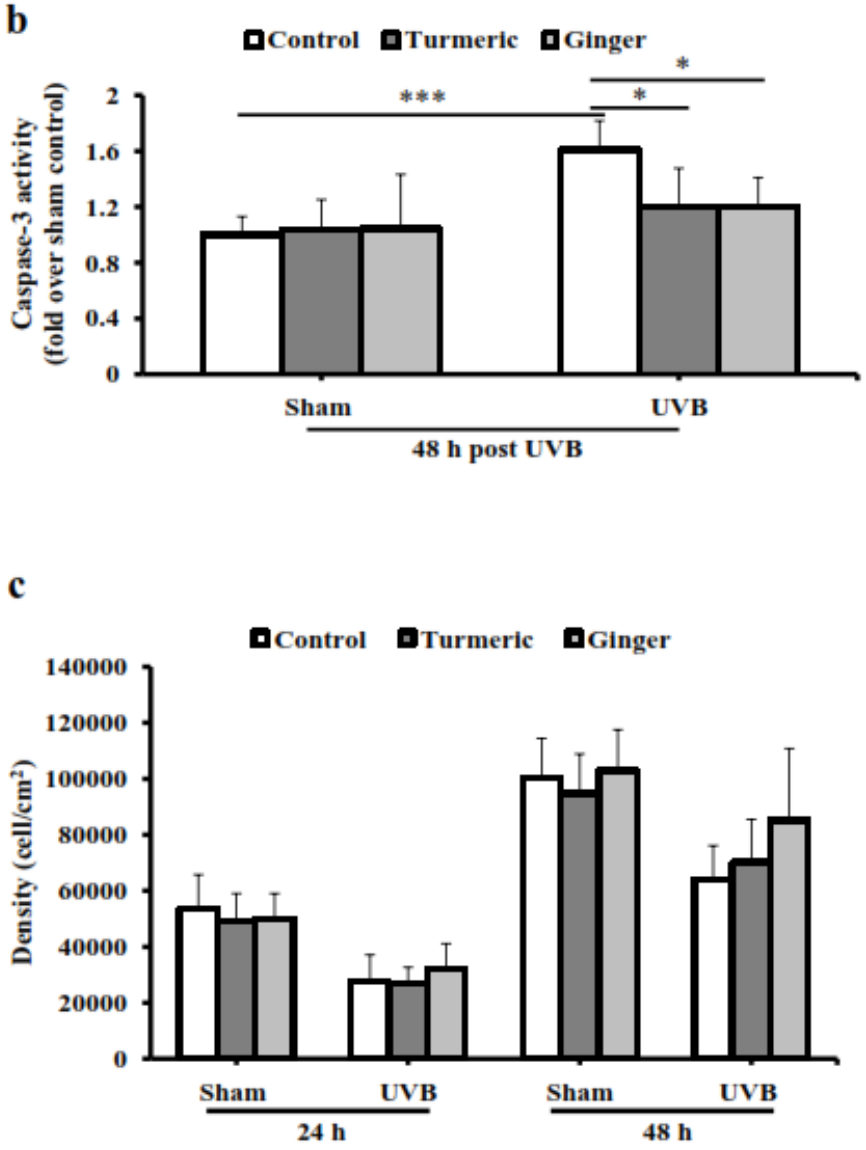


Figure 3.7 Protective activity of Turmeric and Ginger extracts against UVB cytotoxicity. The cells were pre-treated for 8 h with the dichloromethane extracts of Turmeric (at 1 µg/ml) or of Ginger (at 5 µg/ml) and then exposed to UVB (120 mJ/cm^2), followed by incubation in medium contained halved concentration of the herb extract. (a) 24 and 48 h post-UVB, CPDs formation was evaluated in pooled live and dead cells (upper panel), in live cells only (middle panel,), and in dead cells only (lower panel). (b) caspase-3 activity determined in the culture at 48 h post-UVB irradiation. (c) Cells were counted at 24 and 48 h post-UVB. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significant difference between comparison groups.

To gain an insight on the possible mechanisms underlying such protective effect, we checked the expression of anti-oxidant proteins in HaCaT cells exposed to Turmeric and Ginger extracts. We focused on Thioredoxin-1 (TRX), a 12 kDa redox-active protein that protects the cells from oxidative stress [188] and apoptosis [189]. While a 1 h incubation was inefficient (data not shown), a 8 h incubation with 1 μg/ml Turmeric or 5 μg/ml Ginger extract (dichloromethane fraction) was sufficient to determine a strong increase in the expression of Thioredoxin 1 (Figure 3.8).

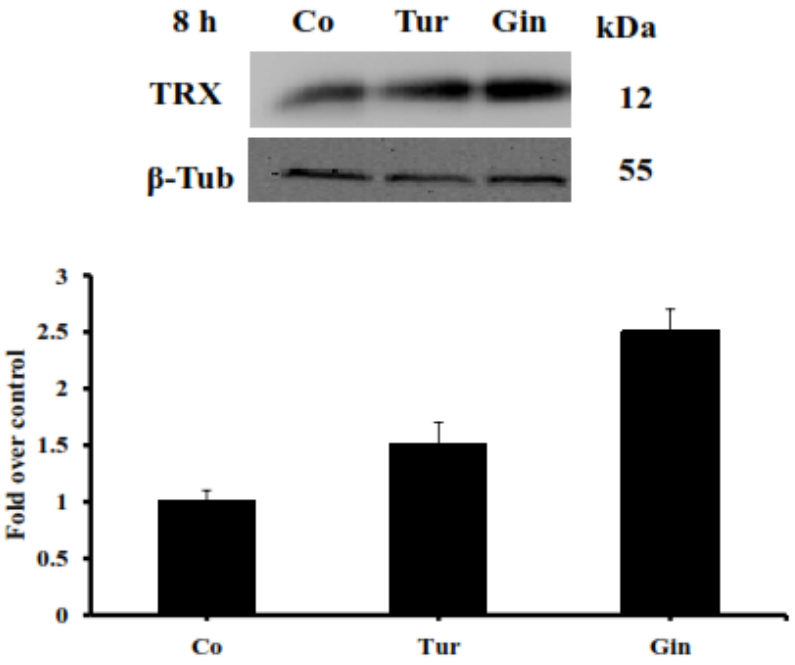


Figure 3.8 Expression of Thioredoxin 1 in HaCaT cells pretreated with Turmeric and Ginger extracts. The cells were plated on Petri dishes, let adhere for 24 h and then incubated for 8 h with the dichloromethane extracts of Turmeric (at 1 μg/ml) or of Ginger (at 5 μg/ml). The cell homogenate was analyzed by western blotting first for the expression of Thioredoxin 1, and then it was stripped and re-probed with anti-b-tubulin antibody for control loading of cell proteins. The experiment was replicated three times (one representative gel is shown). The intensity of bands was evaluated and the relative intensity of Thioredoxin 1 versus b-tubulin was calculated. Data of the three experiments are given as fold-increases (average ± S.D.) in treated versus control cells (assumed as value 1).

3.5 Turmeric and Curcumin induce cell cycle arrest and apoptosis in A431 cells

We first sought to assess the cytotoxic effects of Turmeric and Curcumin in A431 cells. Alterations in cell morphology and cell density, and occurrence of cell death were evaluated in cultures incubated for 24 h with increasing concentrations of Turmeric or Curcumin. Turmeric was un-effective at doses below 20 μg/ml and Curcumin exerted a cytostatic effect at 20 μM, while it produced evident signs of toxicity at 30 μM (Figures 3.9 a-b). In the following experiments we employed the toxic concentrations of both substances, which were shown to affect the mitochondrial integrity as witnessed by the loss of transmembrane potential assessed with the fluorescent dye Rhodamine-123 (Figure 3.9c). Occurrence of apoptotic cell death was evaluated by cytofluorometric analysis of the SubG1 peak (which measures the proportion of apoptotic bodies containing fragmented chromatin) in the cultures exposed to Turmeric or Curcumin in the absence or presence of the pan-caspase inhibitor z-VAD-fmk. After a 24 h exposure to Turmeric or Curcumin, approximately 30 % of the cell population contained a sub-diploid amount of propidium iodide-labelled DNA, and the co-treatment with zVAD-fmk, which inihibits the caspase-dependent apoptosis pathway, nearly completely abrogated the appearance of this sub-population of cells (Figure 3.9d). By cell counting, zVAD-fmk rescued, albeit not completely, cell loss from the monolayers of the cultures incubated with Turmeric or Curcumin (Figure 3.9e). Turmeric and Curcumin also affected the progression in the cell cycle, essentially reducing the proportion of the cells in the S phase (Figure 3.9f). It was found that zVAD-fmk protected the cells by accumulating them in the G2/M phase, thus likely preventing their mitotic cathastrophe, but did not rescue their ability to proceed to the S phase while in the presence of Turmeric or Curcumin (Figure 3.9f). Taken together, both Turmeric and Curcumin, under appropriate time and concentration conditions, can be toxic to A431 cells by inducing apoptosis and cell cycle arrest.

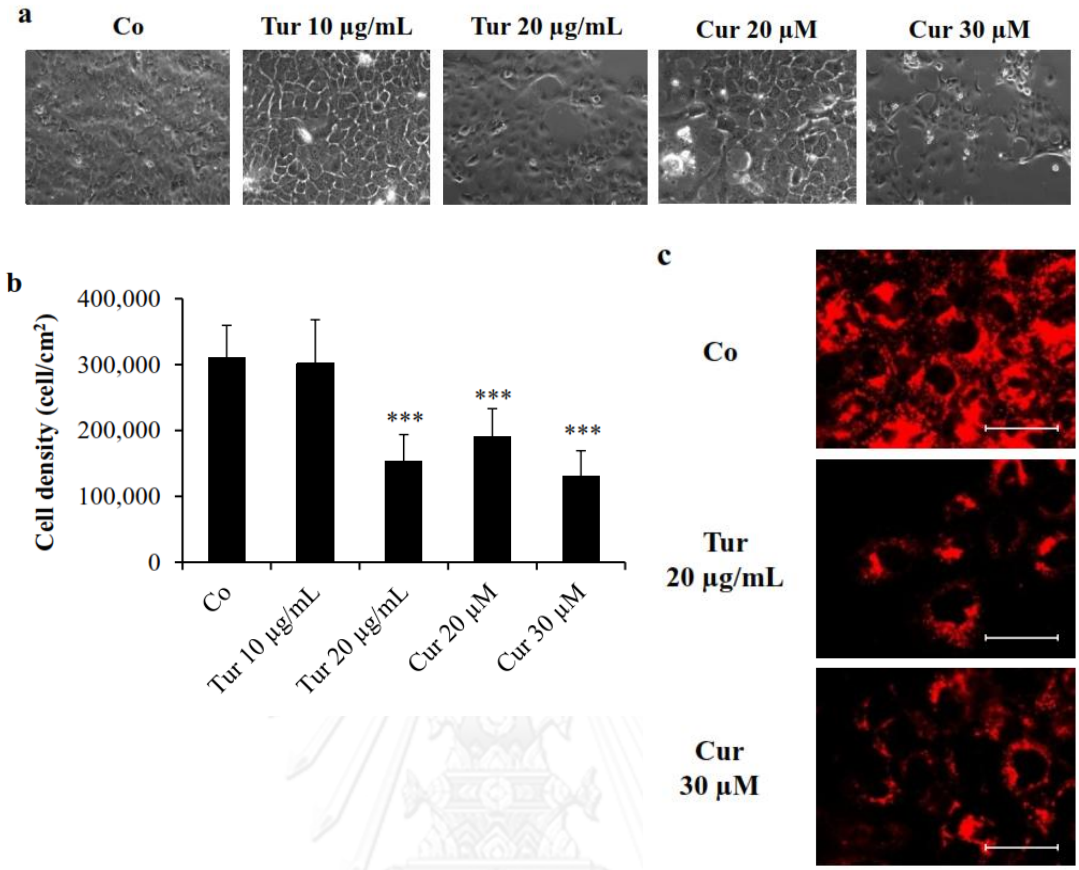


Figure 3.9 Turmeric induces cell cycle arrest and apoptotic cell death in A431 cells. A431 cells were treated with Turmeric (10 and 20 µg/mL) or Curcumin (20 and 30 µM) for 24 h. (a) Morphological alterations were imaged by phase-contrast microscope (40X). (b) Viable cell growth was determined by counting the trypan blue dye-negative cells. (c) Mitochondrial membrane potential was measured by staining with Rho123 at 16 h post-treatment.

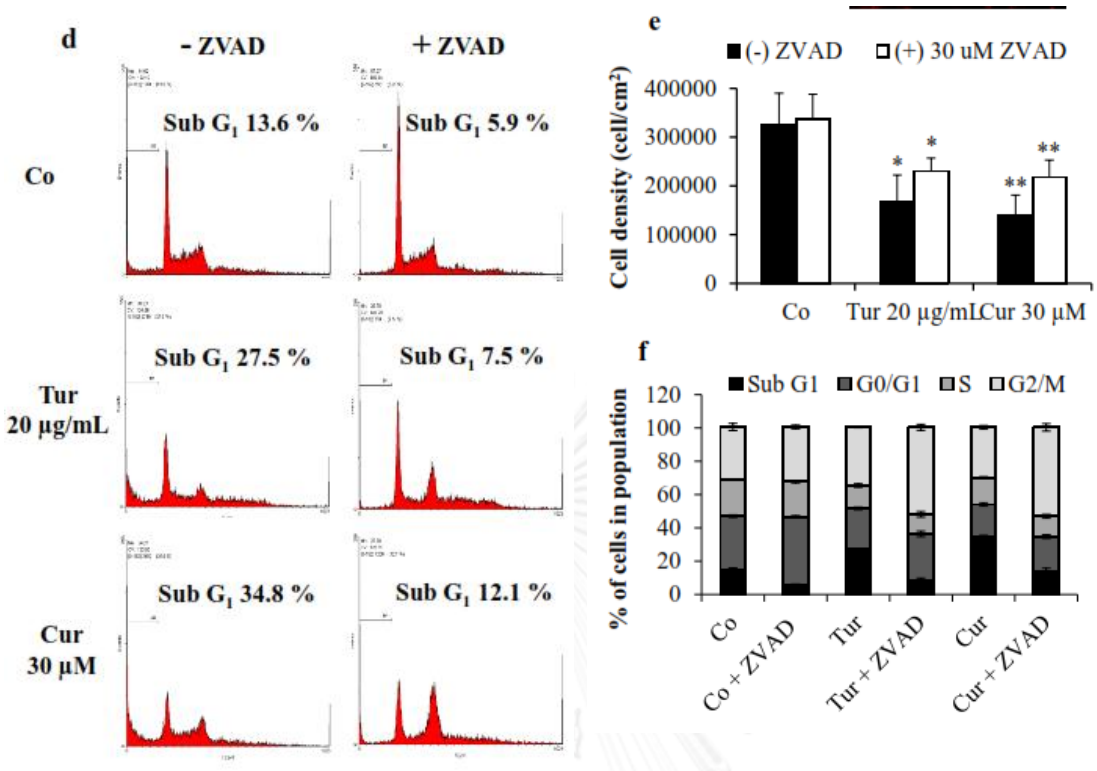


Figure 3.9 (cont.) d-e-f) The cells were pre-incubated 1h with Z-VAD-fmk prior to the treatment with Turmeric 20 µg/mL or Curcumin 30 µM. d) flow cytometry analysis showing the subG1 peak as an indirect measure of apoptosis; e) Cell density in the cultures after counting the viable cells by the trypan blue dye exclusion technique; f) histogram representing the proportion of cells in each phase of the cell cycle. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, significant difference between comparison groups.

3.6 Turmeric and Curcumin induce autophagy in A431 cells

Next, we investigated on the possible induction of autophagy in the cells exposed to Turmeric or Curcumin. First, we assayed by western blotting the presence of the lipidated isoform II of LC3, a protein derived from the microtubule-associated protein MAP-LC3, that associates with autophagosomal membranes [190]. A parallel set of cultures were co-treated with Chloroquine, a lysosomotropic weak base that impairs the lysosomal degradation of autophagosomes. The comparison of the cellular level of LC3-II in the absence and in the presence of Chloroquine allows to measure the autophagy flux, i.e. the ratio of autophagosome formation and consumption under a given treatment [190]. Turmeric and Curcumin per se only slightly increased the actual level of LC3-II in the cells (Figure 3.10a). As expected, in the presence of Chloroquine LC3-II accumulated in the cells, and it increased both in Turmeric- and Curcumin-treated cultures, the latter being the strongest inducer. The cellular level of LC3-II isoform, which is associated to autophagosomal membranes, bona fide reflects the presence of autophagosomes and auophagolysosomes. Ongoing autophagy was further confirmed by immunofluorescence detection of vacuoles positive for LC3. The images in Figure 3.10b reveal indeed the presence of many cells showing many LC3 puncta, which reflect the presence of autophagic vacuoles, in the cultures exposed to Turmeric or Curcumin (again, the latter was the stronger inducer).

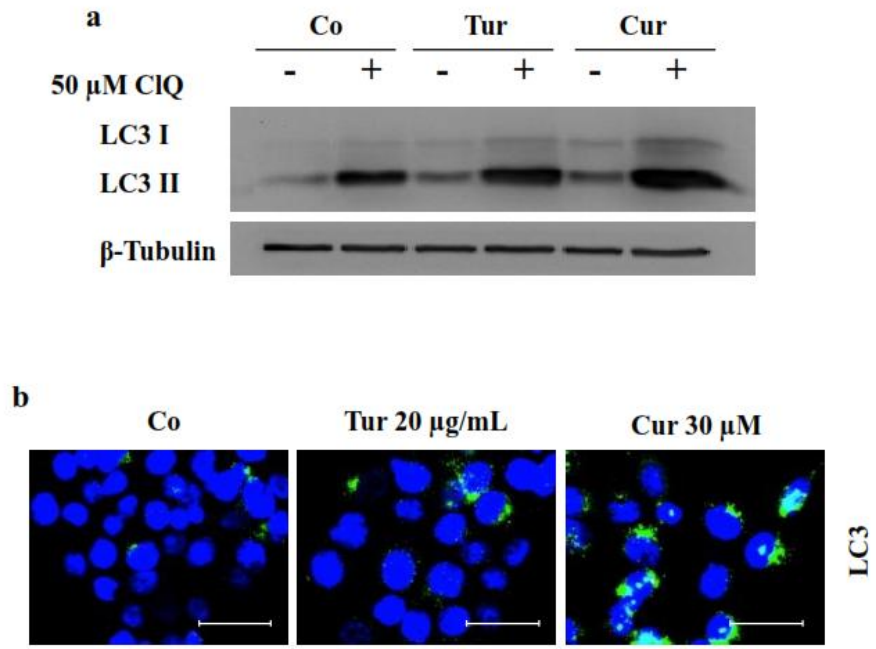


Figure 3.10 Turmeric induces autophagy in A431 cells. A431 cells were treated with Turmeric 20 µg/mL or Curcumin 30 µM and co-treated or not with Chloroquine (ClQ, 50 µM). a) Western blotting analysis of LC3 expression at 24 h post-treatment; LC3-I is the isoform generated when autophagy is triggered, while LC3-II is the lipidated isoform associated with autophagosomal membranes. b) Immunofluorescence staining showing the presence of LC3 puncta associated with autophagic vacuoles.

3.7 Chloroquine-induced accumulation of autophagic vacuoles does not increase Turmeric or Curcumin toxicity in A431 cells

The autophagy-lysosomal degradation pathway plays a fundamental role in cell survival and cell death [191]. We checked whether the toxic effects of Turmeric and Curcumin were associated with induction of autophagy. In the presence of Chloroquine, the autophagosome produced cannot be degraded because of the impaired fusion with lysosomes and the pH-dependent inhibition of the lysosomal hydrolytic enzymes [190]. Consistent with the data in Figure 2A, in the presence of Chloroquine the proportion of cells positive for LC3 and the intensity (number and dimension of LC3-positive vacuoles) of the staining greatly increased in the culture exposed to Turmeric and Curcumin, reflecting the accumulation of the autophagic vacuoles produced during the 24 h of incubation (Figure 3.11a). A parallel, though of much less extent, increase in LC3 staining was also observed in control cultures, reflecting the level of ongoing basal autophagy (Figure 3.11a).

To see whether autophagy degradation was playing a role in Turmeric and Curcumin toxicity, we assayed the cell culture growth upon exposure to these drugs in the presence of Chloroquine. Based on the counting of viable cells, Chloroquine itself was not toxic and had no synergistic effect with nor protected from toxicity of Turmeric or Curcumin (Figure 3.11b). From these data we may conclude that the Chloroquine-induced block of autophagosomal degradation and accumulation of autophagosomes do not exacerbate Turmeric or Curcumin toxicity.

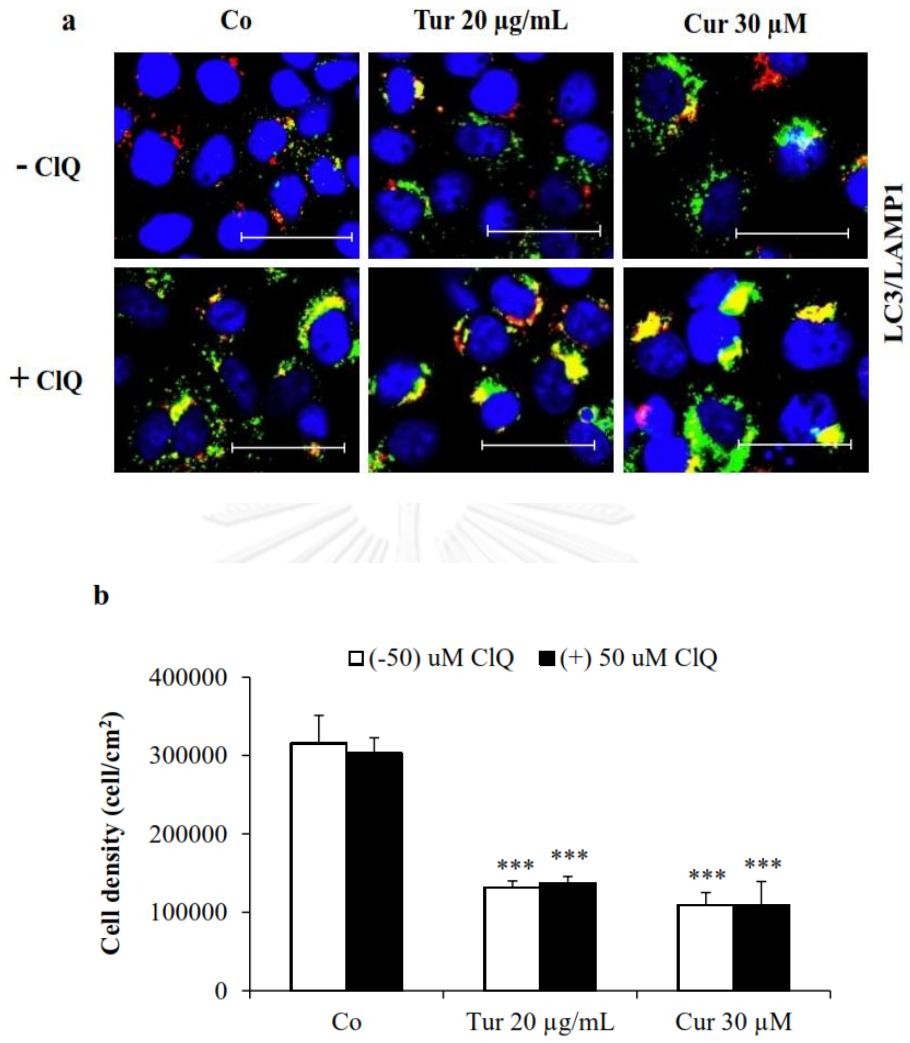


Figure 3.11 Inhibition of the autophagy flux by Chloroquine does not protect from nor synergize with Turmeric or Curcumin. a) Chloroquine was employed to block the autophagy flux and induce the accumulation of undigested autophagosomes and autophagolysosomes, as indicated by the increased immunofluorescence staining with LC3, a marker of autophagosomes, and LAMP1, a marker of lysosomes. b) Cell density determined by counting the adherent viable cells after a 24 incubation with the indicated substances. Statistical significance: *** $p < 0.001$, significant difference between comparison groups.

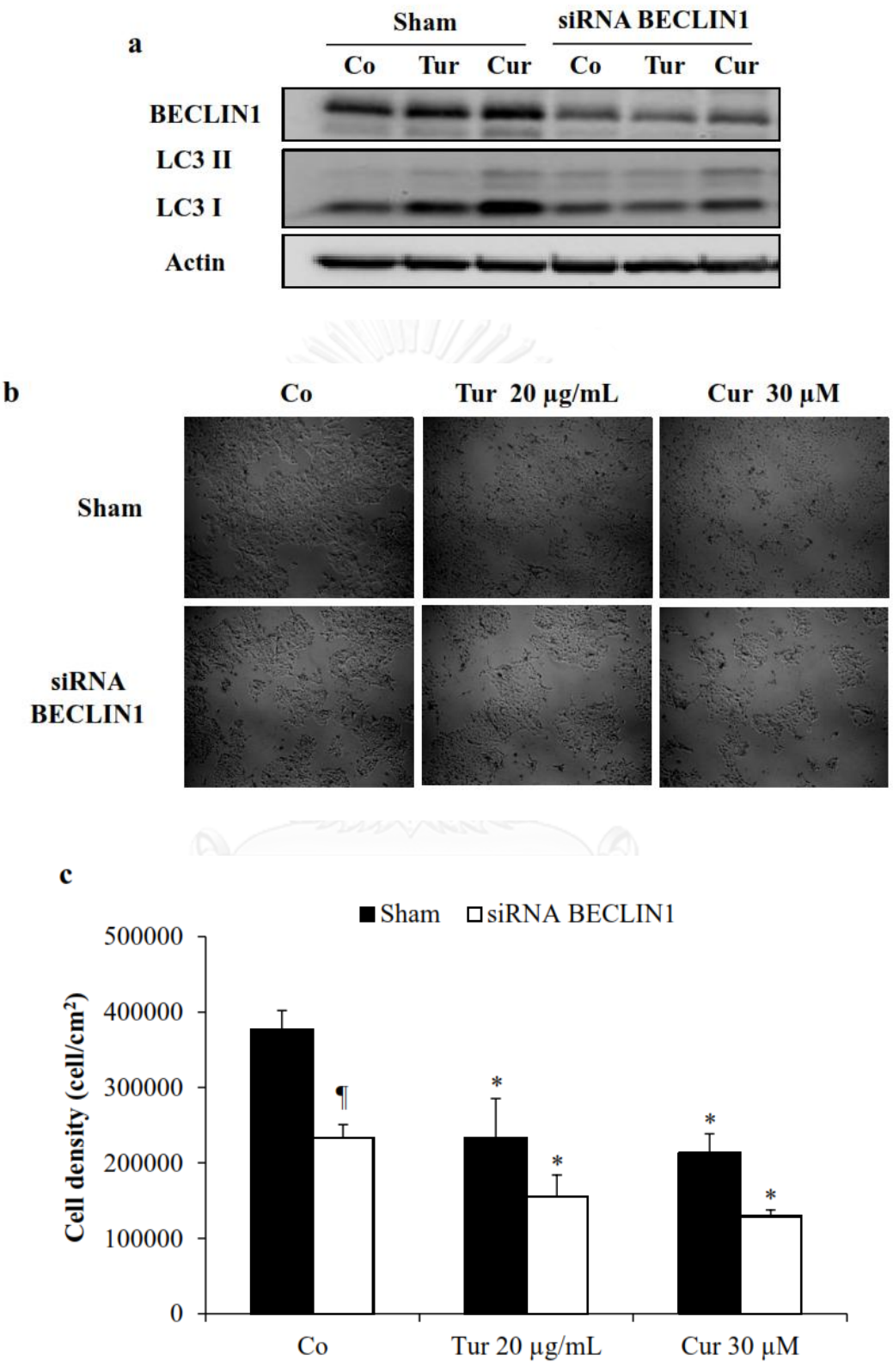
3.8 Knock-down of the autophagy-related protein BECLIN 1 decreases cell growth and synergizes with Turmeric and Curcumin

To better assess the potential involvement of autophagy in the cytotoxic pathway activated by Turmeric and Curcumin in A431 cells we genetically manipulated the induction of autophagy. To this end, the autophagy gene BECLIN 1 was post-transcriptionally silenced with the siRNA technology [114]. By the time the cells were treated with Turmeric or Curcumin, the endogenous level of BECLIN 1 protein in siRNA-transfected cells dropped down to approximately 20% of that in control sham-transfected cells (Figure 3.12a). Silencing of BECLIN 1 expression largely, but not completely, impaired the formation of the autophagosome-associated LC3-II protein upon treatment with Turmeric or Curcumin (Figure 3.12a). Again, at the concentrations used, Curcumin revealed to be the strongest autophagy inducer. At microscope level, no gross morphological alterations, but differences in the cell density, were observed in the cultures subjected to BECLIN 1 knock-down (Figure 3.12b). By cell counting, it was strikingly evident that knocks-down of BECLIN 1 decreased the number of viable cells in the cultures and also synergized with Turmeric and Curcumin (Figure 3.12c). This could result from a block of the cell proliferation and/or induction of cell death. However, based on counting of trypan-blue positive cells and on cytofluorometric assay of the subG1 cell population no signs of necrosis or apoptosis were evident in siRNA-BECLIN 1 cultures (not shown).

We further focused on the proteins mainly implicated in the regulation of apoptosis and of the cell cycle. First, we noted that knock-down of BECLIN 1 resulted in increased levels of the anti-apoptotic protein BCL-2, regardless of the co-treatment with Turmeric or Curcumin (Figure 3.13a). Next, we looked for the expression of the pro-apoptotic protein BAX in its Thr-167 phosphorylated active form. Knock-down of BECLIN 1 did not change per se the level of p-BAX (compared to sham-transfected cells), which increased much upon exposure to Curcumin, and only slightly upon exposure to Turmeric (Figure 3.13a). The relative expression of BCL-2 and p-BAX in siRNA BECLIN 1 cultures could explain the toxic effects of Turmeric and Curcumin, but did not provide any clue to explain the reduced number of viable BECLIN 1-silenced cells as shown in Figure 3.12c. In alternative to cell death, the reduced

number of viable cells in siRNA BECLIN 1 cultures could result from a general block of the cell proliferation. Therefore, we assayed by western blotting the level of p21WAF1/Cip1, that inhibits the progression in the cell cycle. In the cells knocked-down for BECLIN 1 the expression of p21 was greatly increased (Figure 3.13b). This fact indicates that BECLIN 1 is important for progression in the cell cycle and for cell growth.

We have also looked at the status of the mutant p53, which has been shown to play a role in the cytotoxic response to chemotherapeutics [192-194]. Phosphorylation at the Ser15 position in p53 is essential for its stabilization and activation. The level of p53 and of Ser15-p-p53 was drastically reduced upon exposure of the cells to either Turmeric or Curcumin (Figure 3.13c). Strikingly, in BECLIN 1 knocked-down cells, the actual level of p53 was increased, and in general counteracted the action of Turmeric and Curcumin on its Ser15-phosphorylation (Figure 3.13c). These data suggest that induction of autophagy by Turmeric and Curcumin leads to the degradation of the p53 mutant.


a
Sham
siRNA BECLIN1
Co
Tur
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Co
Tur
Cur
BECLIN1
LC3 II
LC3 I
Actin
b
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Tur 20 µg/mL
Cur 30 µM
Sham
siRNA BECLIN1
c
Sham
siRNA BECLIN1
Cell density (cell/cm²)
500000
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300000
200000
100000
0
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*
*
*
*
Co
Tur 20 µg/mL
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Figure 3.12 Post-transcriptional silencing of the autophagy gene *BECLIN* 1 decreases cell growth of A431 cells and synergizes with Turmeric and Curcumin. The transfection in A431 cells of a control (sham) or anti-BECLIN 1 mRNA specific siRNA was followed by 72 h incubation in complete medium before starting the treatment with the drugs. a) Western blotting showing the level of expression of the autophagy protein BECLIN 1 in sham- and in siRNA-BECLIN transfected A431 cells treated or not for 24 h with Turmeric or Curcumin. Based on densitometry, 72 h after the transfection the siRNA reduced the expression of BECLIN 1 of approximately 85 % the value in sham-transfected cells. b) Representative images of the cultures taken under the phase contrast microscope; c) A parallel set of cultures (in triplicate) was used for counting the viable adherent cells upon treatments for 24 h as indicated. Statistical significance: * $p < 0.05$, significant difference between comparison groups; ¶ $p < 0.05$, significant difference between control sham and control siRNA BECLIN1.

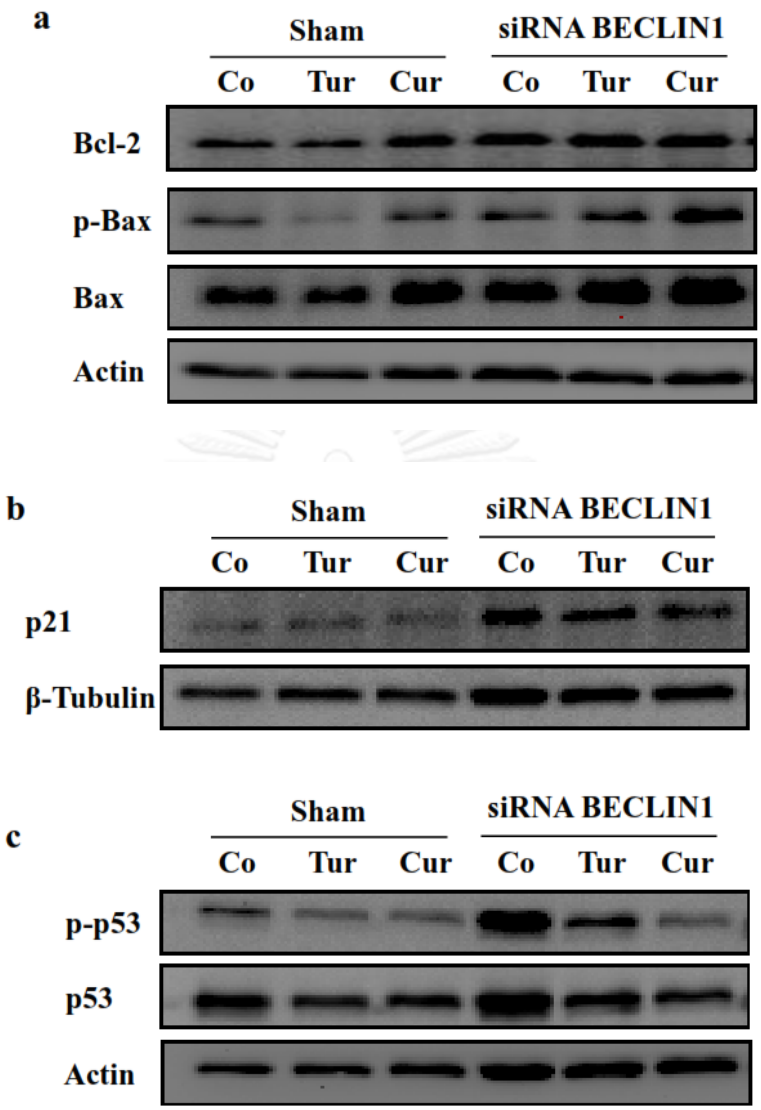

Figure 3.13 Effects of Turmeric and Curcumin on the expression of proteins regulating apoptosis and cell cycle in BECLIN 1-silenced cells. The cells were treated as for the experiment described in Figure 3.12. The homogenate were assayed by western blotting for the expression of key proteins involved in the regulation of apoptosis and cell cycle. a) Expression of the anti-apoptotic BCL-2 and of the pro-apoptotic BAX proteins (total and its Thr167-phosphorylated form). b) Expression of the cell cycle inhibitor protein p21; c) expression of p53 (total and its Ser15-phosphorylated form).

3.9 Turmeric and Curcumin induce mTOR-independent autophagy

The mTORC1 complex plays a pivotal role in the control of cell survival signals by regulating in an opposite fashion the synthetic and the autophagy-degradation pathways [195, 196]. mTOR exerts a tonic inhibition of basal autophagy [196], while it positively regulates protein synthesis [197]. Upon treatment with the mTOR inhibitor Rapamycin (10 μM for 16 h), the down-stream ribosomal protein S6 was inactivated, consistent with a general block of the protein synthesis pathway (Figure 3.14a). Yet, under this condition, the co-treatment with either Turmeric or Curcumin maintained the mTOR-p70S6k pathway slightly active (Figure 3.14a), which is consistent with a pro-anabolic effect of these drugs. Noteworthy, Turmeric and Curcumin could induce autophagy despite the mTOR-p70S6k pathway was still active, as indicated by the increased production of LC3-II in concomitance with S6 phopshorylation (Figures 3.14b and 3.14a). Rapamycin raised up the level of basal autophagy, as expected, and further increased the production of LC3-II in Turmeric- and Curcumin-treated cells (Figure 3.14b). The Rapamycin induction of autophagy was definitively confirmed by the increased immunofluorescence staining for LC3 (as a marker of autophagosome) and for LAMP1 (as a marker of lysosome), as shown in Figure 3.14c.

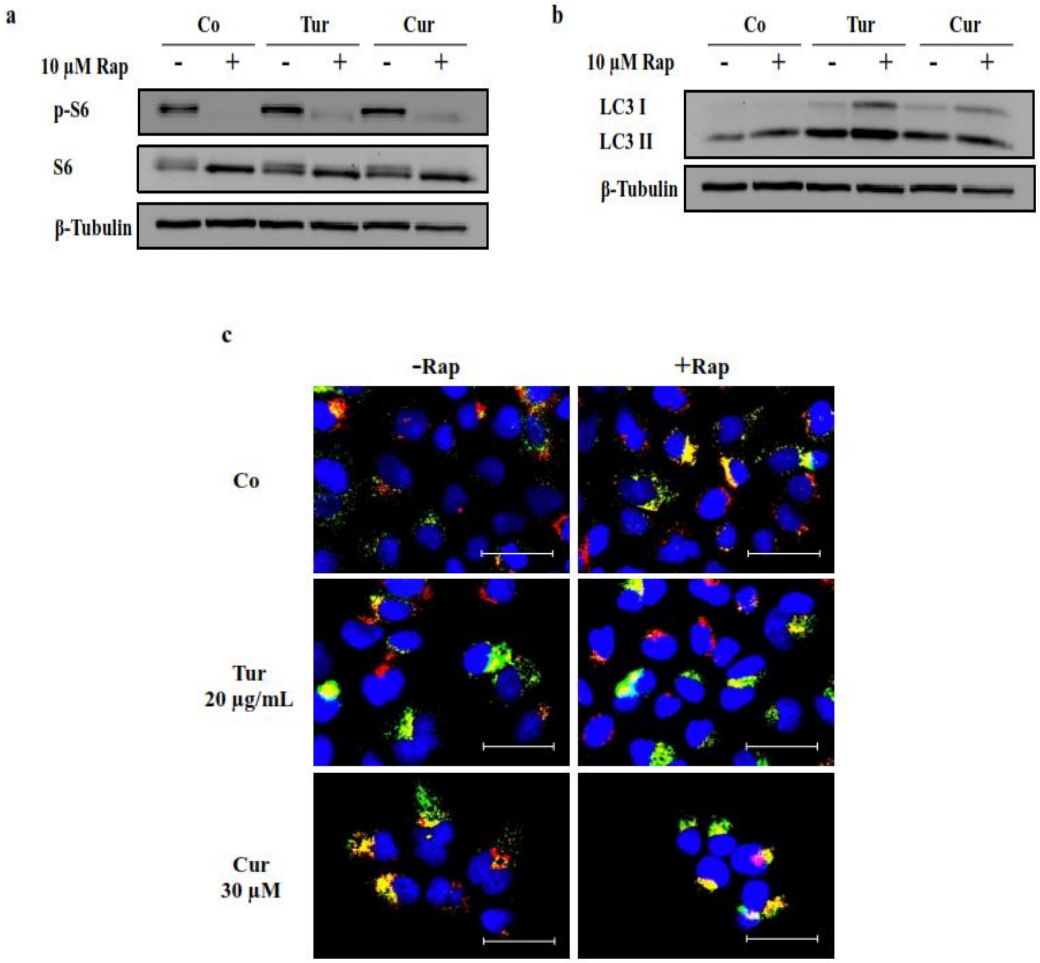

Figure 3.14 Inhibition of mTOR by rapamycin raised basal autophagy and synergizes with Turmeric and Curcumin. A431 cells were pre-treated for 1 h with 10 μM Rapamycin (Rap) to inhibit mTOR, and then exposed for 16 h to Turmeric (20 μg/mL) or Curcumin (30 μM). a) The phosphorylation status of the ribosomal S6 protein, a down-stream substrate of p70S6k, was assumed as a read out of the activity of mTOR (which acts upstream of p70S6k). Western blotting showed the inhibitory effect of Rapamycin and the stimulating effect of Turmeric and Curcumin on mTOR activity. b) Western blotting analysis of the expression of LC3-II as a read out of ongoing autophagy in the cells treated as indicated. c) Immunofluorescence staining of autophagosomes and autophago-lysosomes as detected by anti-LC3 and anti-LAMP1 antibodies. It is evident the hyper-induction of autophagy and the associated toxicity in the cells co-treated with Rapamycin.

3.10 Inhibition of mTOR exacerbates Turmeric and Curcumin cytotoxicity

The above data demonstrated that Turmeric and Curcumin tend to stimulate the S6-mediated synthetic pathway and are capable of inducing an mTOR-independent rise of basal autophagy, which further increases in the presence of the mTOR inhibitor Rapamycin. It was then important to determine the biological consequences of the inhibition of mTOR in the cells treated with Turmeric or Curcumin. While the inhibition of mTOR was *per se* not toxic, it revealed to be extremely toxic when the cells were exposed to Turmeric or Curcumin (Figures 3.15a-b). Of note, while Rapamycin in itself had no effect on p53 stabilization and Ser15-phosphorylation, the combination of Rapamycin with either Turmeric or Curcumin drastically reduced the cellular level of p53 (Figure 3.15c). The monolayer was completely detached by 24 h of incubation with the combination of Turmeric or Curcumin with Rapamycn. We investigated on the type of cell death, and on its relationship with autophagy, caused by the concomitant inhibition of mTOR in Turmeric- and Curcumin-treated cells. Co-staining of LC3, as a marker of autophagy, and of BAX, as a marker of apoptosis, allowed to determine the relationship between these two processes in the cells exposed to Turmeric or Curcumin in the absence or the presence of Rapamycin for up to 16 h. In the combined treatment, the relative proportion of cells stained for LC3 or BAX was increased, while the whole number of cells still adherent to the coverslip was dramatically reduced (Figure 3.15d). The combination Rapamycin plus Curcumin was much more toxic than the combination Rapamycin plus Turmeric. Is to be noted, however, that only a few number of the cells still on the cover-slip showed positive for both LC3 and BAX, while the great majority of the cells positive for LC3 were negative for BAX and vice versa (Figure 3.15d). Besides BAX-mediated apoptosis, we also checked for induction of necrosis. To this end, the cells on cover-slips were stained un-fixed with Propidium Iodide, which enters only discontinued membranes and therefore labels the DNA of necrotic cells. It was found that Curcumin, and to a much lesser extent also Turmeric, induced necrosis, which markedly increased in the presence of Rapamycin (Figure 3.15e). Thus, the inhibition of mTOR increases the toxicity of Turmeric and Curcumin by exacerbating both apoptosis and necrosis.

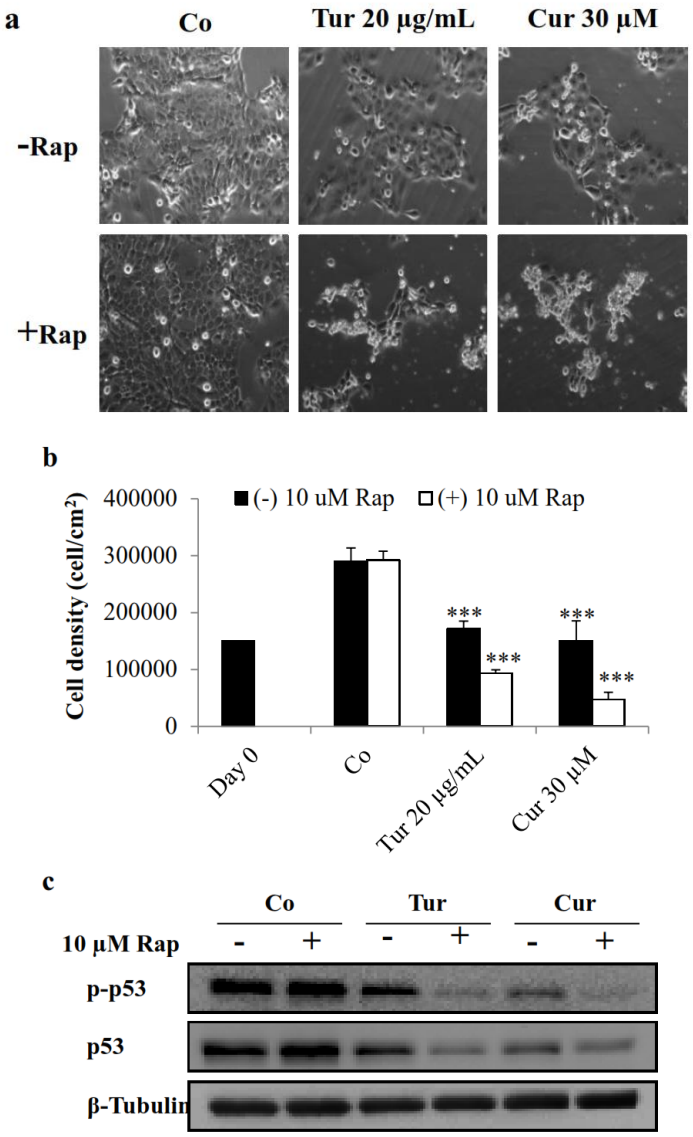

Figure 3.15 Inhibition of mTOR exacerbates Turmeric and Curcumin toxicity and p53 degradation. Biological consequences of the combinatorial treatment of Rapamycin (10 µM) with Turmeric (20 µg/mL) or Curcumin (30 µM). a) Morphological aspect of the monolayer after 16 h of treatment as imaged under the phase contrast microscope; b) Cell count of adherent viable cells at 16 h post-treatment. While Rapamycin alone was not toxic, its combination with Turmeric or Curcumin revealed to be extremely toxic; c) Expression of the Ser15-phosphorylated and total p53 proteins in the cultures. It is evident that Rapamycin synergized with Turmeric and Curcumin in inducing p43 degradation.

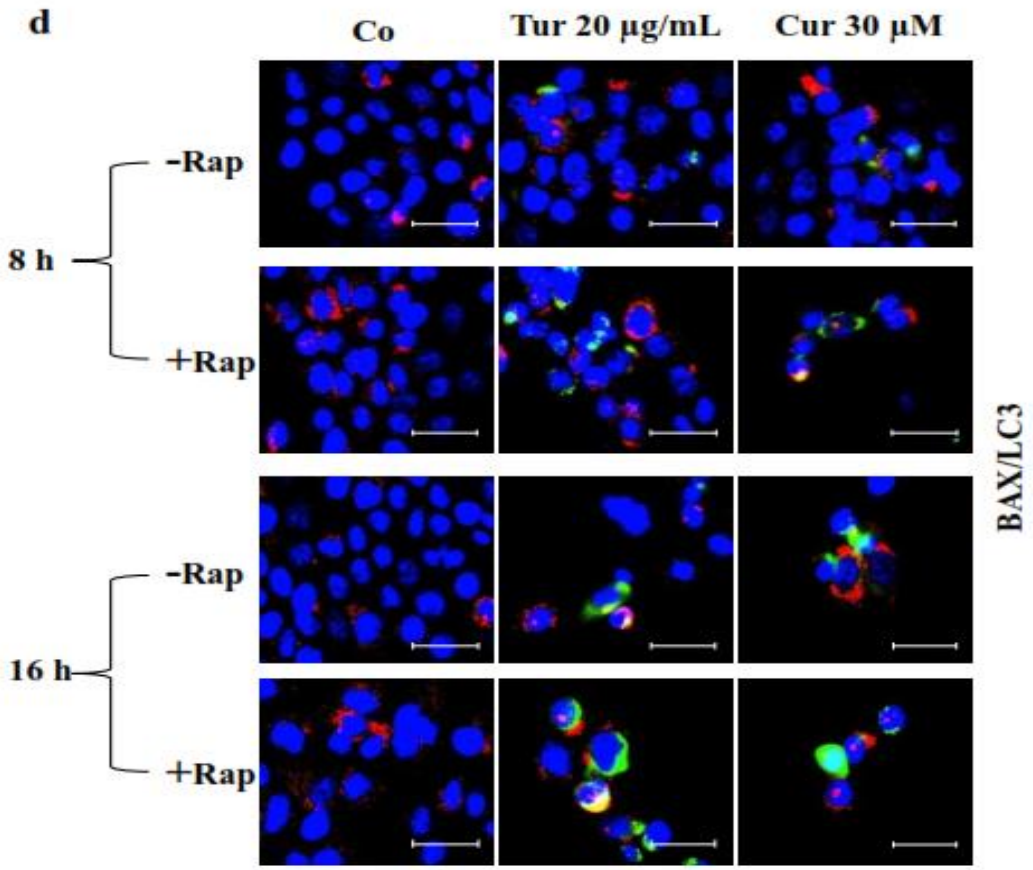

Figure 3.15 (cont.) d) Immunofluorescence co-staining of LC3, as a marker of autophagy, and of BAX, as a marker of apoptosis, in A431 cells treated as indicated. Note that at 16 h of incubation most of the monolayer was detached and only the remnant of the cells could be stained. BAX positivity was more frequently observed in cells negative for LC3, and viceversa.

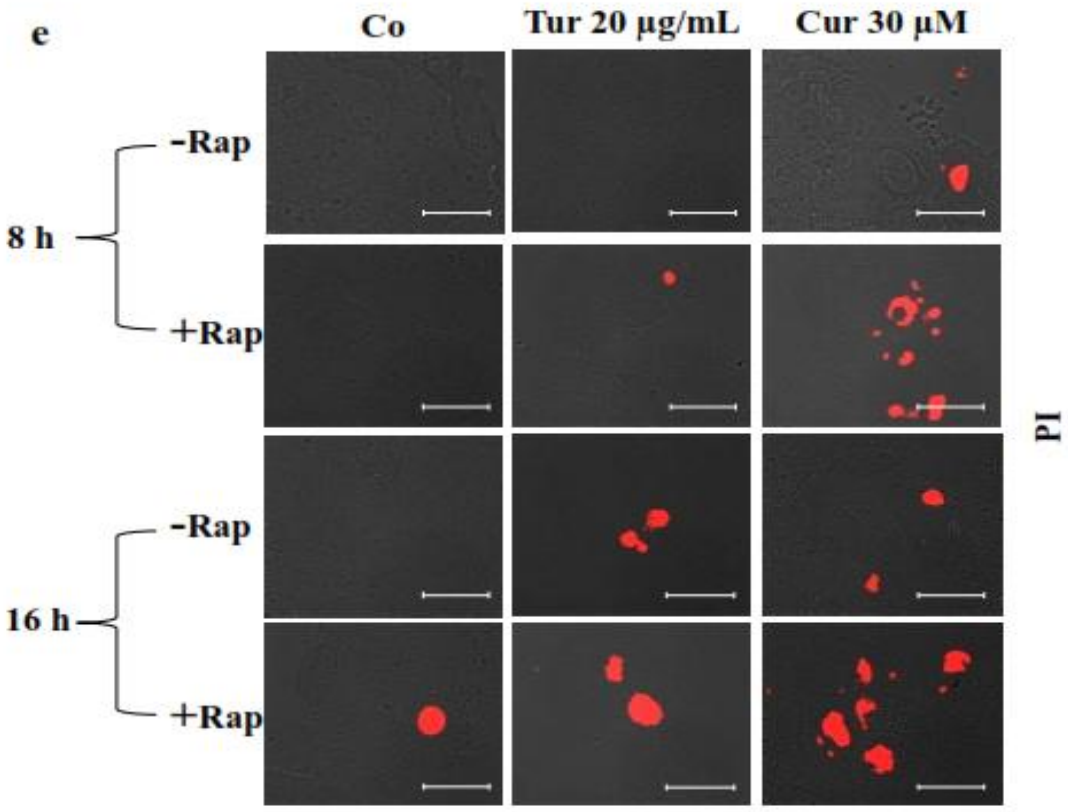

Figure 3.15 (cont.) e) the cells, treated as above were unfixed and stained with Propidium Iodide (PI), which in this conditions enters and labels the DNA of only the necrotic cells. Based on the cell remained attached, it is evident the increase in necrotic cells in the cultures treated with Turmeric or Curcumin along with Rapamycin.

3.16 Turmeric and Curcumin inhibit A431 cell migration

Finally, we tested whether Turmeric and Curcumin could be also effective in inhibiting the migration of A431 skin cancer cells in a classical wound healing assay. The monolayer was scratched and after washing out the debris, the culture was incubated for up to 72 h in the presence or the absence of Turmeric or Curcimin at a non-toxic concentration. Both Turmeric and Curcumin showed the ability to decrease the rate of healing of the wound (Figure 3.16a-b).

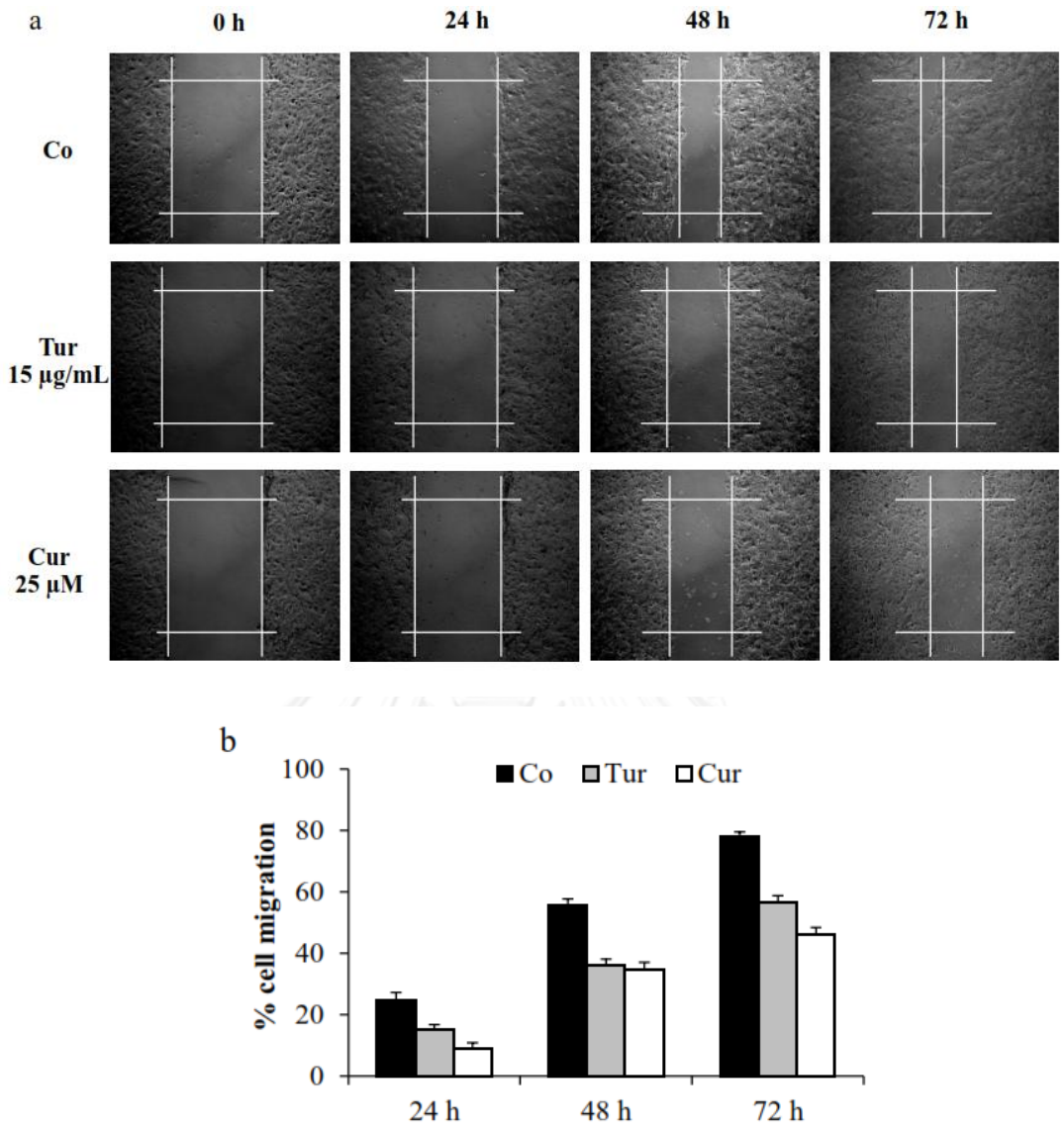

Figure 3.16 Inhibitory effect of Turmeric and Curcumin on A431 cell migration. The effect of Turmeric (15 µg/mL, non-toxic) or Curcumin (25 µM, non-toxic) on cell migration of A431 cells was tested in a classical wound healing assay. a) Representative images at the phase contrast microscope of the cell monolayers; b) percentage of cell migration calculated as described in Materials and Methods.

3.12 **Resveratrol inhibits the growth and migration of skin cancer cells**

Resveratrol, a phytochemical present in the Thai herb, *Morus alba* Linn., also possesses anticancer properties [198]. Previously, Isidoro and collaborators showed that Resveratrol could limit cell migration and invasion in human glioblastoma U87MG cells [123]. Thus, we attempted to investigate the potential of Resveratrol to inhibit cell migration in skin cancer. Human epidermoid carcinoma A431cells were used as *in vitro* cell model for testing the effect of resveratrol on cell migration. Firstly, we used the wound healing assay to assess the inhibitory effect of Resveratrol on cell migration. The A431 cells were exposed to resveratrol at 100 μM for 72 h, and the completed medium with or without Resveratrol was changed every 24 h, and the photograph was taken every 24 h. The cells monolayers images (Figure 3.17a) show that Resveratrol slowed-down A-431 cells migration. The figure 3.17b represents the percentage of cell migration. In control cell, the cells at the migration fronts moved toward each-other and nearly completed the healing at 72 h; by contrast, in the Resveratrol-treated sample, the cells moved much slower than the control cells (the distance of both side of the wound remained unchanged after 48 h), and the wound was not healed at 72 h. We also checked the cytotoxic effect of resveratrol in A431 cells by cell counting. Treatment of Resveratrol at 50 and 100 μM for 24 h decreased the number of cells in a dose dependent manner (figure 3.17c) by inducing cell cycle arrest at S phase, but not apoptosis (figure 3.17d and e).

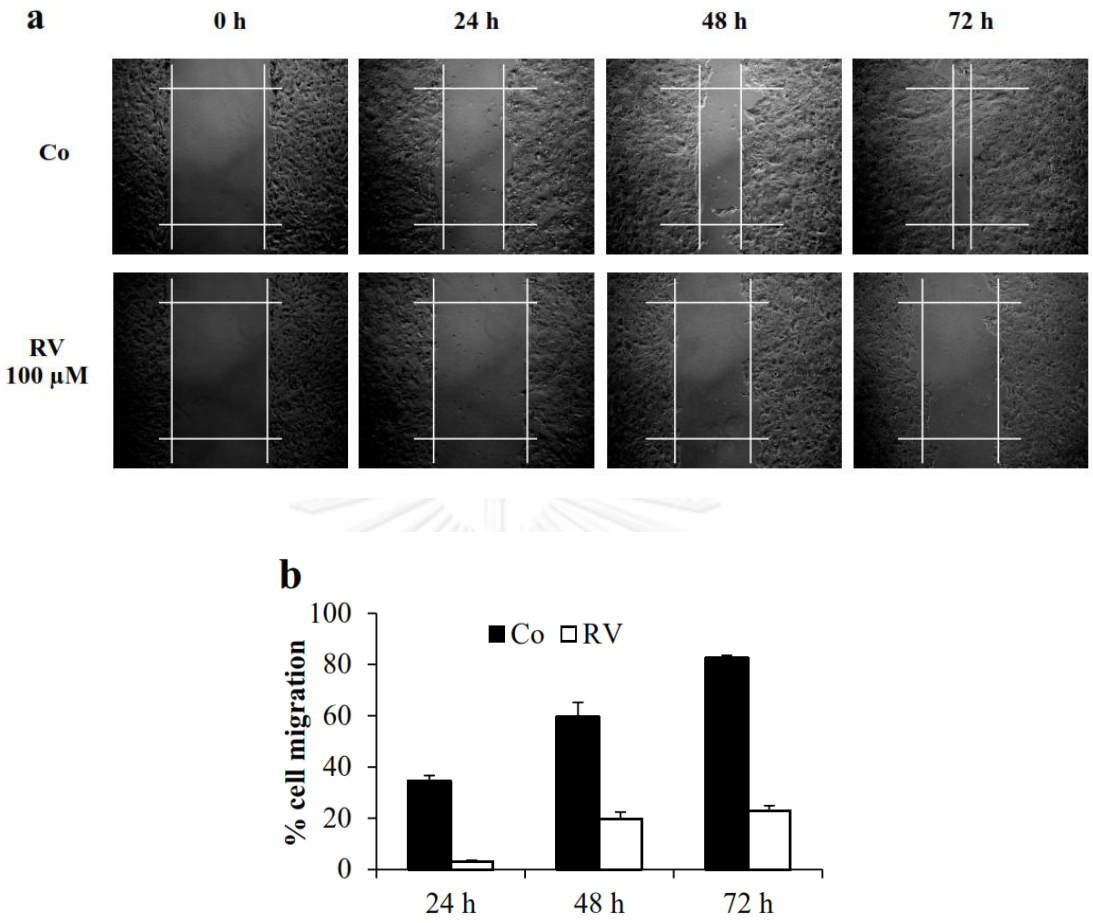

Figure 3.17 Effect of resveratrol on cell migration and cytotoxicity of A431 cells. The inhibitory effect of resveratrol on cell migration in A431 cells was performed by wound healing assay (a) representative cells monolayers images and (b) percentage of cell migration.

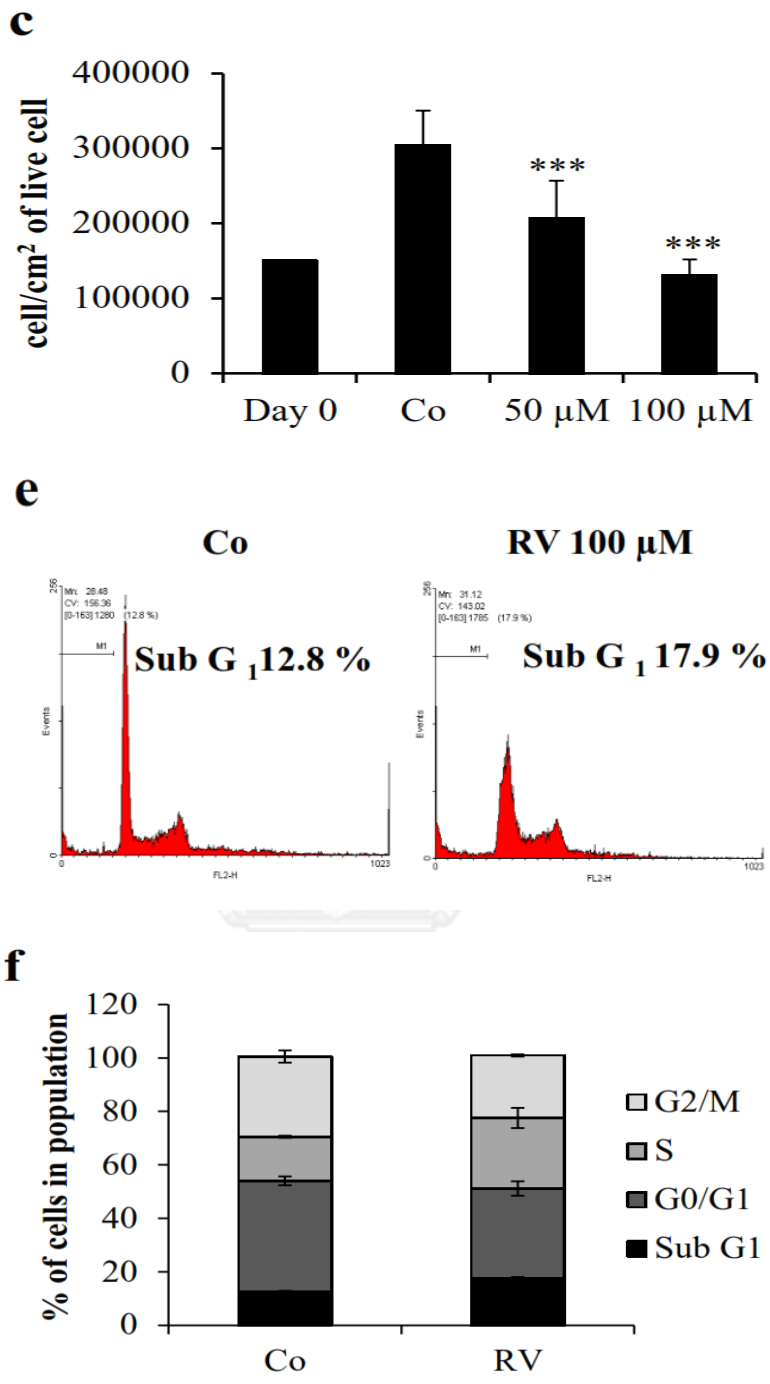

Figure 3.17 (cont.) The cytotoxic effect of resveratrol on A431 cells was investigated by cell counting. Treatment of resveratrol at 50 and 100 µM for 24 h decreased a number of cells in dose dependent manner (c). The flow cytometry shown that resveratrol induce cell cycle arrest at S phase not apoptosis (d and e). (Statistical significance *** $p < 0.001$)

E-cadherin, expressed in most epithelial cells, is known to play a role in cell-to-cell adhesion and also to promote cell polarity [199, 200]. Several studies suggest that loss of E-cadherin associates with tumor cell invasion [201-203]. E-cadherin is therefore considered a key suppressor of cell migration and a marker of the Epithelial-to-Mesenchymal Transition process [204]. A treatment of 8 h with Resveratrol was sufficient to induce the expression of E-cadherin both in the cell located near the wound and far away from the wound, while after 24 h of treatment with Resveratrol only the cells far away from the wound still express E-cadherin at high level (figure 3.18a). This result was confirmed by western blotting, in which however the whole cell population exposed to Resveratrol was assayed for E-cadherin expression (figure 3.18b).

It is well known that activation of AKT mediates cell proliferation, migration, invasion, angiogenesis, and metastasis [205, 206]. It has been demonstrated that inhibition of AKT activation could suppress the cell migration [207]. Therefore, we asked whether the inhibitory effect of Resveratrol on cell migration involved the AKT signaling pathway. Resveratrol could inhibit the (ser473)-phosphorylation of AKT after 8 and 24 h of treatment (Figure 3.18c). We assume that the impairment of AKT phosphorylation in the presence of Resveratrol is likely due to the inhibitory action of this polyphenol on the activity of PI3k class I [208], which provides the PIP3 needed for the mTORC1-mediated phosphorylation of AKT in ser473. The mTOR pathway also has been involved in the signaling associated with cell migration [209]. Though mTOR is placed down-stream to the AKT pathway, we checked for a possible direct effect of Resveratrol on the mTOR pathway. Resveratrol has been reported to inhibit the activity of p70S6Kinase [210], which is down-stream of mTOR and acts on the ribosomal protein S6. We therefore analyzed the phosphorylation status of this substrate. It is to be noted that the phosphorylation of S6 was high in the first 8 h of incubation and decreased to approximately 10% by 24 h of incubation, likely because of the consumption of growth stimulating factors in the medium. At this time, in fact, also pAKT was decreased. Resveratrol inhibited by approximately 50% the phosphorylation of S6 in the first 8 h of incubation, while it paradoxically slightly stimulated the phosphorylation of S6 by 24 h (Figure 3.18c).

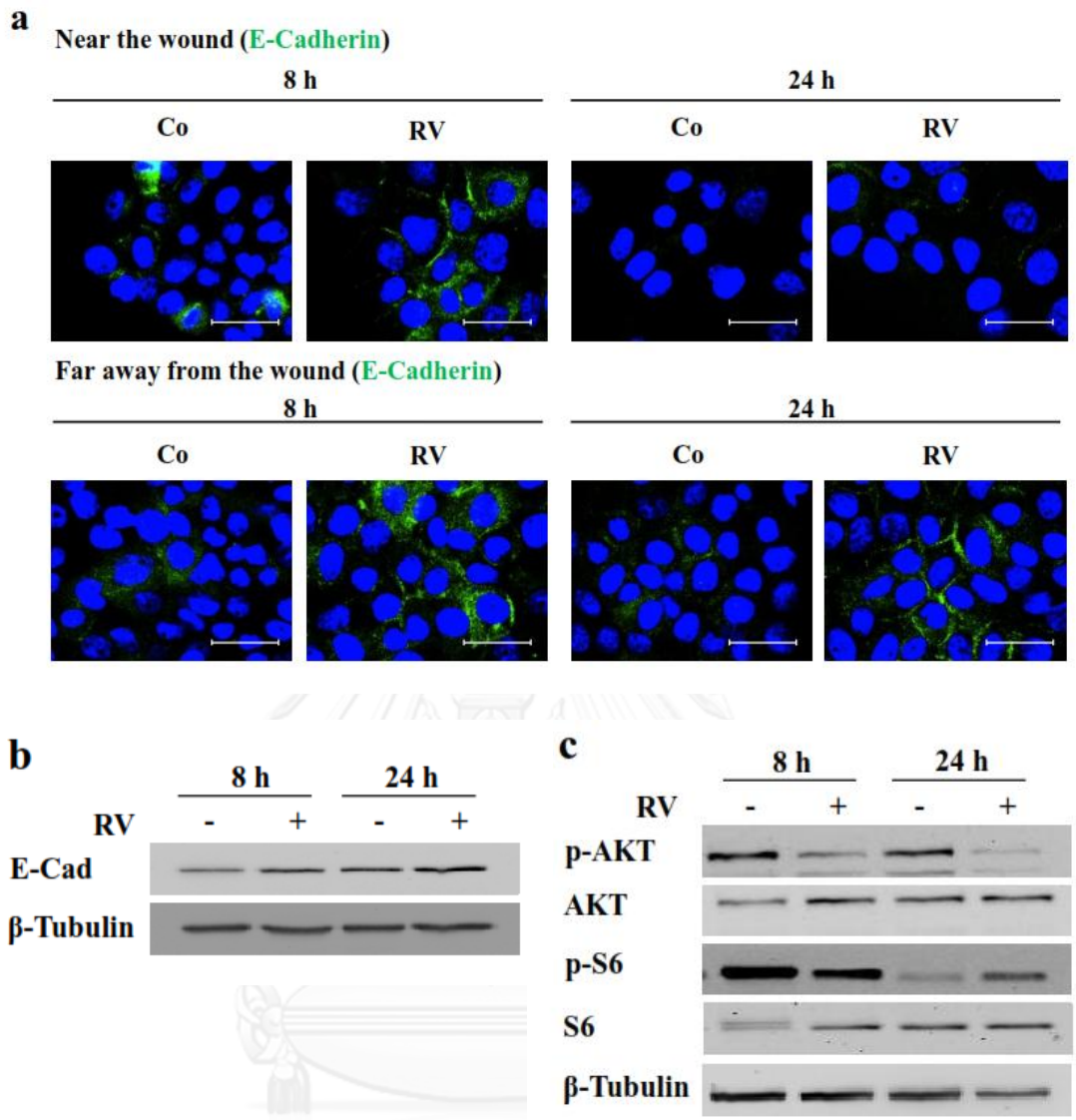

Figure 3.18 Effect of resveratrol on expression of E-cadherin. (a) Resveratrol induce the expression of E-cadherin in A431 cells both the cell near the wound and far away from the wound after 8 h of treatment; whereas, after 24 h treatment of resveratrol, only the cells far away from the wound show highly expression of E-cadherin. (b) E-cadherin expression was confirmed by western blotting. (c) Effect of Resveratrol on cell migration involved the AKT signaling pathway after 8 and 24 h of treatment was performed by western blotting.

Autophagy plays an important role in various physiological and pathophysiological cellular processes including cell migration [211-213]. Previously, we and others have reported that Resveratrol could induce autophagy in several cancer cell types [114, 214-216]. We asked whether autophagy was involved in the cytotoxic effect of Resveratrol. To this end, we co-treated A431 cells with Resveratrol and chloroquine. The latter is a lysosomotropic agent that prevents autophagosome-lysosome fusion and lysosomal degradation, and therefore allows to measurement the rate of autophagosome production and the autophagic flux [190]. Chloroquine inhibits the lysosomal degradation step, thus impairing the recycling of substrates sequestered in the autophagsomes. We first tested the cytotoxic effect of these treatments by cell counting. After 24 h of treatment, the number of cells was not changed; indicating that Resveratrol- induced autophagy was not toxic at this time (figure 3.19a). Induction of Autophagy was confirmed by immunofluorescence and western blotting (figure 3.19b-c). Immunofluorescence showed that Resveratrol triggered the formation and accumulation of LC3-positive vacuoles assumed to be autophagosomes and autophagolysosomes (figure 3.19b). The ubiquitin-binding protein p62 is targeted to autophagosome by binding to Atg8/LC3 [217]. On western blotting, Resveratrol treatment resulted in the accumulation of LC3-II and p62 proteins, which increased upon co-treatment with chloroquine because of the inhibition of the late step of autophagy (figure 3.19c).

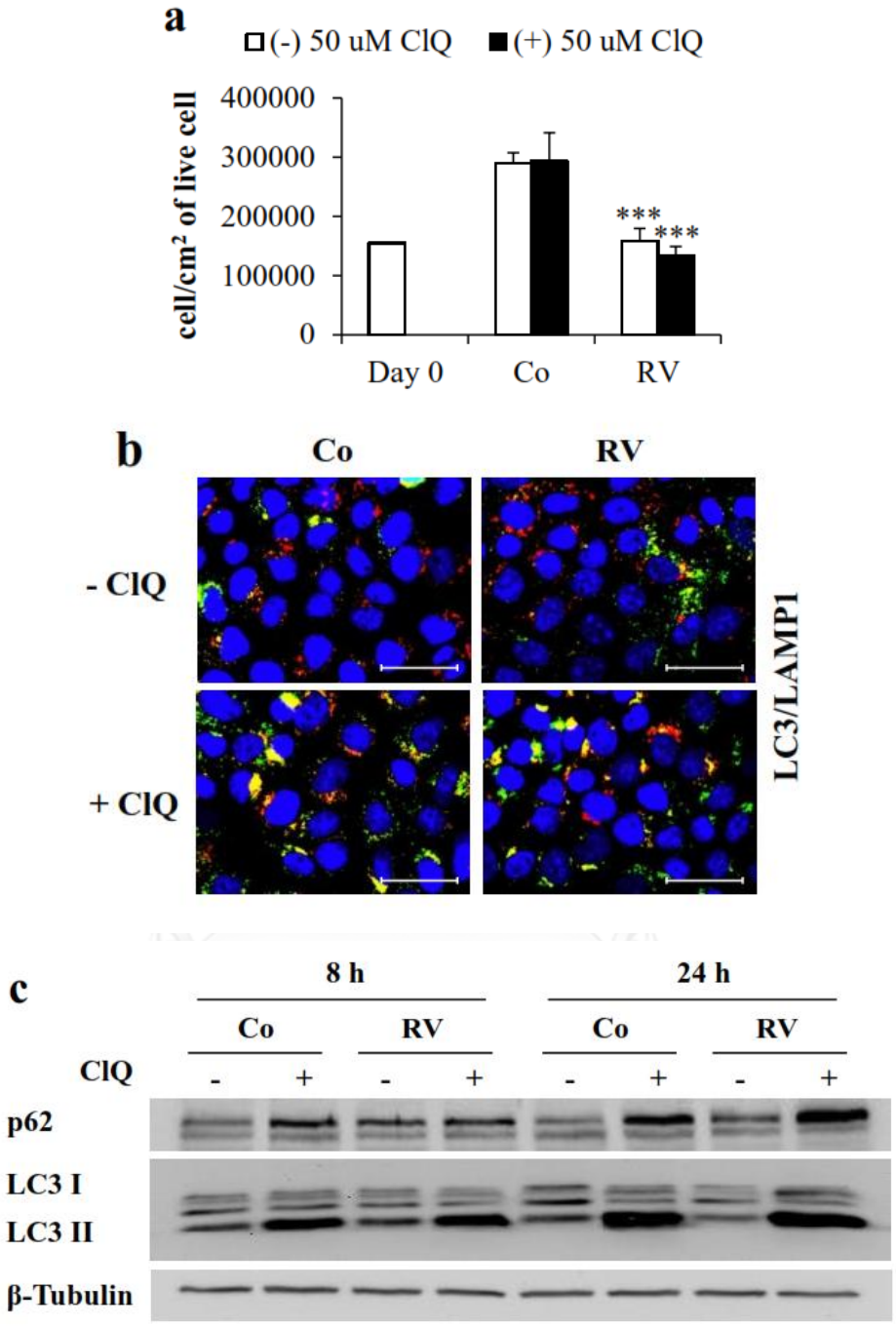

Figure 3.19 Effect of Resveratrol on autophagy induction. (a) Co-treatment between Resveratrol and chloroquine in A431 cells for 24 h, a number of cells did not change (b). Immunofluorescence shown Resveratrol triggered the formation and accumulation of LC3-positive vacuoles. (c) Resveratrol treatment resulted in the accumulation of LC3-II and p62 proteins.

The tumor suppressor p53 protein is known to play an important role in cell cycle checkpoint and cell apoptosis; however it is clear that p53 protein is also involve in cell migration. In fact, loss of p53 influences increasing of cell motility, indicating that this protein contributes to tumor invasion and metastasis [218]. Mutant p53 promotes cell migration, while wild-type p53 is likely to prevent tumor cell invasion [219]. A431 cells have been reported to express p53-mutated [220, 221]. We investigated the effect of Resveratrol on the expression of p53 protein. The western blot analysis showed that the treatment with Resveratrol for 8 and 24 h up-regulated the expression of p53 protein (figure 3.20). Thus, p53 is not involved in the mechanism of migration inhibition by Resveratrol.

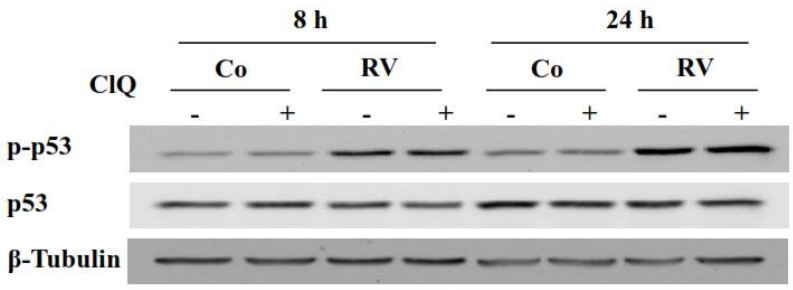

Figure 3.20 The effect of Resveratrol on the expression of p53 protein. Treatment with Resveratrol for 8 and 24 h up-regulate the expression of p53 protein.

We further investigated on the involvement of autophagy in A431 cell migration. To this end, we studied the potential synergistic effects of Resveratrol on autophagy induction together with rapamycin, as mTOR-dependent autophagy inducer [222], or LiCl, as mTOR-independent autophagy inducer [223, 224]. Ribosomal protein S6 kinase-1 (S6K1, also known as p70S6K) is a downstream target of mTOR. To confirm that rapamycin induced mTOR dependent autophagy and LiCl induced mTOR independent autophagy, western blot analysis was performed. The result showed that phosphorylation of S6 was suppressed by treating with rapamycin but not with LiCl (figure 3.21). As already seen in Figure 3.18c, at 24 h Resveratrol slightly stimulated the phosphorylation of S6, and this occurred also in LiCl-treated cells, but not in the presence of rapamycin which completely abrogated the phosphorylation of S6.

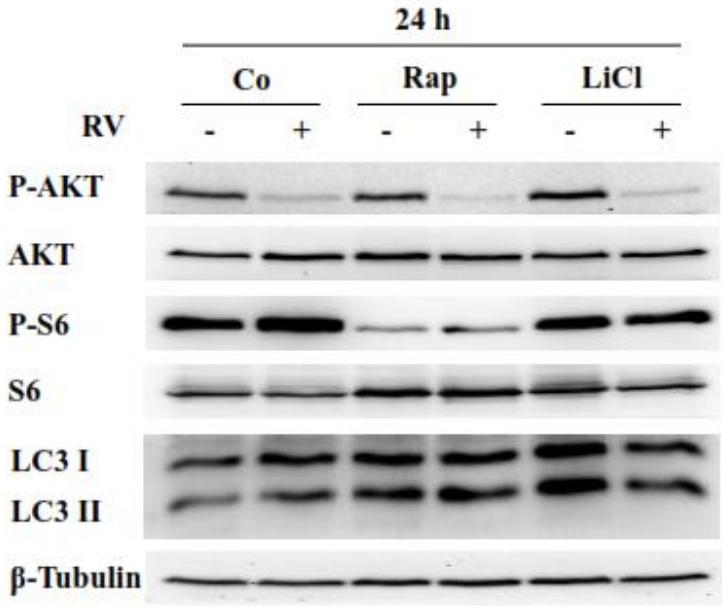

Figure 3.21 Effect of Resveratrol, rapamycin and LiCl in A431 cells on mTOR pathway. Phosphorylation of S6 was suppressed by treating with rapamycin but not with LiCl Resveratrol and LiCl-treated cells slightly stimulated the phosphorylation of S6, and, but not in the presence of rapamycin which completely abrogated the phosphorylation of S6.

Lastly, the effect of Resveratrol in combination with rapamycin, LiCl or Clorochine on cell migration was investigated. We found that after 72 h of treatment, rapamycin and LiCl suppressed cell migration compared to control cell (Figure 3.22). The percentage of closure of wound was calculated for each treatment (see Table 3.5). A synergistic inhibitory effect was observed in the combination Resveratrol plus LiCl. Cloroquine in itself slightly stimulated cell migration, as indicated by the facts that percentage of healing increased at 48 h and that in combination with Resveratrol it slightly attenuated the inhibitory action of the latter. It is to be noted that however the combination Resveratrol plus Chloroquine revealed to be slightly toxic at 72 h. Taken together, the data suggest that autophagy inducers oppose to cell migration.

Immunofluorescence staining confirmed that the treatment with Resveratrol, rapamycin and LiCl were affected in inducing autophagy, as witnessed by the accumulation of LC3-II-positive vacuoles. This effect was more evident in the cells at the migration front than in those located rearward and far away from the wound (figure 3.23). Cloroquine was even more effective in inducing the accumulation of autophagosomes, and nevertheless it was not inhibiting cell migration, rather it stimulated the wound healing. A possible interpretation is that to inhibit cell migration autophagy need to proceed to completion with autophagolysosome formation and degradation of the substrates, whereas in the presence of chloroquine this last step is impaired.

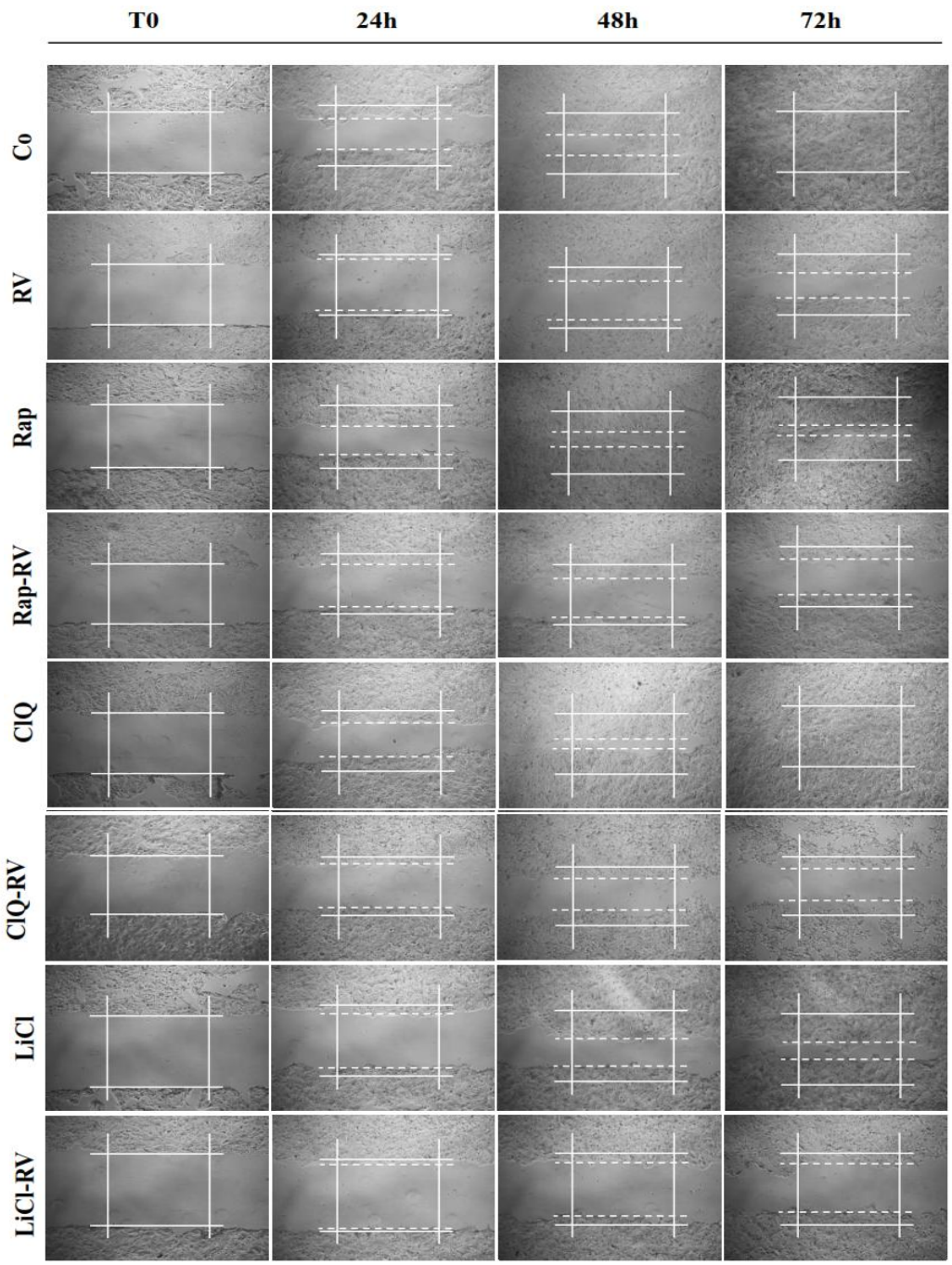

Figure 3.22 The effect of Resveratrol in combination with rapamycin, LiCl or Clorochine on cell migration. After 72 h of treatment, rapamycin and LiCl suppressed cell migration compared to control cell. A synergistic inhibitory effect was observed in the combination Resveratrol plus LiCl. Cloroquine in itself slightly stimulated cell migration, and in combination with Resveratrol it slightly attenuated the inhibitory action of the latter.

Table 3.5 The percentage of closure of wound was calculated for each treatment.

	Co	RV	Rap	Rap-RV	ClQ	ClQ-RV	LiCl	LiCl-RV
24h	50 %	14.29 %	50%	28.57%	42.86 %	25.93 %	21.88 %	9.38 %
48h	64.29 %	35.71 %	75 %	35.71 %	87.5%	44.44 %	59.38 %	21.88 %
72h	100 %	57.14 %	82.14 %	39.29 %	100 %	44.44 %	75 %	31.25 %

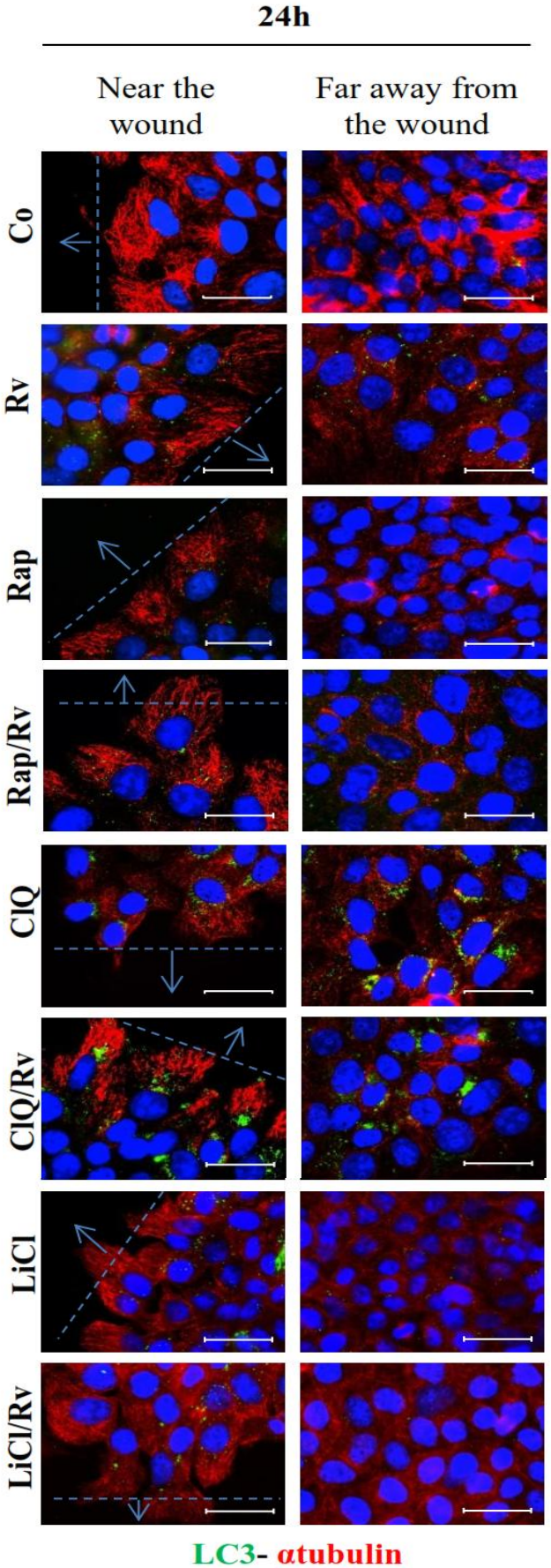

Figure 3.23 The effect of Resveratrol in combination with rapamycin, LiCl or Clorochine on autophagy during cell migration. Immunofluorescence staining confirmed that the treatment with Resveratrol, rapamycin and LiCl were affected in inducing autophagy, by the accumulation of LC3-II-positive vacuoles. This effect was more evident in the cells at the migration front than in those located rearward and far away from the wound. Cloroquine was even more effective in inducing the accumulation of LC3-II-positive vacuoles

CHAPTER IV

DISCUSSION

UVB (280–320 nm) reaches the earth's surface in amounts sufficient to provoke harmful biological effects on the skin that can eventually lead to skin malignancies and non-melanoma cancers [225]. UV is a complete carcinogen. UVB-induced DNA damage generates photoproducts such as cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4) pyrimidone [11, 226, 227]. Cells in which DNA damage is un-repaired should undergo apoptosis [228], as a mechanism to prevent the development of cancer. DNA damage and cytotoxicity occur at UVB intensity of approximately 30 mJ/cm^2, which corresponds to a few minutes of exposition to sunlight [229]. A prolonged exposure to sunlight UVB can cause massive apoptosis in keratinocytes and consequently alters the natural barrier functions of the skin, thus predisposing to inflammation, infections and cancer. Free radicals associated to UV radiation exposure trigger skin inflammation, a condition that contributes to the development of skin cancer [45, 230]. In this regard, cyclo-oxygenase Cox-2 is one of the main player involved in inflammation and skin carcinogenesis induced by UVB exposure [231, 232] and, consistently, natural products able to down-regulate its expression also exert preventive activity against UV injuries [233, 234].

The present study demonstrates that extracts of fifteen species of Thai plant possess varying degrees of total phenolic and total flavonoid contents as well as antioxidant activities, depending on extraction solvents. In particular, extracts derived from ethanol and dichloromethane fractions were found to possess higher total phenolic and total flavonoid contents than those from petroleum ether. A high correlation between total phenolic content and antioxidant activity was found. The highest levels of total flavonoid content were detected in Turmeric extract from dichloromethane fraction, followed by turmeric extract from petroleum ether fraction. In addition, Turmeric and Ginger extracts were shown to possess the highest content of antioxidants and the highest UV-absorping ability. Thus, one first conclusion of the present study is that the method and solvent used for the

preparation of the extract greatly affect the physical-chemical properties, and possibly also the biological activities, of the herb extract. Next, we tested the ability of the extracts to prevent UVB-induced DNA damage and cell death in keratinocytes. In general, low doses of UVB cause DNA mutations that most likely lead to tumor initiation, whereas high doses of UVB directly induce apoptosis as a consequence of oxidative stress [18]. Keratinocytes undergoing apoptosis are known as 'sunburn cells,' and their presence is assumed as indicator of the severity of UVB-induced DNA damage [235]. In the present study we exposed keratinocytes to a relatively high dose of UVB (120 mJ/cm^2), which in fact caused DNA damage (as indicated by a 3-folds increase in CPDs) and apoptosis (approximately 50 % in the culture at 24 h post-irradiation). Remarkably, if prior to exposure to UVB the keratinocytes were incubated with Turmeric or Ginger extracts (dichloromethane fraction) CPDs were found in dead cells but not in living cells. Also, pre-incubation with these extracts protected to some extent from caspase-dependent apoptosis induced by UVB. Taken together, these results suggest that Turmeric and Ginger extracts could save those cells in which UVB-induced DNA damage was prevented, though allowing cell death in those ones bearing DNA mutations. It is conceivable that protection from UVB damage was linked to the anti-oxidant properties of the herbs extracts. In fact, Gingerol, the major component of Ginger, was shown to reduce the UVB induction of ROS, caspases and Cox-2 in HaCaT cells [121], and Curcumin, the major component of Turmeric, also was shown to down-regulate Cox-2 expression [236]. In this study we show that the incubation of HaCaT cells with Turmeric and Ginger extracts increased the expression of Thioredoxin-1, a protein that protects mitochondria from excessive oxidative stress. The primary cytotoxic event in human epidermis associated with UV exposure is the excessive production and accumulation of ROS, which is the consequence of UV inactivation of the thioredoxin system [237]. Therefore, the increased expression of this antioxidant protein achieved by the pre-incubation with the Turmeric and Ginger extracts is likely to confer a protection to keratinocytes exposed to UVB (summarized in Figure 4.1).

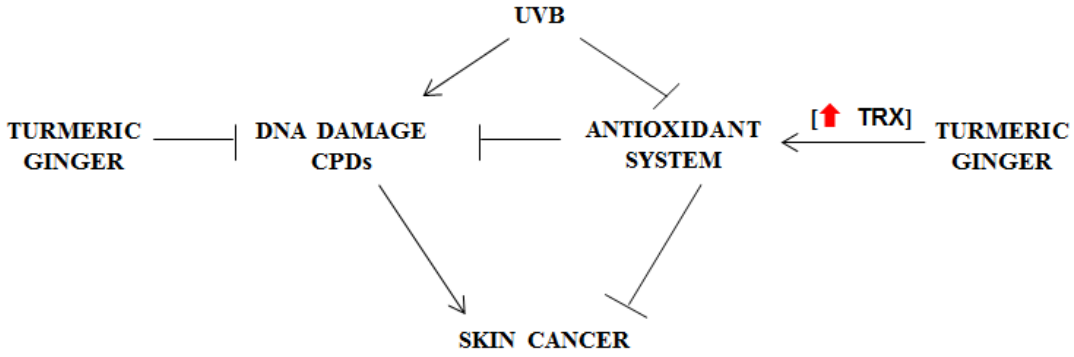

Figure 4.1 Scheme of protective effect of Turmeric and Ginger extracts on UVB-induced skin cancer. Turmeric and Ginger extracts prevent the cells in which UVB-induced DNA damage and increase expression of thioredoxin, antioxidant protein.

In the Asiatic traditional medicine, Turmeric is by large the true spice employed for the preparation of topical medicaments, including cosmetics [238], as compared to its component Curcumin. The latter has been shown to exert beneficial and preventive effects toward skin cancer [239-242]. Curcumin was shown to inhibit the cell proliferation of epidermoid carcinoma A431 cells by down-regulating EGFR [243]. Further, Curcumin (at a dose of 50 μM) was shown to induce p53-dependent apoptosis in Basal Cell Carcinoma cells, whereas primary keratinocytes were shown to resist to higher doses (up to 100 μM) [34]. While the genetic and epigenetic pathways triggered by pure Curcumin have been widely explored [244, 245], much less is known about the mechanisms through which Turmeric exerts its anticancer activity [246, 247].

Here we have investigated the potential of Turmeric to inhibit the growth and the migration of cultured skin SCC-type A431 cells. The effects of Turmeric were compared with those of pure Curcumin. We found that both the Turmeric extract and pure Curcumin elicited anti-cancer effects of A431 cells (summarized in Figure 4.2a), through the activation of multiple pathways that involved essentially the signaling molecules mTOR, BECLIN 1 and p53 mutant (as shown in Figure 4.2b). Both these substances were capable of inducing cell cycle arrest and apoptosis, in accord to previous observations in other cancer cell types [248, 249]. In addition, we found that both Turmeric and Curcumin strongly induced the production of

autophagosomes and also stimulated their consumption. At the doses used, Curcumin was shown to accelerate the autophagic flux more than Turmeric, as indicated by the stronger accumulation of LC3-positive vacuoles and of lipidated LC3-II in the cells co-treated with Chloroquine. The latter, at the same doses as used in the present study was shown to induce vacuoalization and autophagy-associated cell death in human normal dermal fibroblasts and immortalized keratinocytes [250]. However, a 24 h co-treatment of Turmeric or Curcumin along with Chloroquine revelead to be non-toxic to A431 cells, despite the accumulation of undigested autophagic vacuoles. It is likely that these cells are resistant to Chloroquine inhibition of autophagy degradation even in combination with Turmeric or Curcumin, possibly because of pro-survival signals triggered by the latter. Yet, it cannot be excluded that a prolonged incubation under such stressful conditions would eventually result in cell death.

The epidermoid cancer A431 cell line has harbours the R273H mutant of the oncosuppressor p53. p53 is a master regulator of the cell fate in cells subjected to genotoxic stress and prevents carcinogenesis. Accordingly, the combined topical application of Resveratrol and Black Tea Polyphenols was shown to suppress mouse skin carcinogenesis through the activation of p53 [251]. In addition, polyphenol-induced Ser15-phosphorylation and increase in the expression level of p53 correlated with apoptosis and inhibition of chemically-induced skin carcinogenesis [251-253]. The anti-proliferative activities of wild-type p53 extend from its nuclear transcription to its cytosolic pro-apoptotic functions. While nuclear p53 transcribes, among others, the pro-apoptotic BAX protein and the cell cycle inhibitor p21WAF1/Cip1 [254, 255], the cytosolic p53 associates with the mitochondria, sequesters the anti-apoptotic BCL-2 proteins and promotes BAX oligomerization [256], thus allowing cell death through the intrinsic pathway. However, the p53R273H mutant is likely unable to direct the transcription of anti-proliferative proteins (such as BAX or p21) and, accordingly, it has been shown to confer resistance to drug-induced apoptosis [192, 257]. Yet, in response to natural polyphenols, A431 cells undergo cell cycle arrest associated with a (p53-independent) up-regulation of p21 [193, 194]. Here we found that in A431 Turmeric and Curcumin induced both

autophagy and cell death, though only slightly induced the expression of p21 and of phospho-BAX and also of BCL-2, and that the modulation of p21 was BECLIN 1-dependent. Turmeric and Curcumin promoted the Ser15 phosphorylation of the mutant p53 (likely mediated by AMPk) and also its degradation. These effects were clearly autophagy-dependent. In fact, in BECLIN 1 knocked-down cells, the actual level of p53 was increased, and in general counteracted the action of Turmeric and Curcumin on its Ser15-phosphorylation. This finding indicates that induction of autophagy by Turmeric and Curcumin triggers the (MDM2-mediated) degradation of p53. Other autophagy triggers have been shown to direct the proteasome-mediated degradation of p53 [258]. Further, degradation and inactivation of the p53 mutant in A431 has also been observed upon treatment with alpha-Santalol [194]. It is known that degradation of wild-type p53 occurs through the MDM2-proteasome pathway [259], and is triggered during induction of autophagy [258]. Recently it has also been reported the degradation of p53 mutants through the chaperon-mediate autophagy pathway [260]. Here we show that the activation of mTOR-independent (macro) autophagy-lysosomal pathway by Turmeric or Curcumin leads to the degradation of the mutant p53, a necessary step to allow onset of cell death through apoptosis and necrosis. When in the cytosol, wild-type p53 has been shown to also inhibit basal autophagy, and this correlates with increased mTOR activity and concomitant inactivation of the AMPk pathway [258]. Consistently, the p53R273H mutant, which shows a preferential cytoplasmic localization, has been proven to inhibit BECLIN 1-dependent autophagy [261]. Surprisingly, we found that in A431 cells (expressing the p53R273H) Turmeric and Curcumin are capable of inducing a rise of basal autophagy, which occurs (at least partly) independently of mTOR (as indicated by its slight activation), very likely through activation of the AMPk pathway (as suggested by the Ser15-p53 phosphorylation). Consistent with the activation of a pathway alternative to the AKT-mTOR, Rapamycin further increased the level of autophagy in the cells exposed to Turmeric and Curcumin. Noteworthy, while in itself not toxic at the concentration used, Rapamycin synergized with Turmeric and Curcumin in causing the degradation of the p53R273H mutant and greatly exacerbated cell death triggering both apoptosis and necrosis, likely as a result of an over-stimulation of

autophagy. A two-hits stress that hyper-induces autophagy may in fact lead to cell death [108, 259]. An interpretative scheme of the present findings is reported in Figure 4.2b.

Thus, it is likely that the pathway triggered by these drugs is cell context dependent, as suggested by the apparent contradictions present in the literature. For instance, in glioblastoma cells Curcumin was shown to inhibit the AKT-mTOR pathway and to induce a G2/M cell cycle arrest and autophagy but not apoptosis [248]. In human Basal Cell Carcinoma, Curcumin (at 50 μM) was shown to induce apoptosis in association with increased expression and nuclear translocation of p53 along with p21, while no changes in the level of BCL-2 and BAX were observed [34]. These findings contrasted with other studies in which p53-dependent apoptosis was associated with a decrease in the expression of BCL-2. However, in human Basal Cell only 50% of cell death could be ascribed to p53-dependent apoptosis [34]. In MCF7 breast cancer cells Curcumin was shown to induce apoptosis through a p53-dependent activation of BAX [249], but in MDA-MB-231 breast cancer cells Curcumin decreased the level of p53 and of BCL-2, and increased the expression of p21 [262].

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a naturally phytoalexin found in several plants such as grapes, peanuts, multiberries. Resveratrol is also present in the Thai herb, *Morus alba* Linn. [198]. Several studies have shown that Resveratrol possesses antioxidant, cardioprotective, anti-diabetic, and anticancer properties [263-266]. Previously, Isidoro and collaborators showed that Resveratrol could limit cell migration and invasion in human glioblastoma U87MG cells [123]. In this study, we have shown that Resveratrol has the capability to inhibit cell migration in human epidermoid carcinoma A431cells. it has been reported that Resveratrol induced G1-phase arrest, followed by inducing cell apoptosis [267]. However, in our cell model, Resveratrol induced cell cycle arrest at S phase, but not apoptosis at 24 h post-treatment. In agreement with our finding, Nifli *et al*., found that Resveratrol induced growth arrest at S phase of the cycle in T47D human breast cancer cells [268]. E-cadherin, expressed in most epithelial cells, is known to play a role in cell-to-cell adhesion and also to promote cell polarity [199, 200]. Several studies suggest that

loss of E-cadherin associates with tumor cell invasion [201-203]. E-cadherin is therefore considered a key suppressor of cell migration and a marker of the Epithelial-to-Mesenchymal Transition (EMT) process [204]. Recently, Li et al. reported that Resveratrol modulated the expression of EMT-related genes such as E-cadherin, N-cadherin, vimentin, MMP-2, and MMP-9, which are known to play a role in cancer cellular motility, invasiveness and metastasis during tumorigenesis [269]. Our data also showed that Resveratrol induced the expression of E-cadherin in human epidermoid carcinoma A431cells. It has been reported the correlation between the expression of tumor suppressor p53 and of E-cadherin. Chemically-induced papillomas in p53+/- mice, is associated with loss of E-cadherin expression in tumors with undetectable level of p53, and with normal E-cadherin expression in tumors that retain wild-type p53 expression [270]. Shen et al. found that Resveratrol could suppress EMT through reactivating p53 [271]. The tumor suppressor p53 protein is known to play an important role in cell cycle checkpoint and cell apoptosis; however it is clear that p53 protein is also involved in cell migration. In fact, loss of p53 increases of cell motility, indicating that this protein opposes to tumor invasion and metastasis [218]. Mutants p53, including mutants Arg175His and Arg273His, promote cell migration, while wild-type p53 prevent tumor cell invasion [219]. A431 cells have been reported to express p53-mutated (His273 mutation) [219, 272]. We showed that Resveratrol up-regulated the expression of p53 protein. Thus, p53 is not involved in the mechanism of migration inhibition by RV and other mechanisms are likely to contribute in its effect.

It is well known that activation of AKT mediates cell proliferation, migration, invasion, angiogenesis, and metastasis [205, 206]. It has been demonstrated that inhibition of AKT activation could suppress the cell migration [207]. Our data showed that Resveratrol could inhibit the (ser473)-phosphorylation of AKT. We assume that the impairment of AKT phosphorylation in the presence of Resveratrol is likely due to the inhibitory action of this polyphenol on the activity of PI3k class I [208], which provides the PIP3 needed for the mTORC2-mediated phosphorylation of AKT in ser473. The mTOR pathway also has been involved in the signaling associated with cell migration [209]. Though mTOR is placed down-stream to the AKT pathway, we

checked for a possible direct effect of Resveratrol on the mTOR pathway. Resveratrol has been reported to inhibit the activity of p70S6Kinase [210], which is down-stream of mTOR and acts on the ribosomal protein S6. We also showed that Resveratrol inhibits the phosphorylation of S6 in the first 8 of treatment, while it slightly stimulated the phosphorylation of S6 by 24 h. At present, we have not an explanation for this effect.

Autophagy plays an important role in various physiological and pathophysiological cellular processes including cell migration [211-213]. AKT-mTOR pathway is a negative regulator of autophagy [83]. Previously, we and others have reported that Resveratrol could induce autophagy in several cancer cell types [114, 214-216]. In the work reported here, we show that autophagy is induced by Resveratrol and this induction was not toxic at this time. Recently, it has been reported that autophagy is downregulated during cell migration and this downregulation is associated with induction of mTOR in migrating cells [213]. Therefore, we suggest that autophagy may be involved in the inhibitory effect of Resveratrol on cell migration in human epidermoid carcinoma A431cells. To this end, we studied the potential synergistic effects of Resveratrol on autophagy induction together with rapamycin, as mTOR-dependent autophagy inducer [222], or LiCl, as mTOR-independent autophagy inducer [223, 224]. We found that rapamycin and LiCl suppressed cell migration compared to control cell. Similar results were reported in human cervical carcinoma HeLa cells [213]. A synergistic inhibitory effect was observed in the combination Resveratrol plus rapamycin or plus LiCl. Nevertheless, chloroquine was not inhibiting cell migration; rather it stimulated the wound healing. A possible interpretation is that to inhibit cell migration autophagy needs to proceed to completion with autophagolysosome formation and degradation of the substrates, and in the presence of chloroquine this last step is impaired. Taken together, the data suggest that autophagy inducers oppose to cell migration (summarized in Figure 4.2a).

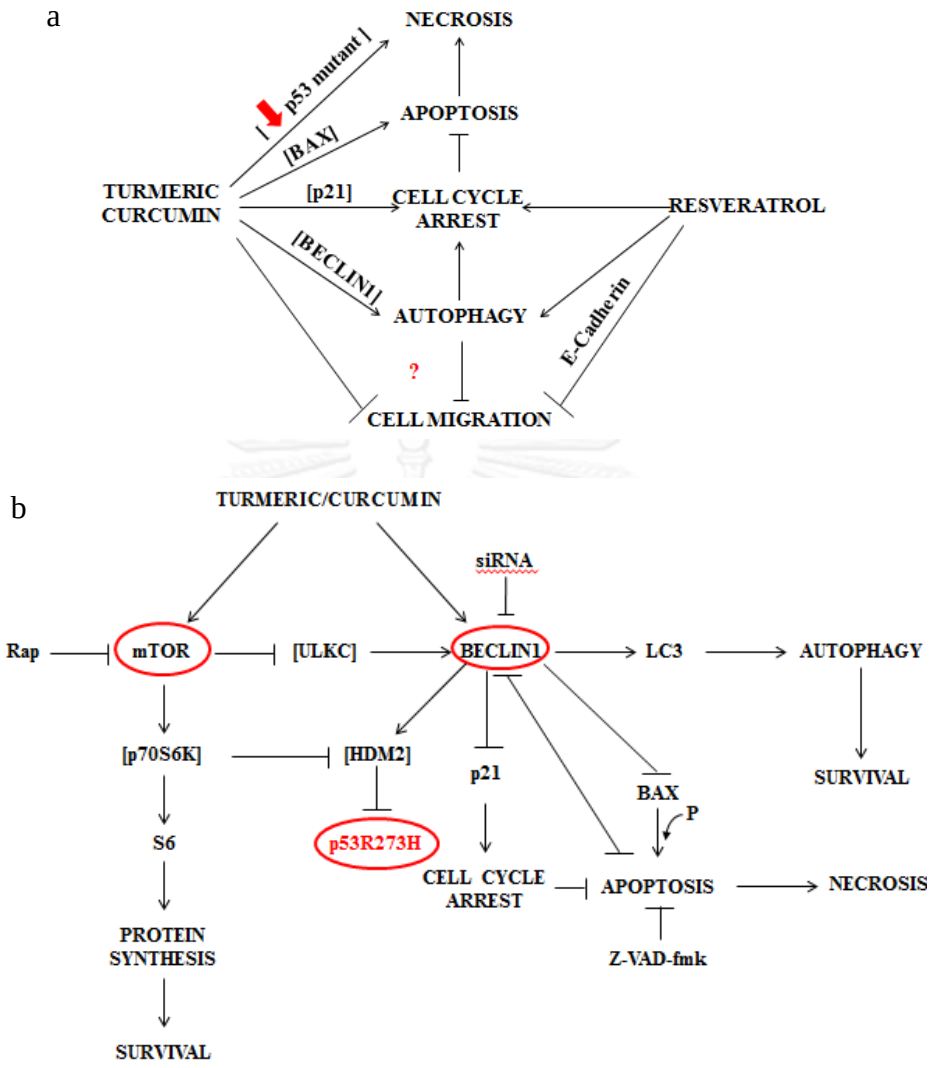

Figure 4.2 Interpretative schemes of the mechanisms of Turmeric and Curcumin toxicity in A431 cells. (a) Anti-cancer effects of Turmeric extract, Curcumin and Resveratrol in A431 cells. (b) Turmeric and Curcumin induce autophagy and cell death in A431 cells. Autophagy occurs through activation of both mTOR-dependent and mTOR-independent pathways. Autophagy leads to degradation of the p53R273H mutant, which was reported to oppose both apoptosis and autophagy. Knocking down the autophagy protein BECLIN 1 leads to a (partial) stabilization of Ser15-phospho-p53 and accumulation of p21, which results in cell proliferation arrest but not increased cell death. Concomitant inhibition of mTOR by Rapamycin increases autophagy, degradation of the mutant p53 and exacerbates cell death with onset of apoptosis and necrosis.

CHAPTER V

CONCLUSION

In conclusion, we propose that the pre-treatment with Turmeric or Ginger extract renders skin cells more competent to face the oxidative stress induced by UVB through the up-regulation of diverse anti-oxidant systems, including thioredoxin1. Additionally, our data also demonstrate that in A431 cells Turmeric extract and Curcumin tend to stimulate the S6-mediated synthetic pathway and are capable of inducing an mTOR-independent rise of basal autophagy, which likely has a pro-survival function. In parallel, Turmeric and Curcumin also activate a cell death program, which is exacerbates in the presence of the mTOR inhibitor Rapamycin. In addition, at non-toxic concentrations Turmeric, Curcumin, and Resveratrol also inhibited A431 cell migration. Further, Resveratrol induce cell growth arrest and downregulate AKT-mTOR pathway which lead to modulate autophagy. Moreover, Resveratrol plus autophagy inducers, as Rapamycin and LiCl show synergistic inhibitory effect. Therefore, in A431 cells, autophagy also plays a role in cell migration.

Cosmetic pharmaceuticals are demanded for chemoprevention of damaging effects of UVB exposure. A plethora of compounds naturally found in edible and non-edible vegetables and fruits have been shown to exert a protective action against UV skin damages, including inflammation and cancer [184, 233, 234, 273-275], and many of these constitute the active pharmacologic ingredient of cosmetic formulations that can prevent skin injuries [276] or improve skin repair [182, 277]. The present data showing that Turmeric and Ginger extracts could prevent UV-induced DNA and cellular toxicity in keratinocytes support their utilization in cosmetic biopharmaceuticals. Given that the cancer phenotype results from alterations in the expression of more than 500 genes, combinatorial drugs treatments are supposed to be more efficacious. Accordingly, herb extracts that contain a mix of substances could elicit a more powerful synergistic effect than their single purified components. We propose the combination of Turmeric and Rapamycin (rapalogs) at low

concentrations as a formula for a topic medicaments finalized to the cure of skin cancer.

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APPENDIX

VITA

My name is Visa Thongrakard. I graduated the bachelor degree, the master degree and doctoral degree from faculty of Allied Health Science, Chulalongkorn University. My field of study is molecular of medicinal plants, especially on cell apoptosis and autophagy.