ผลการต้านออกซิเดชัน การเพิ่มจำนวน และการตายแบบอะพอพโทซิสของสารสกัดผักพื้นบ้านไทยบางชนิดต่อเซลล์สายพันธุ์มะเร็งของมนุษย์

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THE ANTIOXIDATION, PROLIFERATION AND APOPTOSIS EFFECTS OF SOME LOCAL THAI VEGETABLE EXTRACTS ON HUMAN CANCER CELL LINES

Jirayus Woraratphoka

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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(THE ANTIOXIDATION, PROLIFERATION AND APOPTOSIS EFFECTS OF SOME LOCAL THAI VEGETABLE EXTRACTS ON HUMAN CANCER CELL LINES)
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สารพฤกษเคมีที่ใช้เป็นอาหารได้รับความสนใจเนื่องจากเป็นแหล่งของสารต้านอนุมูลอิสระ และเป็นสารเคมีป้องกันที่มีประสิทธิภาพ ดังนั้น สารสกัดจากผักพื้นบ้านของไทย 6 ชนิด ได้แก่ ผักจิก ผักติ้ว ผักแขยง ผักแพว ผักเม็ก และผักมะตูมแขก จึงนำมาตรวจสอบชนิดสารพฤกษเคมี รวมทั้งคุณสมบัติในการต้านอนุมูลอิสระ ความเป็นพิษ และวิถีการตายของเซลล์ ผลจากการทดลองพบว่า สารสกัดผักจิก ผักเม็ก และผักมะตูมแขกมีปริมาณสารฟีนอลิกทั้งหมดสูงซึ่งส่วนใหญ่เป็นสารเทนนินฟีนอลิก แต่มีปริมาณสารฟลาโวนอยด์ทั้งหมดต่ำ ขณะที่สารสกัดผักติ้ว ผักแขยง และผักแพวพบปริมาณสารฟลาโวนอยด์ทั้งหมดในปริมาณสูง สารสกัดผักจิก ผักเม็ก และผักมะตูมแขกพบสารฟีนอลิก 377.28 ± 11.57 303.70 ± 19.18 และ 438.73 ± 14.06 μg GAE/mg ตามลำดับ สารสกัดผักติ้ว ผักแขยง และผักแพวพบสารฟลาโวนอยด์ 251.36 ± 1.83 112.35 ± 0.50 และ 117.30 ± 0.68 μg CAE/mg ตามลำดับ คุณสมบัติในการต้านอนุมูลอิสระของสารสกัดผักจิก ผักเม็ก และผักมะตูมแขกแสดงผลใกล้เคียงกับสารคาเทชิน โทรล็อกซ์ และวิตามินซี และต้านอนุมูลอิสระสูงกว่าสารสกัดจากผักอีกสามชนิดอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ในด้านความสามารถในการรีดิวซ์เฟอริก ความสามารถในการจับอนุมูลอิสระ 2, 2-diphenyl-1-picrylhydrazyl (DPPH) และการยับยั้งการเกิดออกซิเดชันของไขมัน ส่วนในระบบที่ใช้เซลล์ในการทดสอบฤทธิ์ต้านอนุมูลอิสระ สารสกัดผักจิก และผักมะตูมแขกที่ความเข้มข้น 20 μg/mL และผักเม็กที่ความเข้มข้น 25 μg/mL แสดงการลดลงของการผลิตอนุมูลอิสระอนุพันธ์ของออกซิเจนที่ว่องไว อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ซึ่งแสดงค่าร้อยละการยับยั้งเท่ากับ 26.40 12.34 และ 17.99 ส่วนสารสกัดผักติ้ว ผักแขยง และผักแพวที่ความเข้มข้น 100 μg/mL แสดงค่าร้อยละการยับยั้งเท่ากับ 35.60 29.00 และ 28.30 ตามลำดับ

ผักจิกแสดงความเป็นพิษต่อไรทะเล เซลล์เม็ดเลือดขาวปกติ เซลล์มะเร็งเต้านมสายพันธุ์ MCF7 เซลล์มะเร็งตับสายพันธุ์ HepG2 โดยมีค่า IC_{50} ใกล้เคียงกันซึ่งอยู่ระหว่าง 112.70-196.40 μg/mL อย่างไรก็ตาม ผักจิกและผักมะตูมแขกมีความเป็นพิษที่จำเพาะต่อเซลล์มะเร็งเม็ดเลือดขาวสายพันธุ์ Jurkat ซึ่งมีค่า IC_{50} 66.71 และ 75.36 μg/mL ตามลำดับ คุณสมบัติในการทำให้เซลล์เม็ดเลือดแดงแตกของสารสกัดผักจิก และผักมะตูมแขก อาจจะสัมพันธ์กับความเป็นพิษต่อเซลล์ สารสกัดผักติ้วแสดงความเป็นพิษที่เด่นชัดต่อไรทะเลที่ค่า IC_{50} 37.00 μg/mL สารสกัดผักเม็กเป็นพิษต่อเซลล์มะเร็งสายพันธุ์ MCF7 ที่ค่า IC_{50} 66.71 μg/mL และมีผลเล็กน้อยต่อเยื่อหุ้มเซลล์เม็ดเลือดแดง

สารสกัดผักเม็กชักนำให้เกิดการตายแบบอะพอพโทซิสของเซลล์มะเร็งเต้านมมนุษย์สายพันธุ์ MCF7 ดังที่พบการแตกหักของนิวเคลียส (Hoechst) มีค่า EC_{50} เท่ากับ 136.60 118.20 และ 112.80 µg/mL ที่ 12 24 และ 48 ชั่วโมง ตามลำดับ และพบรูปแบบการแตกหักของดีเอ็นเอแบบขั้นบันไดเมื่อแสดงโดยอะกาโรสเจลอิเล็กโทรโฟไรซิส การวิเคราะห์โดยวิธีโฟลไซโทเมทรี แสดงให้เห็นว่า สารสกัดผักมะตูมแขกกระตุ้นการตายแบบอะพอพโทซิสผ่านวัฏจักรเซลล์ของเซลล์สายพันธุ์ MCF7 โดยหยุดยั้งการเจริญของเซลล์ที่ระยะ G_0/G_1 ขณะที่สารสกัดอื่นๆ ทำให้ตายแบบไม่ขึ้นกับวัฏจักรเซลล์ การหลุดของไซโทโครมซีจากไมโทคอนเดรีย พบในสารสกัดทุกชนิด โดยพบมากในสารสกัดผักเม็กที่ความเข้มข้น 200 µg/mL มีการหลุดของไซโทโครมซีเท่ากับร้อยละ 39.29 และร้อยละ 71.94 ที่ 12 และ 24 ชั่วโมง ตามลำดับ สารสกัดผักทั้ง 6 ชนิดยับยั้งการเจริญเติบโตของเซลล์ MCF7 ผ่านกลไกการตายแบบไม่ขึ้นกับเอนไซม์คาสเพสเป็นหลัก ซึ่งตรวจสอบได้จากการตัดของโปรตีน PARP แบบไม่ปกติ (~ 60 kDa) ซึ่งอาจจะเป็นการทำงานของเอนไซม์ย่อยโปรตีน เช่น คาลเพนและคาเทปซิน การศึกษาครั้งนี้ทำให้ได้ข้อมูลเชิงลึกของผักพื้นบ้านไทยโดยเฉพาะผักเม็กซึ่งอาจใช้บริโภคสำหรับเป็นแหล่งของสารต้านอนุมูลอิสระและสารป้องกันการเกิดมะเร็ง

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาชีววิทยา
ปีการศึกษา 2554

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JIRAYUS WORARATPHOKA : THE ANTIOXIDATION, PROLIFERATION AND APOPTOSIS EFFECTS OF SOME LOCAL THAI VEGETABLE EXTRACTS ON HUMAN CANCER CELL LINES. THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D. 209 PP.

LOCAL THAI VEGETABLE/ PHENOLIC COMPOUNDS/ ANTIOXIDANT/ CYTOTOXICITY/ CELL DEATH

Dietary phytochemicals have been of interest as source of active antioxidants as well as chemopreventive agents. Six local Thai vegetable extracts of *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) were investigated for phytochemicals availability, antioxidative properties, cytotoxicity effects and the pathway of cell death. The results revealed that BaLE, SgLE, and StLE contained high TPC, mainly tannin phenolic, but low in TFC, whereas CfLE, LaLE, and PoLE were rich in TFC. TPC of BaLE, SgLE, and StLE were 377.28 ± 11.57, 303.70 ± 19.18, and 438.73 ± 14.06 µg GAE/mg and TFC of CfLE, LaLE, and PoLE were 251.36 ± 1.83, 112.35 ± 0.50, 117.30 ± 0.68 µg CAE/mg, respectively. The antioxidant of BaLE, SgLE, and StLE exhibited similar properties to catechin, trolox, and ascorbic acid and significantly higher than that of the other three extracts ($p < 0.05$) in ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, and inhibition of lipid peroxidation activities. In cell-base antioxidant assay, BaLE and StLE at 20 µg/mL, and StLE at 25 µg/mL elicited significant reduction ($p < 0.05$) of ROS production with 26.40%, 12.34%, and 17.99% inhibition, whereas LaLE, PoLE, and CfLE at 100 µg/mL was 35.60%, 29.00%, and 28.30%, respectively.

BaLE demonstrated cytotoxic against *Artemia salina*, human normal lymphocytes, MCF7 human breast cancer, and HepG2 human liver cancer cell lines with similar IC_{50} values ranging from 112.70-196.40 µg/mL. However, BaLE and StLE had more selective inhibitory effects on Jurkat human leukemia cells with IC_{50} values of 66.91 and 75.36 µg/mL, respectively. The lytic properties of BaLE and StLE on erythrocytes might relate to their cytotoxic effects. CfLE demonstrated cytotoxicity prominently on *A. salina* with IC_{50} values of 37.00 µg/mL. SgLE was toxic on MCF7 cells with IC_{50} values of 66.71 µg/mL, and had a slight effect on erythrocyte membrane.

SgLE actively induced apoptotic cell death on MCF7 cells as observed in the fragmented nuclei (Hoechst) with EC_{50} values of 136.60, 118.20, and 112.80 µg/mL at 12, 24, and 48 h, respectively and the pattern of DNA ladder shown by agarose gel electrophoresis. Flow cytometry study revealed that StLE could trigger cell cycle apoptotic cell death of MCF7 cells (G_0/G_1 cell cycle arrest), while the others were cell cycle independence. The release of cytochrome c was observed in all extracts, highly in SgLE 200 µg/mL with 39.29% and 71.94% at 12 h and 24 h, respectively. The 6 extracts inhibited MCF7 cell growth prominently *via* caspase-independent cell death, as observed by the atypical cleaved of poly-(ADP)-ribose-polymerase (PARP) (~ 60 kDa) which might be the action of proteolytic enzyme such as calpain and cathepsin. These studies provide the in depth data on local Thai vegetables, especially SgLE, which may possibly be consumed as sources of potential antioxidant and chemopreventive agents.

School of Biology

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มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS

µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolar
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BaLE	*Barringtonia acutangula* leaf ethanolic extract
Bp	Basepair
BSA	Bovine serum albumin
CAE	Catechin equivalent
CfLE	*Cratoxylum formosum* leaf ethanolic extract
CI	Confidence interval
CLS	Cell line services
DI water	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
EC_{50}	Half maximal effective concentration or Median effective concentration
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum

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LIST OF ABBREVIATIONS (Continued)

FITC	Fluorescein isothiocyanate
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IC_{50}	Half maximal inhibitory concentration or Median inhibition concentration
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
LaLE	*Limnophila aromatica* leaf ethanolic extract
LD50	Median lethal dose or Lethal concentration for 50% mortality
min	Minute
mL	Milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NaCl	Sodium chloride
Na_2HPO_4	Sodium phosphate dibasic
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
PoLE	*Polygonum odoratum* leaf ethanolic extract
Rf	Retention factor
RNase	Ribonuclease
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute number 1640
SD	Standard deviation

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SgLE	*Syzygium gratum* leaf ethanolic extract
StLE	*Schinus terebinthifolius* leaf ethanolic extract
TFC	Total flavonoid contents
TLC	Thin layer chromatography
TPC	Total phenolic contents
TRE	Trolox equivalent
v/v	volume by volume
w/v	weight by volume

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CHAPTER I

INTRODUCTION

1.1 Introduction

Cancer is one of the major causes of death worldwide including Thailand. In 2010, the highest incidence of cancer in Thai male fell into bronchus and lung cancer followed by colon and rectum and liver and bile duct whereas in Thai women, it was breast cancer followed by cervix uteri and colon and rectum (National Cancer Institute, www, 2010).

The epidemiological evidence suggests that diet is a significant factor in the development of cancer, high consumption of fruits and vegetables significantly decrease the risk of cancer and other chronic diseases (Steinmetz and Potter, 1996). Furthermore, the additive and synergistic effects of whole-food consumption are considered for more beneficial effects (Liu, 2004). It has been recognized that phytochemicals including flavonoids and terpenoids in diets have a great deal of attention on their antioxidative roles and also their anticancer roles (Aggarwal and Shishodia, 2006).

Cancer development is a multistage processes, consists of at least three processes: initiation, promotion, and progression, in which a normal cell is initiated and transformed into a malignant phenotype (uncontrolled proliferation). Chemoprevention could intervene on this sequence of events in multiple mechanism of action which are mainly divided into 2 categories: blocking and suppressing mechanisms. Blocking mechanism prevents the DNA damage during initiation phase, and also prevents further DNA damage during the promotion and progression phases that results from reactive oxygen species (ROS) or carcinogens. Suppressing mechanism could halt or slow down the growth of tumor cell by

inducing the apoptosis, modulation of cell cycle arrest, and inhibition of angiogenesis (Manson, 2003). Chemoprevention which involves the use of pharmacological, phytochemicals, and whole plant extracts has been proposed to prevent or arrest the cellular and molecular process of carcinogenesis in both mechanisms.

Excessive of ROS, includes superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), with the cellular antioxidant depletion, lead to the biological stress and can subsequently damage the crucial molecules such as proteins, lipids, carbohydrate and can cause DNA damage leading to mutation (Waris and Ahsan, 2006). Thus, ROS has been widely accepted as a major cause of cancer and several degenerative diseases. To prevent cancer, antioxidants are needed to operate during harmful action by blocking the accumulative effects of ROS. The potential antioxidant can react by directly scavenging ROS, chelating metal ions (the catalyzer of lipid oxidation), and upregulation of cellular antioxidant defense system (Halliwell and Gutteridge, 1999).

Induction of cell death is one of the potential useful strategies for eliminating tumor cells. Key feature would be the ability to compromise only tumor cells but not normal cells. Apoptosis or programmed cell death is a controlled mechanism for removing redundant, infected, or damaged cells without giving the inflammatory response. Cells undergo apoptosis show typical morphological changes including chromatin condensation, membrane blebing, and formation of apoptotic bodies that allows for removing by macrophages. Several anticancer agents and dietary phytochemicals have been reported to activate the apoptotic cell death by regulating on multiple genes and proteins involved in apoptosis (Aggarwal and Shishodia, 2006). Understanding of the molecular mode of cell death will support the selection of appropriate agents or extracts for study in an *in vivo* animal model and further on clinical trials.

In recent years, the great attentions have been investigated on natural antioxidant and anticancer agent due to the safety and compatibility with human physiology. Furthermore,

the use of total plant extracts has also been investigated because of the synergistic effects of the combination in plant phytochemicals and the multiple points of intervention in chemoprevention (Neergheen, Bahorun, Taylor, Jen, and Aruoma, 2010). Tropical plants have been recognized as an abundant source of highly effective phytochemicals. Six local Thai vegetables, *Barringtonia acutangula*, phak jig; *Cratoxylum formosum*, phak tiew; *Limnophila aromatica*, phak khayaeng; *Polygonum odoratum*, phak paew; *Syzygium gratum*, phak mek; and *Schinus terebinthifolius*, phak matumkhaeg, are widely grown in the Northeast of Thailand. They are of our interest to investigate since their young leaves have been commonly consumed as vegetables in traditional daily diet. Moreover, some of them are used as dietary plants in Southeast Asian countries and claimed for health promotion in traditional treatment of various symptoms, such as inflammation, asthma, hypertensive, indigestion, laxative, peptic ulcer, diarrhea, and antibacterial infection (Panthong, Kanjanapothi, Taesotikul, and Taylor, 1991). The leave extracts of these plants have been reported to possess antioxidant (Rahman et al., 2010; Bureemas, Kukongviriyapan, Kukongviriyapan, Senggunprai, and Jetsrisuparb, 2007; Maisuthisakul, Suttajit, and Pongsawatmanit, 2007), antimicrobial (Lokaewmanee, Chandang, Khumpeerawat, and Jantajaroonpong, 2009; Phongmanee and Sanampol, 2007; Martínez, Bentancourt, Alonso-González, and Jauregui, 1996), antifungi (Johann, Pizzolatti, Donnici, and Resende, 2007), anti-inflammatory (Turntipopipat, Muangnoi, and Failla, 2009), and antimutagenic activities (Nakahara et al., 2002). Crude extracts from the leaves of *C. formosum*, *S. gratum* and *L. aromatica* had antioxidant and vascular protective activities *in vitro* and *in vivo* (Kukongviriyapan, Luangaram, Leekhaosoong, Kukongviriyapan, and Preeprame, 2007). The antitumor properties and cytotoxicity on cancer cells have been reported in some of these vegetables. The methanolic extract of *L. aromatica* and *P. odoratum* had potent antitumor-promoting activity determined by Epstein-Barr virus activation assay (Murakami, Jiwajinda, Koshimizu, and Ohigashi, 1995). Crude extract from leaves of *C. formosum*

showed cytotoxic effect on HepG2 liver cancer cell line without giving any harm to the normal cell (Prayong, Barusrux, and Weerapreeyakul, 2008). Moreover, Nanasombat and Teckchuen (2009) demonstrated that *P. odoratum* leave extract had an active cytotoxic effect against MCF7 breast cancer proliferation. In addition, the polyphenols, isoquercitrin, purified from *S. terebinthifolius* leaves inhibit cell proliferation by induction of cell cycle arrest, apoptotic and autophagic cell death of the androgen-insensitive DU145 prostate carcinoma cells (Queires et al., 2006). There is little information available at the present time regarding their cytotoxic or anticancer effects of the leaves of six local Thai vegetables, especially on the molecular mechanism of cell death.

1.2 Research objectives

The main objectives of this study are as followings:

1.2.1 To determine total phenolic contents (TPC), total flavonoid contents (TFC), and phytochemicals availability of six local Thai vegetable extracts.

1.2.2 To determine the antioxidative properties of six local Thai vegetable extracts on chemical and cell-base assay.

1.2.3 To investigate the cytotoxic effects of six local Thai vegetable extracts on *Artemia salina*, human normal lymphocytes, erythrocytes and three cancer cell lines: Jurkat leukemia, MCF7 breast cancer and HepG2 liver cancer cell line.

1.2.4 To observe the effects of six local Thai vegetable extracts on apoptotic induction of MCF7 human breast cancer cell line.

1.3 Research hypotheses

1.3.1 Six local Thai vegetable extracts contain high total phenolic contents and total flavonoid contents as well as the active phytochemicals.

1.3.2 Six local Thai vegetable extracts have high antioxidative activities as compared to standard antioxidants.

1.3.3 Six local Thai vegetable extracts are toxic to cancer cells, but show less cytotoxic effect in normal cells.

1.3.4 Six local Thai vegetable extracts have the antiproliferative activity and apoptotic induction properties on human MCF7 breast cancer cell line.

1.4 Scope and limitations of the study

Six local Thai vegetables were purchased from local markets in Nakhon Ratchasima, during October to February of 2008-2009. Phytochemical availability was observed by thin layer chromatography and testing with specific reagents. Antioxidant activities of six local Thai were determined by chemical and cell-base assays (using HepG2 human liver cancer cell line). Cytotoxicity was investigated on human normal lymphocytes, erythrocytes and three cancer cell lines. MCF7 breast cancer cell line was used as a model in observing the effects of six local Thai vegetable extracts to inhibit cell proliferation and induce apoptosis. Apoptosis was assessed by DNA fragmentation in chromatin staining, DNA ladders formation in agarose gel, increasing the subG_0 phase in cell cycle, and reduction of the intensity of intact cytochrome c in flow cytometry. Apoptotic proteins were observed using Western blot assay.

1.5 Expected results

1.5.1 The outcomes from the study of phytochemicals and antioxidative properties of six local Thai vegetables can provide the valuable information for supporting the benefit of these plants and the relation between phytochemicals and antioxidative activities.

1.5.2 The cellular and molecular data of anticancer properties provide the putative mechanism on which the vegetables induce for apoptosis in cancer cells. This can be applicable for cancers that could be prevented and cured by local Thai vegetables.

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มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER II

LITERATURE REVIEWS

2.1 Phytochemicals in dietary plants

It has been recognized that healthy diets link with biological and physiological benefits. Epidemiological studies have been evidently reported that high consumption of polyphenol-rich foods such as fruits and vegetables associate with the lower risk of cancers and other degenerative disease (Steinmetz and Potter, 1996). Apart from the antioxidant vitamins and minerals these diets contain a mixture of phytochemicals such as polyphenol, terpenoids and other natural antioxidant which attributed for the antioxidative and anticancer properties (Liu, 2004).

Phytochemicals are biologically-active, non-nutritive secondary metabolites which come from various plant families and variety in chemical constituents. The classification of phytochemicals is still debated, but can be classified as alkaloid, phenolics, terpenoids, and organosulfur compounds (Liu, 2004) as shown in Figure 2.1. The most found and studied of the phytochemicals are the terpenes and phenolic compounds.

Terpenes are simple classified by the number of five-carbon isoprene units which are hemiterpenes C_5, monoterpenes C_{10}, sesquiterpenes C_{15}, and diterpenes C_{20}, triterpenes C_{30} and tetraterpenes C_{40} (e.g. carotenoids).

Phenolic compounds constitute a large class of compounds in which a hydroxyl group (-OH group) is bound to an aromatic ring. More than 8,000 compounds are known with different chemical structures and activities. The main classes of phenolic in diet are shown in Figure 2.1.

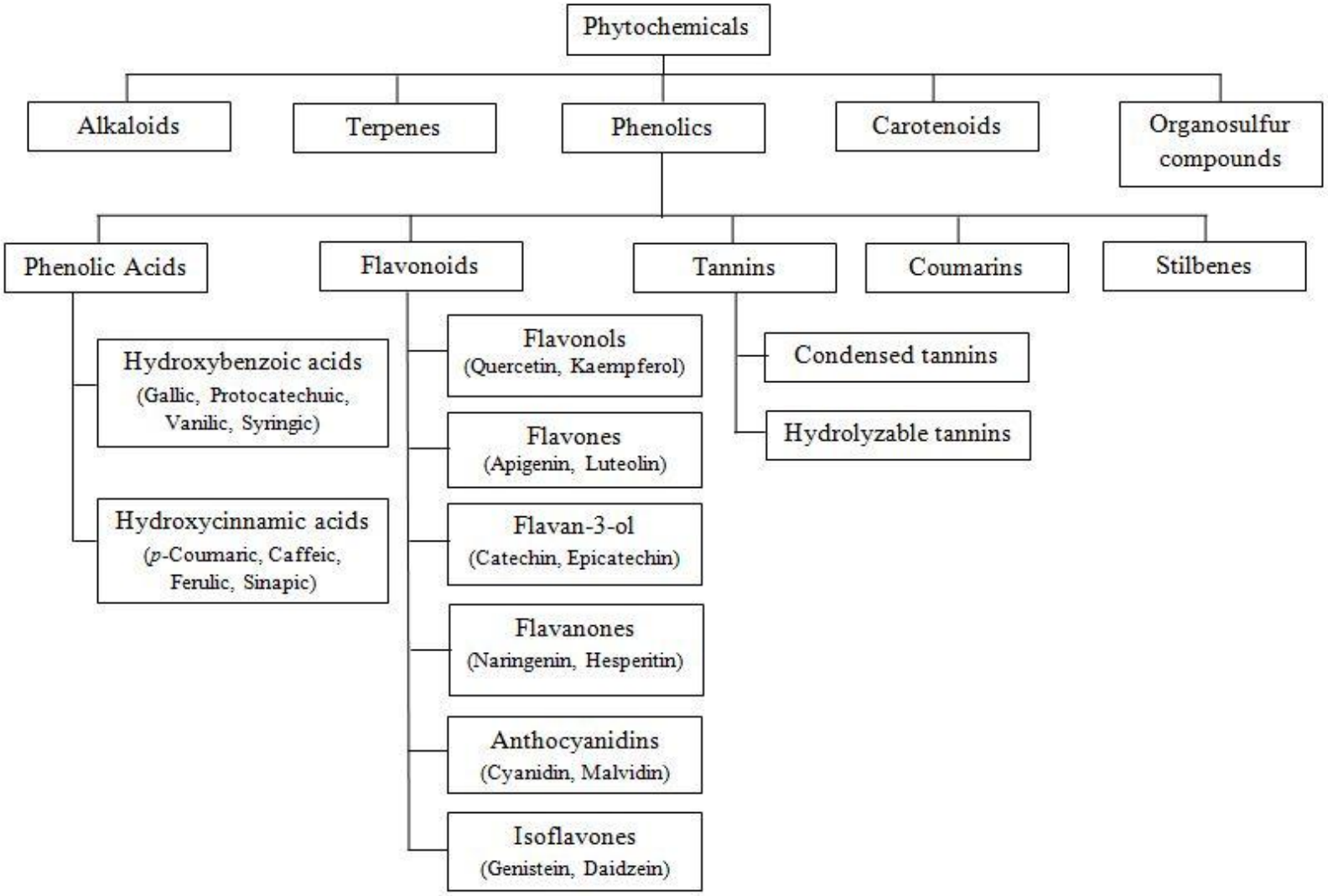


Figure 2.1 Classification of dietary phytochemicals (adapted from Liu, 2004).

2.2 Carcinogenesis and mechanism for interventions

Carcinogenesis is a multistage processes, consists of at least three processes: initiation, promotion, and progression, in which a normal cell is transformed into a lineage with a malignant phenotype. Initiation is defined as an irreversible modification of the target cell DNA whereas promotion represents the expansion of initially damaged or mutated cells to form a clone of an actively proliferating multicellular premalignant/benign tumor cell population, which eventually progresses to the malignant cells characterized by increased invasiveness and metastatic potential. Fortunately, this sequence of events presents many opportunities for intervention (Figure 2.2). The targets for chemoprevention could be multiple and varied from the initiation to progression phases. *In vitro* studies elucidating the mechanisms of action of putative chemopreventive agents can be divided into 2 categories: blocking agents and suppressing agents (Manson, 2003). Chemoprevention which involves the use of pharmacological, phytochemicals and whole plant extracts can block the initiation of carcinogenesis and also suppress or inhibit the growth of transformed cells into malignant ones.

2.2.1 Blocking initiation or other DNA-damaging events

The most effective way of preventing cancer is to block its initiation by preventing the DNA damage that results from reactive oxygen species (ROS) or carcinogens. Many dietary phytochemicals possess antioxidant properties which can directly scavenged ROS or induced the cellular defense enzymes (Table 2.1). Some of them prevent carcinogens to attack the cellular targets by enhancing carcinogen detoxification. They altered the metabolism of procarcinogenic molecules that either they are not converted to carcinogenic species by phase-I drug-metabolizing enzymes (particularly cytochrome P450) or they are safely removed from the cell by a secondary line of defense that involves phase-II conjugating enzymes (e.g. glutathione-S-transferases (GST), glucuronidases and sulphotransferases). Blocking mechanisms could also be

effective in slowing down the later stages of cancer progression and in preventing the development of second primary tumors following removal of the initial cancer (Manson, 2003).

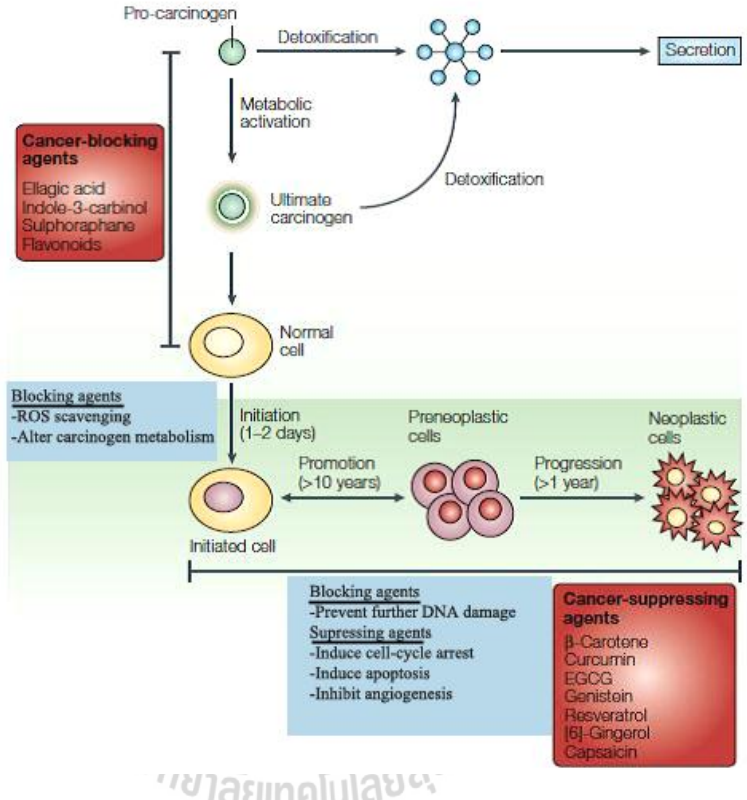


Figure 2.2 Potential of chemopreventive agents for intervention in carcinogenesis. Carcinogenesis is initiated with the transformation of the normal cell into cancer cells (initiated cell) then promotion and progression into neoplastic cells. Dietary phytochemicals can block or suppress multistage of carcinogenesis. (adapted from Surh, 2003).

Table 2.1 Possible mechanisms of phytochemicals on cancer prevention (Blocking mechanism) (adapted from Huang, Cai, and Zhang, 2010).

Mechanisms of Action	Phytochemicals (Representative compounds)
Antioxidant activity	
Scavenging free radicals and ROS, such as superoxide anions, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide and peroxynitrite; increase SOD activities; decrease lipoperoxidation.	Phenolic acids and analogs (caffeic acid, gallic acid, chlorogenic acid, and ellagic acid), flavonoids (catechin and quercetin), tannins (proanthocyanidins, corilagin), stilbenes (resveratol), curcuminoids, coumarins, lignans, quinones, others. Saponins, terpenoids.
Enzyme inhibition	
Phase I enzyme (block activation of carcinogens); COX-2; iNOS; XO; signal transduction enzymes, such as PKC and PTK; topoisomerase I and II; telomerase; urease; lipase; angiotensin I-converting enzyme; DNA methyltransferases (consequent reactivation of key tumor suppressor gene p16).	Phenolic acids and analogs (chlorogenic acid, caffeic acid, ellagic acid, and hydroxytyrosol), flavomoids and analogs (apigenin, luteolin, quercetin, and EGCG), tannins (proanthocyanidins, corilagin), stilbenes (resveratrol), curcuminoids (curcumin), coumarins, lignans (podophyllotoxin), and quinones.
Enzyme induction and enhancing detoxification	
Phase II enzymes, such as UDP-glucuronosyl transferase and quinine reductases; glutathione peroxidase; catalase; SOD; cytochrome P450 epoxide hydrolase; NADPH: quinone reductase.	Phenolic acids and analogs (protocatechuic acid and ellagic acid), flavonoids (hesperidin and anthocyanins), tannins, stilbenes (resveratrol), curcuminoids (curcumin), lignans, and quinones.

ROS, reactive oxygen species; SOD, superoxide dismutase; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; XO, xanthine oxidase; PKC, protein kinase C; PTK, protein tyrosine kinase, UDP, uridine diphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; EGCG, epigallocatechin gallate.

2.2.2 Suppressing mechanism

Because tumors result from an imbalance between proliferative and apoptotic processes. They are characterized as uncontrolled proliferation, any mechanism that halts or slows down inappropriate cell division, or that induces damaged cells to undergo apoptosis, is potentially useful. A key feature would be the ability to compromise only tumor cells but not normal cells. Many dietary phytochemicals such as phenolic acids, flavonoids, tannins and terpenes have been demonstrated the effective capacity in suppressing mechanisms by regulation of cell cycle arrest, induction of apoptosis as shown in Table 2.2 and inhibition of angiogenic process (Ren, Qiao, Wang, Zhu, and Zhang, 2003).

Table 2.2 Possible mechanisms of phytochemicals on cancer prevention (Suppressing mechanism) (adapted from Huang et al., 2010; Rabi and Bishayee, 2009; Singh, Bhat, Singh, 2003).

Mechanisms of Action	Phytochemicals (Representative compounds)
Induction of cell-cycle arrest and induction of apoptosis	
Inhibit different cell cycle at different cell phases: G1, S, S/G2 and G2; direct or indirect effect on cell cycle arrest; subsequently induce apoptosis, involving p53, the Bcl-2, and caspase families.	Phenolic acids (ferulic acid, caffeic acid and its phenethyl ester, ellagic acid, and hydroxytyrosol), flavonoids (quercetin and EGCG), tannins (proanthocyanidins, gallotannin and curcuminoids (curcumin), coumarins (coumarin and 7-hydroxycoumarin), lignans (sesamin), quinones, and others (eugenol). Monoterpene (*d*-limonene and perillyl alcohol), diterpenes (retinoic acid), triterpenes (asciatic acid, pristimerin, withaferin A, and betulinic acid), tetraterpenes (β-carotene, lycopene, and lutein), saponins

EGCG, epigallocatechin gallate.

2.3 Reactive oxygen species (ROS) and antioxidants

2.3.1 Major types of free radicals in living organisms

The diatomic molecule of oxygen contains two uncoupled electrons and can therefore undergoes reduction, yielding several different oxygen metabolites which are called reactive oxygen species (ROS), for example, superoxide anion (O_2^-), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are invariably generated in cells by several pathways. ROS normally exist in all aerobic cells in balance with efficient biochemical antioxidant mechanism such as superoxidase dismutase (SOD), glutathione (GSH) peroxidase and catalase (Figure 2.3).

Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidants depletion, or both. These biological stresses can initiate the peroxidation of polyunsaturated fatty acids in biological membranes leading to accumulation of lipid peroxide. They are also capable of damaging crucial molecules such as DNA, proteins, lipids, and carbohydrate and can cause DNA damage that can lead to mutation. Excessive of ROS may contribute to diverse pathologies, such as Alzheimer s disease, atherosclerosis, diabetes, Parkinson’s disease and cancers (Waris and Ahsan, 2006; Halliwell, 1997).

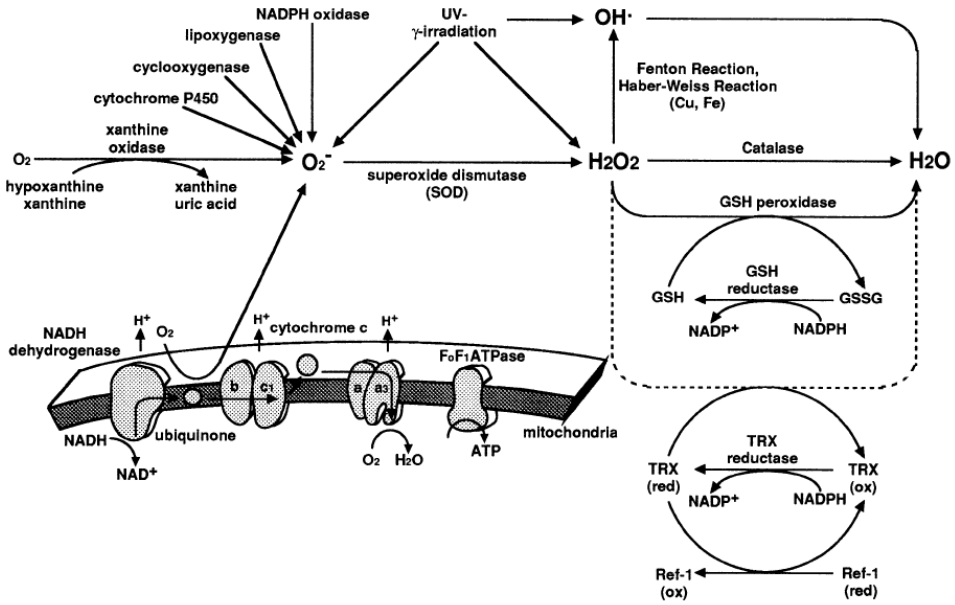


Figure 2.3 Metabolic mechanisms of reactive oxygen species (ROS). ROS; H_2O_2, O_2^- and $OH^{\cdot}$ are generated in cells by several mechanisms such as byproduct from mitochondria, distinct enzyme systems and UV radiation. Superoxide dismutase converts O_2^- into H_2O_2, and then H_2O_2 is mostly degraded to H_2O by glutathione (GSH) peroxidase and catalase (Kamata and Hirata, 1999).

2.3.2 ROS related to cancer

DNA damaged by ROS has been widely accepted as a major cause of cancer. The changes in DNA such as base modification, rearrangement of DNA sequence, miscoding of DNA lesion, gene duplication and activation of oncogenes may be involved in the initiation of various cancers. ROS can cause DNA lesion and when the cell divides with an unpaired or misrepaired DNA, these lead to mutations. The majority of mutations induced by ROS appear to involve modification of quinine, causing G-T transversion (Waris and Ahsan, 2006; Jackson and Loeb, 2001). If DNA damage relates to critical genes such as oncogenes or tumor suppressor genes, the result of initiation/progression can occur (Ames, Shigenaga, and Gold, 1993). Since, ROS can act at several steps in multistage of

carcinogenesis. It is now assumed that ROS are potential carcinogens which involved in initiation, promotion and progression of cancers (Moller and Wallin, 1998).

2.3.3 ROS and antioxidants

2.3.3.1 Cellular antioxidants

ROS involving in both initiation and promotion of carcinogen, are minimized by the antioxidant capacity and repair mechanism within the cells. Antioxidants are substances that deactivate or prevent the oxidation of cellular oxidizable substrates. The body has an effective antioxidative system includes non-enzymatic and enzymatic systems for eliminating the harmless. Antioxidants which constitute the defence system *in vivo* can be classified into preventive antioxidant and free-radicals scavenging antioxidant as shown in Table 2.3.

1) Preventive antioxidants

Antioxidant enzymes, including catalase, glutathione peroxidase, peroxidase, and glutathione-S-transferase, are the first defense line for suppressing ROS formation by decomposition of hydrogen peroxide (H_2O_2) and hydropeoxides into non-reactive oxygen molecules. Catalase decomposes H_2O_2 to water and oxygen. Glutathione peroxidase catalyses the reduction of H_2O_2 and lipid hydroperoxide, generated during lipid peroxidation, to water using reduced glutathione as substrate. Sequestrants or chelating agents are capable to bind with transition metal ions in the forms that will not stimulate free radical formation. Transferrin and lactoferin, can block the pro-oxidant metal ions, and thus reduce the formation of chain initiator. Antioxidants, superoxide dismutase (SOD) mainly acts by quenching of superoxide (O_2^-). Carotenoids and vitamins are an excellent quencher of singlet oxygen (Shi, Noguchi, and Niki, 2001).

Table 2.3 Defense systems *in vivo* against oxidative damage (adapted from Shi et al., 2001).

1. Preventive antioxidant: suppress the formation of free radicals.	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide:	
- Catalase	decomposition of hydrogen peroxide $2H_2O_2 \longrightarrow 2H_2O + O_2$
- Glutathione peroxidase	decomposition of hydrogen peroxide and Free fatty acid hydroperoxide $H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$ $LOOH + 2GSH \longrightarrow LOH + H_2O + GSSG$
- Peroxidase	decomposition of hydrogen peroxide and lipid hydroperoxides $LOOH + AH_2 \longrightarrow LOH + H_2O + A$ $H_2O_2 + AH_2 \longrightarrow 2H_2O + A$
- Glutathione-S-transferase	decomposition of hydrogen peroxides
(b) Sequestration of metal by chelation:	
- Transferrin, Lactoferin	sequestration of metal ions
(c) Quenching of active oxygens:	
- Superoxide dismutase (SOD)	disproportionation of superoxide $2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$
- Carotenoids, vitamin E	quenching of singlet oxygen
2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation.	
Hydrophilic: vitamin C, uric acid, bilirubin, albumin	
Lipophilic: vitamin E, ubiquinol, carotenoids, flavonoids	

GSH, reduced glutathione; GSSG, glutathione disulfide; LOOH, lipid peroxide; AH_2, oxidized molecule; $O_2^{\bullet-}$, singlet oxygen species.

2) Radical scavenging antioxidant

The radical-scavenging antioxidants: vitamin C, vitamin E, function as the second line of defense which directly scavenge the free radicals and inhibit chain initiation and/or break the chain propagation. Vitamin C interacts directly with radical such as O_2^-, $OH^{\cdot}$. Vitamin E scavenges peroxyl radical intermediates in lipid peroxidation and responsible for protecting poly unsaturated fatty acid (PUFA) present in cell membrane and low density lipoprotein (LDL) against lipid peroxidation.

2.3.3.2 Dietary antioxidants

The additional source of antioxidant compounds, phytochemicals, from diets is an important factor in additive and synergetic effects for health protection. Natural antioxidants have received considerable interest because of their safety and potential nutritional and therapeutic effects (Steinberg, 1991). Dietary phytochemicals in fruits and vegetables, flavonoids (flavones, isoflavones, flavonones, anthocyanins and catechin), tannins (gallic and ellagic tannins), possess remarkable antioxidants activity (Table 2.1). Phytochemicals, play an importance role for preventing the formation of oxidative stresses by (1) directly scavenging ROS; (2) chelating trace elements; and (3) upregulating the cellular antioxidant defense systems (Halliwell and Gutteridge, 1999).

The antioxidant activity of flavonoids depends on their chemical structure. Generally, there are three structure groups in the determination, the free radical scavenging and/or antioxidative potential of flavonoids (Figure 2.4): (a) a catechol moiety of the B-ring, (b) the 2,3-double bond in conjugation with a 4-oxofunction of a carbonyl group in the C-ring and (c) presence of hydroxyl groups at the 3 and 5 position. Flavonoids can also bind to chelate irons, due to their chemical structures (Pietta and Simonetti, 1999). Quercetin, flavonols, possesses all the three structure groups and thus usually gives a higher antioxidant potential both in free radical scavenging activity and metal-induced lipid peroxidation (Sugihara, Arakawa, Ohnishi, and Furuno, 1999).

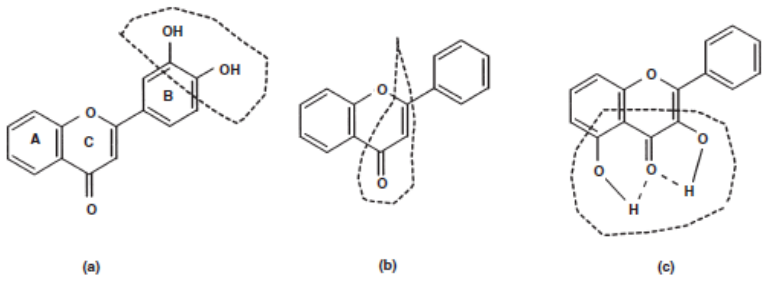


Figure 2.4 Antioxidant activity-structure relationships of flavonoids (Shi et al., 2001).

Tannins are powerful free radical antioxidant activity which more effective than flavonoids (Yokozawa et al., 1998), because they have many hydroxyl groups, especially many *ortho*-dihydroxyl or galloyl groups. Bigger tannin molecules possess more galloyl and *ortho*-dihydroxyl groups and stronger in their activities (Yokozawa et al., 1998).

2.4 Programmed cell death (PCD)

Balance between cell division and cell death is of fundamental importance for the development of multicellular organisms and homeostasis of their tissues. The equilibrium is tightly controlled and faulty elements can effectively be eliminated by a process called "programmed cell death". Aberrant in PCD can lead to many human diseases, participating to tumor initiation and cancer development. Programmed cell death (PCD) is genetically regulated, controlled and conserved form of death for eliminating of unwanted cells. Therefore, it is a critical therapeutic interest of drug or treatment to convey PCD induction in order to act on cancer cells and thus lead to tumor regression. In past decade, PCD have been morphologically classified into three major types: Type I, known as apoptotic cell death, Type II or lysosomal cell death or autophagy, and Type III PCD, or cytoplasmic cell death (nerosis) (Figure 2.5).

Apoptosis mediated by caspases activity results in the morphological change into apoptotic bodies with no inflammatory response. Autophagy features the degradation of cellular components within the dying cell in autophagic vacuoles with no inflammation. In contrast, necrosis is accompanied by cellular and organelle swelling (oncosis) and membrane breakdown with release of inflammatory cellular contents (Fink and Cookson, 2005). Furthermore, there is now accumulating evidence indicating that cell death can occur in a programmed fashion but in complete absence and independent of caspase activation. Alternative models of PCD have therefore been proposed, including paraptosis, mitotic

catastrophe, and descriptive model of apoptosis-like and necrosis-like PCD (Bröker, Kruyt, and Giaccone, 2005).

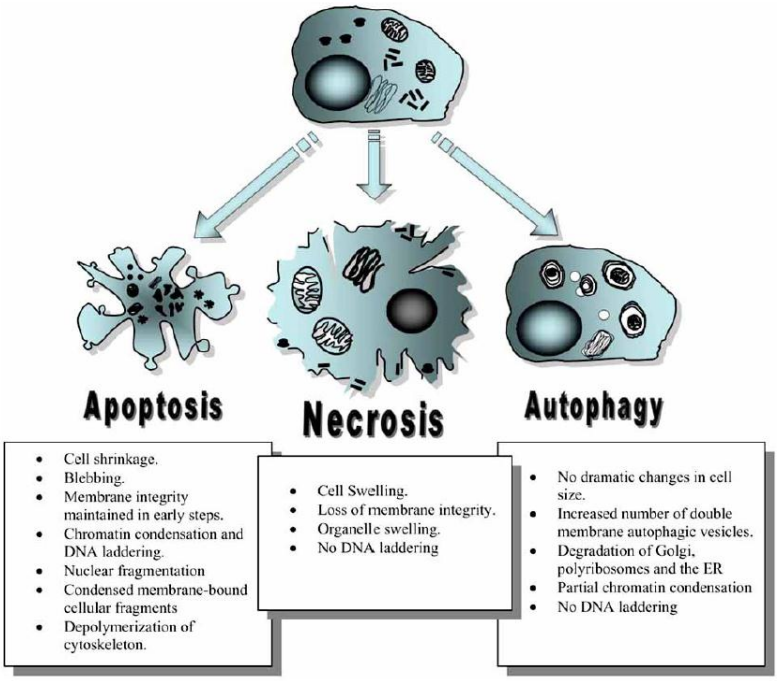


Figure 2.5 Morphological changes associated with the three modes of programmed cell death (PCD) (Henriquez, Armisén, Stutzin, and Quest, 2008).

2.4.1 Apoptotic cell death (Caspase-dependent pathway)

Apoptosis is a genetically controlled, energy-dependent process which characterized by distinct morphological changes (Figure 2.5) such as cell shrinkage, chromatin condensation, chromosomal DNA fragmentation and disintegration of the cell into small fragments (so-called “apoptotic bodies”) which then be phagocytosed by nearby cells (macrophages or epithetial cells) without associated inflammation. The process of apoptotic cell death is responded to a variety of stimuli and involved cellular mechanisms which controlled and regulated cascade. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and undergo serious health problems.

2.4.1.1 Apoptosis signaling pathway

The apoptotic cascade can be divided into two pathways which are intrinsic and extrinsic signaling pathway as shown in Figure 2.6. The intrinsic or mitochondrial pathway is activated by several of stimuli including DNA-damaging agents or cytotoxic stresses. These stress signals modulate member of Bcl-2 family such as anti-apoptotic proteins: Bcl-2, Bcl-x_L and pro-apoptotic proteins: Bax, Bak, resulting in the disruption of the mitochondrial membrane and subsequent leakage of inner proteins such as cytochrome c into the cytosol. An association of cytochrome-c, apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9 forms an apoptosome, which activates of caspase-9 and then downstream effector caspases including caspase-3, -6, and -7, leading to apoptosis.

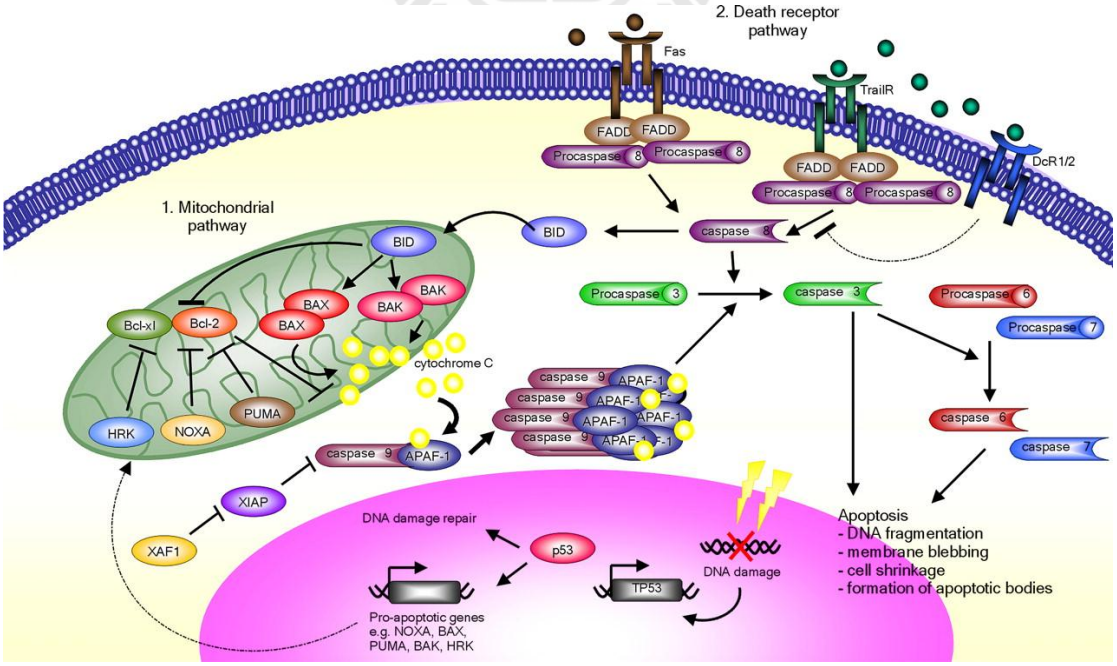


Figure 2.6 Initiation and regulation of apoptosis. The two principal known pathways of apoptosis are mediated via the mitochondria and the death receptors (e.g. Fas/APO1/CD95 and the TNF family) pathways (Murphy, Perry, and Lawler, 2008).

The extrinsic signaling pathway or death receptors, are cell surface receptors belonging to the TNF super family such as tumor necrosis factor (TNF) receptor, Fas/APO1/CD95, which trigger apoptosis upon ligand binding. Death receptors contain an intracellular death domain associates with an adaptor protein called Fas-associated death domain (FADD) directly or indirectly *via* TNF-R-associated death domain (TRADD). FADD recruits procaspase-8 and it is activated to caspase-8. The activated caspase-8 activates the downstream effector caspase-3, -6, and -7, which then cleave multiple substrates within the cells. Caspase-8 also activates the protein Bid, which translocates to mitochondria, activates Bak or Bax and leads to release of cytochrome c, in the way able to be inhibited by Bcl-2. Caspase activity is responsible, either directly or indirectly, for cleavage of cellular proteins which are characteristically proteolysed during apoptosis.

2.4.1.2 The Bcl-2 family proteins

The Bcl-2 family consists of the proteins involved in promoting or inhibiting the permeabilization and disruption of the mitochondrial outer membrane (MOM), and response to apoptotic process. The Bcl-2 family can be divided into two groups: anti-apoptotic and pro-apoptotic members (Figure 2.7). Anti-apoptotic members, such as Bcl-2 and Bcl-xL, have BH1, BH2, BH3, and BH4 domains and a carboxy-terminal hydrophobic transmembrane tail domain, which localizes the proteins to the MOM (Bras, Queenan, and Susin, 2005). Bcl-2 resides in the MOM and also endoplasmic reticulum and nuclear membranes and translocates to MOM upon an apoptotic signal, whereas Bcl-x_L resides only in the MOM. Pro-apoptotic members are further classified into multidomain and BH3-only proteins. Multidomain pro-apoptotic members, i.e. Bax and Bak have BH1, BH2, and BH3 domains and the transmembrane tail domain. BH3-only pro-apoptotic members, i.e. Bid and Bad, contain only the BH3 domain and are cytosolic. Bax localization is controversial; it is likely to reside as a monomer in cytosol, whereas Bak is

already an integral protein of the MOM (Mohamad et al., 2005). Anyhow, Bax or Bak are activated by the cleaved form of the BH3-only proteins, Bid (tBid). Bax changes its conformation, translocates, inserts into MOM, oligomerizes and promotes MOM permeabilization for cytochrome c release. Bak could also oligomerize (Bras et al., 2005). The sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti-apoptotic Bcl-2 family members. The interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins resulting in the formation of pores in the mitochondrial membrane. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, whereas an excess of anti-apoptotic proteins the cells will tend to be more resistant.

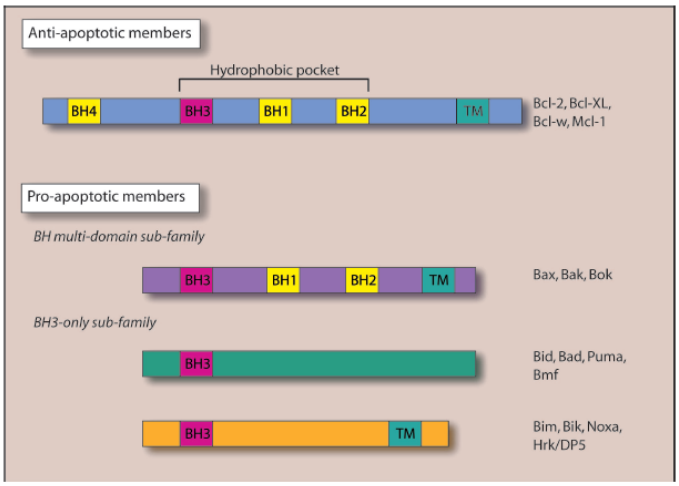


Figure 2.7 The Bcl-2 family members. The Bcl-2 family could be divided into two groups: anti-apoptotic members and pro-apoptotic members, according to their Bcl-2 homology (BH) domains. Most of the Bcl-2 family member possess a carboxy-terminal transmembrane domain (TM) implicated in their targeting to intra-cellular membrane (Bras et al., 2005).

2.4.1.3 Mitochondria and apoptogenic IMS proteins

Mitochondria are very specialized organelles containing an outer membrane (OM) separated from an inner membrane (IM) by an intermembrane space (IMS) containing many apoptosis mediated proteins. Maintenance of the permeability of the permeability of the OM, primarily through voltage dependent anion channel (VDAC), is required to keep the integrity of this membrane and to prevent the release of apoptogenic proteins from the IMS. Release of proteins form the IMS of the mitochondria triggered by permeabilization of OM constitutes the point of no return in PCD. The mechanisms of OMM permeabilization have been controversial, and there are 2 proposed models: firstly, MOM permeabilization is regulated by the Bcl-2 family proteins, and secondly, by the permeability transition pore (PTP) (Figure 2.8).

First mechanism of MOM permeabilization involves members of the Bcl-2 family of proteins, notably Bax and Bak, and t-Bid as described above in 2.4.1.2. The second involves the opening of the permeability transition pore (PTP), which consists of voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin D (CyD), as well as several other proteins. Pore opening is followed by swelling of the mitochondrial matrix, rupture of the OMM and the release of cytochrome c (Cyt c) and other apoptogenic proteins such as Smac/DIABLO, Omi/HtrA2, endonuclease G (Endo G) and apoptotic inducing factor (AIF) and from the IMS, the process can be blocked by cyclosporin A, which binds to CyD and prevents pore opening.

Cytochome c, an essential component of the cellular respiratory chain, is the first protein shown to be released from mitochondria in apoptotic induction. Upon release from mitochondria, cytochrome c complexes with Apaf-1, dATP, and procaspase-9 form apoptosome. This triggers the classic apoptotic cascade, leading to apoptotic cell death. The catalytic function of cytochrome c is safeguarded by members of the inhibitor of apoptosis protein (IAP) family such as Smac/DIABLO and Omi/HtrA2

which antagonize IAP inhibition of caspases (directly binding caspase) also release upon apoptotic stimulation.

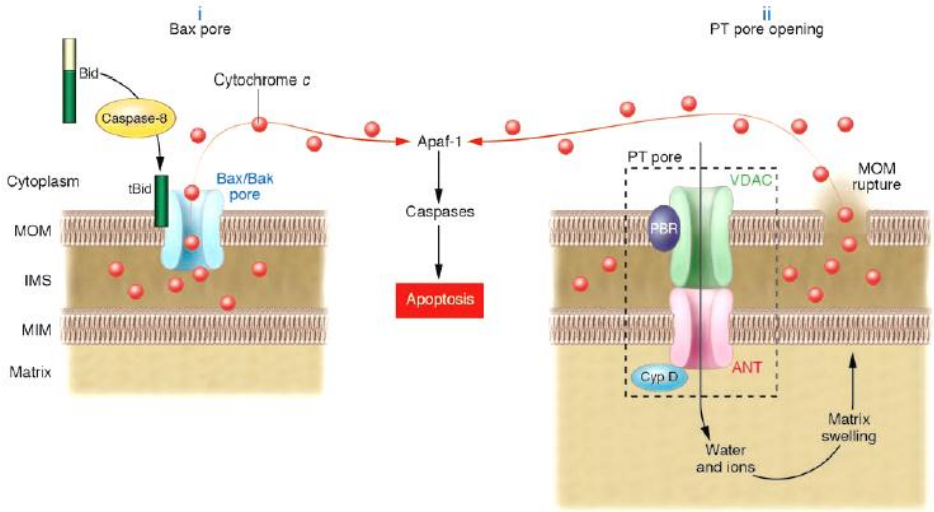


Figure 2.8 Molecular mechanism of MOM permeabilization. The proposed models of MOM permeabilisation leading to cytochrome c release are presented. (i) Bax or Bak form a pore in the MOM after activation by a BH3-only protein such as Bid. (ii) Opening of the permeability transition (PT) pore allows an influx of water and ions into matrix, causing matrix swelling; this lead to rupture of the MOM, releasing IMS proteins such as cytochrome c. (Bouchier-Hayes, Lartigue, and Newmeyer, 2005).

2.4.1.4 Role of caspases

Caspases belong to a group of enzymes known as aspartate-specific cysteine proteases and exist within the cell as inactive forms or zymogens known as procaspase. These procaspase can be cleaved to form active enzymes following the induction of apoptosis. The caspases can be devided into two subgroups based on their aspects of their activation during cell death (Figure 2.9). Initiator caspases (caspase-2, -8, -9, and, -10) are primarily responsible for initiating caspase activation cascades. Effector or

executioner caspases (caspase-3, -6, and -7) are responsible for the actual dismantling of the cell by cleaving cellular substrate.

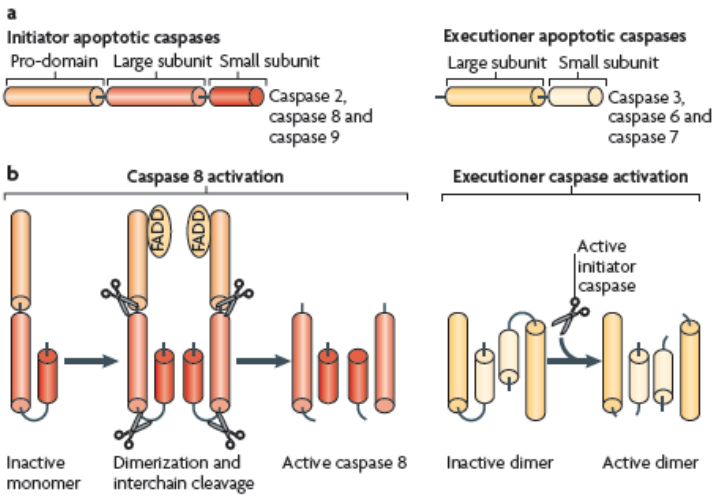


Figure 2.9 Caspase classification and activation. Apoptotic caspases can be divided into two classes: initiator and executioner caspases (a); Initiator, caspase-8, activation and executioner caspase activation (b) (adapted from Tait and Green, 2010).

Activated executioner caspases selectively cleave a restricted set of target proteins to produce the morphological and biochemical features associated with apoptosis. The caspases play an important role in this process by activating DNases, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus (Dash, www, 2011).

(a) Activation of DNase; a DNA fragmentation factor 45 kDa subunit normally exists as an inactive complex with its inhibitor (ICAD) (CFF45/ICAD). During apoptosis, ICAD is cleaved and inactivated by caspases, such as caspase-3 and -7. The active form of DFF40/CAD subunit (DNase) plays a critical role in the internucleosomal DNA degradation, generating fragments with lengths corresponding to multiple integers of approximately 180 base pairs (Figure 2.10).

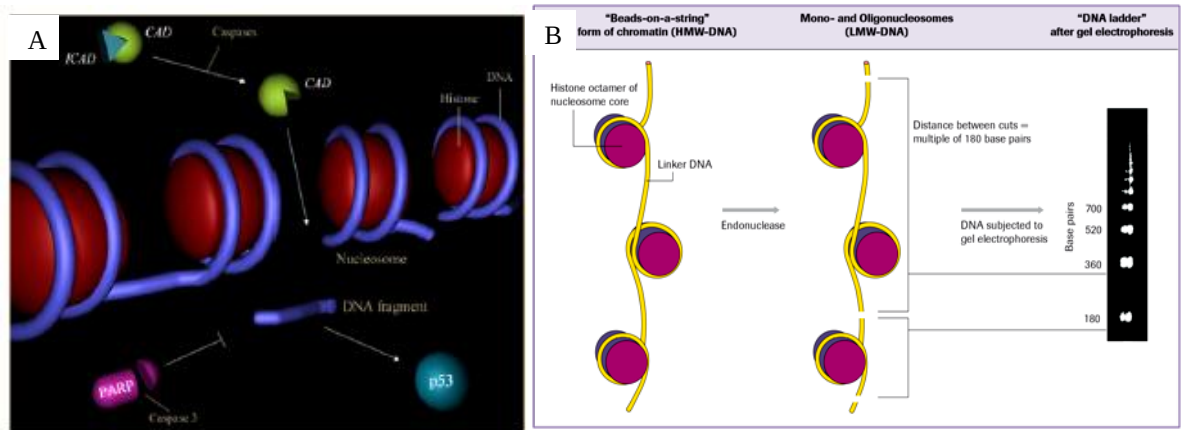


Figure 2.10 Activation of DNase. During apoptosis, ICAD (inhibitor of CAD) is cleaved by caspases to release CAD (Caspase-activated DNase), fragmentation of nucler DNA follows (A); the fragmentation of DNA into nucleosomal units as seen in DNA ladder (B) (Dash, www, 2011).

(b) Inactivation of enzymes involved in DNA repair; the enzyme poly (ADP-ribose) polymerase (PARP) is an important DNA repair enzyme known as a substrate for caspases. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3. The typical cleavage of PARP into C-terminal 89 kDa and N-terminal 24 kDa fragment is the hallmark for caspase-dependent apoptotic induction.

(c) Breakdown of structural nuclear protein; lamins are intra-nuclear proteins that maintain the shape of the nucleus and mediate interactions between chromatin and the nuclear membrane. Lamins cleavage by caspase-6 and -3 results in the chromatin condensation and nuclear fragmentation.

2.4.2 Caspase-independent cell death pathway

The various types of PCD have been proposed, including autophagy (Type II PCD as described above), parapoptosis, mitotic catastrophe, and descriptive model of apoptosis-like and necrosis-like PCD. Parapoptosis has recently been characterized by cytoplasmic vacuolation that begins with progressive swelling of mitochondria and the endoplasmic reticulum (ER). It typically does not involve activation of caspases or other characteristic of apoptotic morphology. Mitotic catastrophe, is not typical for apoptosis, is triggered by mitotic failure caused by defective cell cycle checkpoints and the development of aneuploidy cells that are doomed to die. In addition, Leist and Jäättelä (2001) proposed a descriptive model, which classifies cell death into four subclasses, according to their nuclear morphology (Figure 2.11).

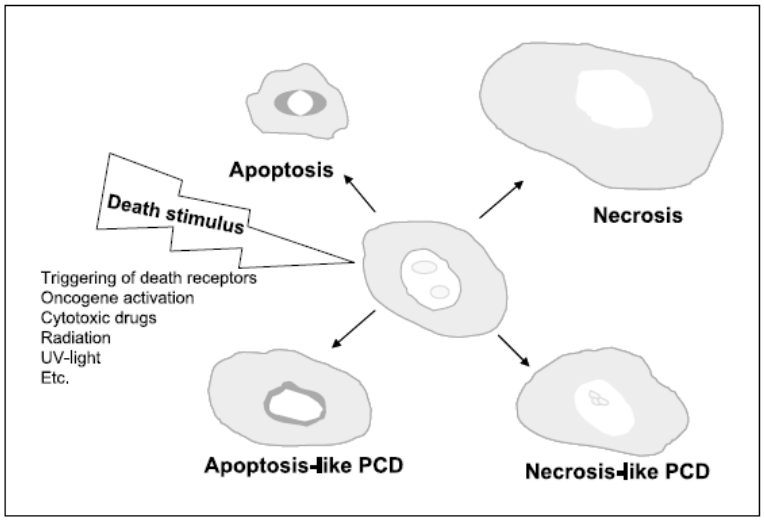


Figure 2.11 Classification of cell death according to the nuclear morphology of the dying cell. Upon a lethal stimulus, a cell can die in different ways that can be classified according to their nuclear morphology. (adapted from Bröker et al., 2005).

In apoptosis, there is chromatin condensation into compact figures, which are often globular or crescent shaped. Apoptotic morphology further includes shrinkage of the

cell, blebbing, and the formation of apoptotic bodies, dependent on caspase-3 and caspase-activated DNase. Apoptosis-like PCD is characterized by chromatin condensation that is less compact but which gives more complex and lumpy shapes and is caused by apoptosis inducing factor (AIF), endonuclease G (Endo G), cathepsins, or other proteases. In necrosis-like PCD, no chromatin condensation is observed, but chromatin clustering to loose speckles, whereas necrosis is associated with cytoplasmic swelling and cell membrane rupture. Any degree or combination with other apoptotic features can be found (Bröker et al., 2005).

Organelles involved in caspase-independent cell death are mitochondria, lysosome and endoplasmic reticulum (ER) as described following:

1) Mitochondria

Upon apoptotic stimuli that insulting in mitochondria outer membrane (MOM) permeabilization, the apoptogenic proteins, cytochrome c, Smac/DIABLO, Omi/HtrA2 release into the cytosol and regulate in caspase-dependent cell death. In this way, Omi/HtrA2 can induce caspase-independent cell death via its serine protease activity and direct association with cell surface death receptors. Another mitochondrial protein contributes to caspase-independent cell death: AIF and Endo G are also released from mitochondria. The flavoprotein AIF translocates from the mitochondria to the nucleus and triggers peripheral chromatin condensation and high molecular weight DNA (50 kb). Moreover, Endo G appears to work in conjugation with both AIF and the caspase-activated DNase CAD/DFF40 in chromatin condensation and nuclear degradation (Bras et al., 2005).

2) Lysosome

Lysosomes are intracytoplasmic organelles defined by an acidic milieu (pH around 4.5) and surrounded by a single membrane that comprises a variety of proteases, nuclease, glycosidase, sulfatase and lipases for digestion of macromolecules. The main class

of lysosomal proteases is represented by the cathepsins. They are subdivided in three subgroups according to the amino acids of their active sites that confer catalytic activity: cysteine (mainly cathepsins B and C), aspartyl (cathepsins D and E) and serine cathepsins (cathepsins A and G). In the classic apoptosis-necrosis, lysosomes are solely considered involving in necrotic and autophagic cell death and the lysosomal proteases are believed to be responsible for nonspecific protein degradation within the lysosome. In recent years, however, it has become evident that the role of lysosomes in cell death is sophisticated (Bröker et al., 2005). Several mechanisms have been proposed that may connect the lysosomal pathway(s) of cell death to other well-documented death pathways as shown in Figure 2.12.

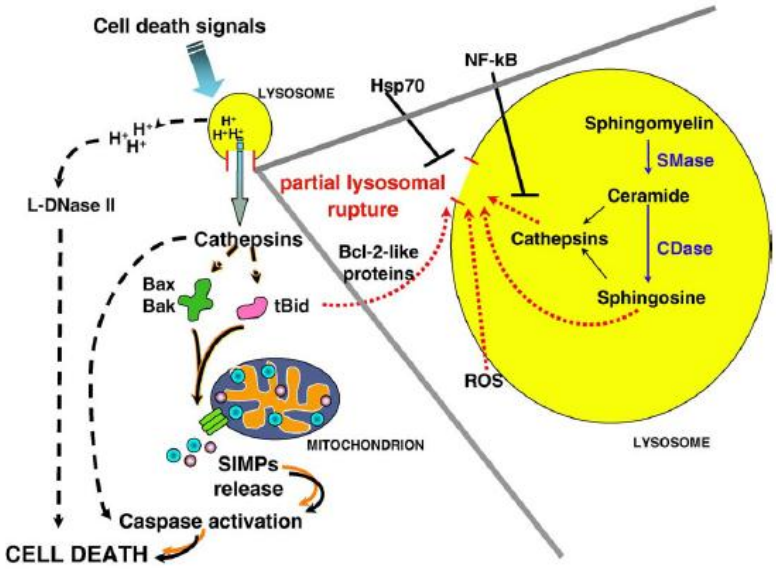


Figure 2.12 The lysosomal pathway of cell death: hypothetical mechanisms. Stress agents can trigger cytosol acidification and a limited permeabilization of lysosomes. CDase, ceramidase; SIMPs, soluble intermembrane mitochondrial proteins (adapted from Tardy, Codogno, Autefage, Levade, and Andrieu-Abadie, 2006).

The partial lysosomal rupture can be promoted by some sphingolipids, lysosomal proteases, ROS or, possibly, by members of Bcl-2 family that translocate to the

lysosomal membrane. The proteases, cathepsins, which are released from the lysosomal compartment to the cytosol, seem to be able to activate some steps of the death cascade leading to apoptosis. Of note, a necrotic cell death can be triggered by a too strong lysosomal membrane permeabilization. Lysosomal proteases rather promote cell death more indirectly by triggering mitochondrial proteins. This may occur *via* the Bcl-2 family protein Bid which is cleaved and translocated to the mitochondria after lysosomal disruption by lysosomotropic agents. In addition, cathepsin D can trigger activation of Bax, leading to selective release of AIF from the mitochondria and PCD in T lymphocytes. Finally, lysosomal proteases have been reported to directly cleave and activate caspases, thereby event in the apoptotic cascade (Tardy et al., 2006).

3) Endoplasmic reticulum (ER)

The ER is an important sensor of cellular stress that can withhold protein synthesis and metabolism to restore cellular homeostasis. If the damage to the ER is too extensive, this can initiate PCD *via* the unfolded protein response or *via* release of calcium into the cytoplasm. This leads to activation of caspase-12, possibly *via* translocation of the Bcl-2 family member Bim to the ER. The activated caspase-12 triggers downstream caspases and then undergoes PCD. ER stress can induce permeabilization of the mitochondrial membrane and thus activate the classical apoptotic pathway as well as other mitochondrial pathways. Bcl-2 family proteins as well as cytoplasmic calcium shifts orchestrate the cross talk between the mitochondria and the ER (Figure 2.13) (Bröker et al., 2005).

In addition, intracellular calcium influx caused by ER stress induces activation of a family of cytosolic proteases, the calpains (calcium-activated neural proteases), which normally reside in the cytosol as inactive zymogens. Calpains have been shown to act downstream of caspase activation and to contribute to the degradation phase of

campthotecin-induced apoptosis in HL-60 cells. Interestingly, vitamin D compounds have been reported to trigger cell death in MCF7 cells executed by calpains in complete absence and independent of caspase activation, indicating that the ER may play a key role in certain types of caspase-independent cell death (Mathiasen et al., 2002).

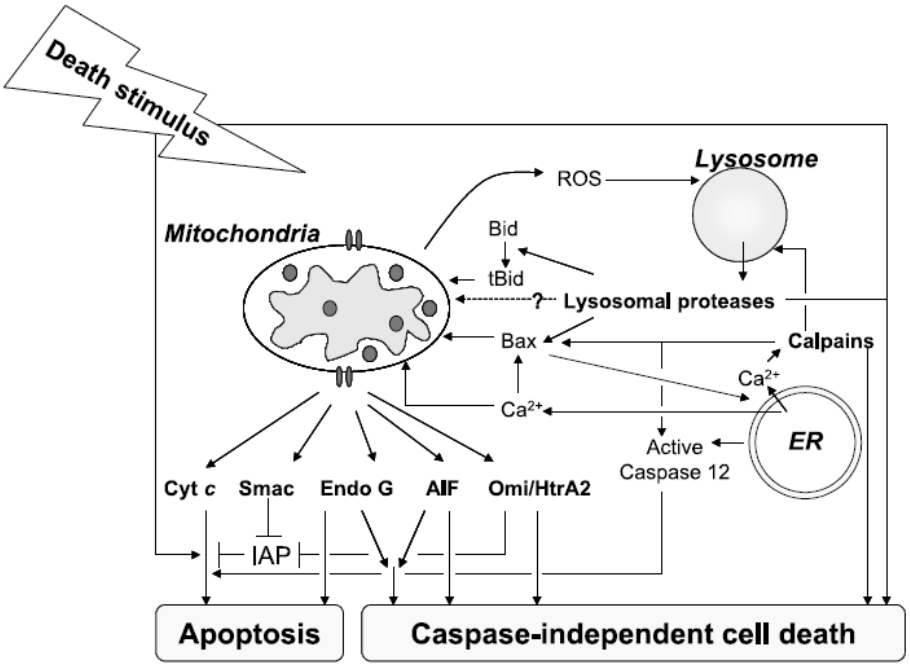


Figure 2.13 Cross-talk between cellular organelles during cell death. Upon a lethal stimulus, a cell has access to different death programs that can be executed *via* caspases (apoptosis) or independent of caspases (adapted from Bröker et al., 2005).

Mitochondria, lysosomes and the ER can be involved in various pathways but may play a more prominent role in certain types of PCD. As depicted here, the signals from the different organelles are liked and may act both upstream and downstream of each other. It has been postulated that the dominant cell death phenotype is determined by the relative speed of the available death programs and only the most effective pathway is usually evident (Bröker et al., 2005).

2.5 Local Thai vegetables

Local Thai vegetables are mostly grown in the Northeast of Thailand and their young leaves commonly consumed in daily diet as fresh (with chili paste), cooked vegetable, or ingredients in Thai cuisine. Some of them are traditionally used as an herbal plant for reliving pains and inflammation. The description, chemical constituents and properties of six local Thai vegetables are described as the followings:

Figure 2.14 The leaves of the 6 local Thai vegetables.

2.5.1 *Barringtonia acutangula* (L.) Gaertn.

Family : LECYTHIDACEAE

Name : Indian Oak (English), Phak Jig (Thai, local)

Use : It has been used as bitter tonic, fish poison, and also as folklore medicine for the treatment of dysentery, diarrhea, and gonorrhea (Panthong, Kanjanapothi, Taesotikul, and Taylor, 1991; Sahoo et al., 2008). Leaves are used to treat diarrhea, wound and dysentery.

Chemical constituents : Leaves contain a trihydroxy triterpene monocarboxylic acid, (acutangulic acid), and steroid compounds (barringtogenic, tangulic and oleanolic acids); saponins and sapogenins, acutagenol A and acutagenol B, three triterpenoid sapogenols, barringtogenols, B, C, and D, E, two triterpenoid acid sapogenins, stigmasterol glucoside, β-sitosterol and β-amyrin, and phenolic acid such as vanillic, syringic, gallic, melilotic and ρ-coumaric acid, gossypetin and 3′-4′ diOMe quercetin (Daniel, 2011).

Scientific studies : The leaf extracts of *B. acutangula* have a strong antioxidant property (Rahman et al., 2010) and moderately exhibited antimutagenic effect against heterocyclic amines such as 3-amino-1,4-dimethyl-5*H*-pyrido[*4,3-b*] indole (Trp-P-1) in the Ames Test (Nakahara et al., 2002).

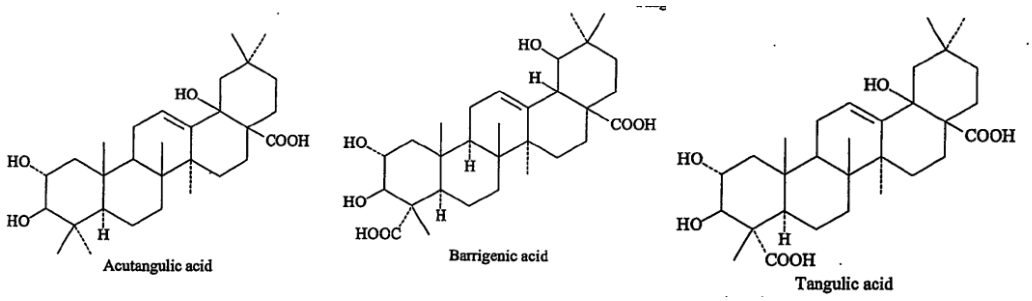


Figure 2.15 The chemical structure of some compounds in *B. acutangula* (Quinn and Mills, 2007).

2.5.2 *Cratoxylum formosum* Dyer subsp. pruniflorum Gogel.

Family : GUTTIFERAE

Name : Phak Tiew (Thai, local)

Use : The plant's leaf and bark had been used as traditional medicine for the treatment of food poisoning, diarrhea, and internal bleeding (Anderson, 1986).

Chemical constituents : Leaves contain chrologenic acid, dicaffeoylquinic acid and ferulic acid derivatives (Maisuthisakul, Pongsawatmanit, and Gordon 2007). Root contains xanthones, flavonoids and anthraquinone (Boonsri, Karalai, Ponglimanont, Kanjana-Opas, and Chantrapromma, 2006).

Scientific studies : The leave crude extracts of *C. formosum* had antioxidant and vascular protective activities in the *in vitro* and *in vivo* (Kukongviriyapan, Luangaram, Leekhaosoong, Kukongviriyapan, and Preeprame, 2007). Furthermore, *C. formosum* extract scavenged both free radicals (DPPH and ABTS) more strongly than α-tocopherol and BHT (Maisuthisakul et al., 2007). In addition, Prayong, Barusrux, and Weerapreeyakul (2008) reported the cytotoxicity effect of leave crude extract of *C. formosum* on HepG2 cells without any harm to the normal Vero cells.

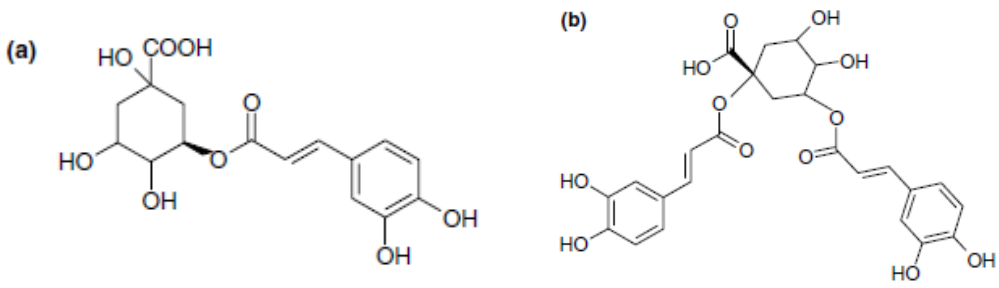


Figure 2.16 Chemical structures of 5-caffeoylquinic acid (Chlorogenic acid) (a); dicaffeoylquinic acid (1,3-diCQA) (b) in *C. formosum* (Maisuthisakul et al., 2007).

2.5.3 *Limnophila aromatica* (Lam.) Merrill.

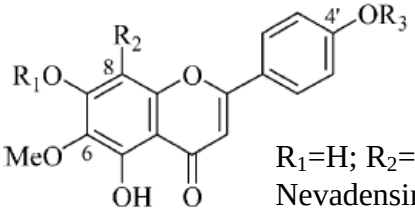
Family : SCROPHULARIACEAE

Name : Shisokusa (Japanese), Phak Kha-yaeng (Thai, local)

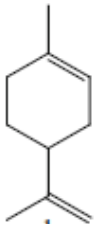
Use : The young shoots and leaves have a fragrant smell and are eaten with the fermented fish and chili sauce, *nam phrik plaa raa*, or are put into curries such as bamboo shoot curry or the coconut milk curry, *kaeng om*.

Chemical constituents : Aerial part of its contains several flavonoids, such as nevadensin, nevadensin-7-*O*-β glycopyranoside, gardenin B and other flavones (Bui et al., 2004) and essential oils such as limonene (15.09%), trans-isolimonen (14.52%) and humulene (6.04%) (Sribusarakum, Bunyapraphatsara, Vajragupta, and Watanabe, 2004). Another study reveals that the essential oils of *L. aromatic* rich in Z-ocimene (39.21%), terpinolene (17.24%) and camphor (12.89%) (Bhuiyan, Akter, Chowndhury, and Begum, 2010).

Scientific studies : The methanol and essential oils of *L. aromatica* exhibit weak antioxidant activities when compare to trolox by DPPH, nitric oxide radical scavenging, and inhibition of lipid peroxidation (Sribusarakum et al., 2004), but show high antimicrobial activities (Phongmanee and Sanampol, 2007). However, the methanolic extract of *L. aromatica* had potent antitumor-promoting activity determined by Epstein-Barr virus activation assay (Murakami, Jiwajinda, Koshimizu, and Ohigashi, 1995). Moreover, *L. aromatica* have anti-inflammatory activities by strongly suppressed LPS-induced production of NO and TNF-α (Turntipopipat, Muangnoi, and Failla, 2009) and moderate antimutagenic activity (Nakahara et al., 2002).



R_1=H; R_2=OMe, R_3=Me,
Nevadensin



Limonene

Figure 2.17 Chemical structures of some compounds in *L. aromatica* (Bui et al., 2004).

2.5.4 *Polygonum odoratum* Lour.

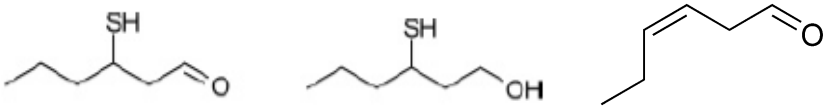
Family : POLYGONACEAE

Name : Vietnamese coriander (common), Phak Paew (Thai, local)

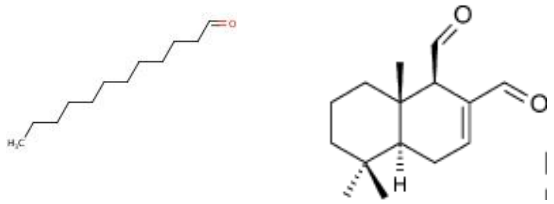
Use : Leaves, has a strong coriander leaf odor, are used in Southeast Asian cooking and herb. In Vietnamese cuisine, it is commonly eaten fresh in salads (including chicken salad) and in raw summer rolls as well as in some soups. In Thailand, they are eaten fresh with cooked mince, *larb*.

Chemical constituents : Leaves contain flavonoids such as rutin (3.77%), catechin (0.34%), quercetin (0.079%), kaempferal (0.009%), and isorhamnetin (0.007%) (Nanasombat and Teckchuen, 2009) and aromatic organic compounds, (Z)-3-hexenal, (Z)-3-hexenol, decanal, undecanal, and dodecanal, as well as 3-sulfanyl-hexanal and 3-sulfanyl-hexan-1-ol, and polygodial, a 1,4-dialdehyde derived from drimane terpenoids (Starkenmann, Luca, Niclass, Praz, and Roguet, 2006). Essential oil of leaves contain long-chain aldehydes e.g. decanal (28%), dodecanal (44%), and decanol (11%) and sesquiterpenes (α-humulene, β-caryophyllene) accounted for about 15% of the essential oil (Hunter, Brophy, Ralph, and Bienvenu, 1997).

Scientific studies : The methanolic extract of *P. odoratum* exhibited strong antioxidant and antibacterial activities. *P. odoratum* extract was moderately cytotoxicity against MCF7 with IC_{50} value of 6.01 µg/mL at 72 h, rutin was a major found compound (Nanasombat and Teckchuen, 2009).



3-Sulfanylhexanal 3-Sulfanyl-hexan-1-ol (Z)-3-hexenal



Dodecanal Polygodial

Figure 2.18 Chemical structures of some compounds in *P. odoratum* (Starkenmann et al., 2006).

2.5.5 *Syzygium gratum* (Wight) S.N. Mitra synonym : *Eugenia grata* Wight.

Family : MYRTACEAE

Name : Phak Mek, Samet Chun (Thai, local)

Use : The shoots and young leaves are eaten raw with *nam phrik*, with vermicelli or with southern food. They are also served blanched with *nam phrik*. The tannic taste is helpful for stomach disorder.

Chemical constituents : No report about active compounds in *S. gratum*, several plants from *Syzygium* spp. contain high total phenolic contents, such as gallic acid, proanthocyanidins, and conjugated flavonoids, which suggest that they are the rich sources of antioxidant compounds (Neergheen, Soobrattee, Bahorun, and Aruoma, 2006).

Scientific studies : The leaves of *S. gratum* possess strong antioxidant and vascular protective activities both *in vitro* and *in vivo* study models. The water extracts of *S. gratum* showed relatively strong radical scavenging activities by DPPH and intracellular oxygen radical (Kukongviriyapan et al., 2007). *S. gratum* exhibits potent antioxidant and intracellular oxygen radical scavenging activities and can induce cytoprotective enzyme *in vivo* (Senggunprai, Kukongviriyapan, Prawan, and Kukongviriyapan, 2010). Moreover,

short term intake of *S. gratum* water extract enhances plasma antioxidant capacity (Bureemas, Kukongviriyapan, Kukongviriyapan, Senggunprai, and Jetsrisuparb, 2007).

2.5.6 *Schinus terebinthifolius* Raddi.

Family : ANACARDIACEAE

Name : Brazilian pepper (common), Phak Matoom Khaeg (Thai, local)

Use : Almost all parts of *S. terebinthifolius*, including leaves, bark, fruit, seeds, and resin have been medicinally used throughout the tropical region. In some country, the leaf tea is used to treat colds and a leaf decoction is inhaled for hypertension, depression, and irregular heartbeat. Traditionally, leaf and bark of *S. terebinthifolius* have antibacterial, antiviral, diuretic, anti-inflammatory, balsamic and hemostatic activities and also used as tonic to treat urinary and respiratory infections (De Lima et al., 2006).

Chemical constituents: Leaves contain saponins, flavonoids, triterpenes or steroid, and tannins (Johann, Pizzolatti, Donnici, and Resende, 2007), quinic acid esters (5-O-caffeoylquinic acid and 5-O-coumaroylquinic acid) and three myricetin glycoside, and tannic acid (Farag, 2008), gallic acid (4%), methyl gallate (28%), and 1,2,3,4,6-pentagalloylglucose (26%) (Cavalher-Machado et al., 2008). Moreover, the phenolic compound such as caffeic acid (5.07 mg/100 mg extract), syringic acid (1.59 mg), coumaric acid (1.64 mg), ellagic acid, and gallic acid were identified in *S. terebinthifolius* leaf extract and terpenes, 56.96% sesquiterpenes, 34.37% oxygenated monoterpenes, in its essential oils (El-Massry, El-Ghorab, Shaaban, and Shibamoto, 2009).

Scientific studies : *S. terebinthifolius* exhibited antioxidant and antimicrobial activities against food-pathogen microorganism (El-Massry et al., 2009), as well as anti-allergic activity (Cavalher-Machado et al., 2008). Futhermore, the polyphenol from *S. terebinthifolius* and their fractions (Isoquercitrin) were demonstrated to induce G_0/G_1 cell

cycle arrest and cell apoptosis on the androgen-insensitive DU145 human prostatic carcinoma cell line (Queires et al., 2006).

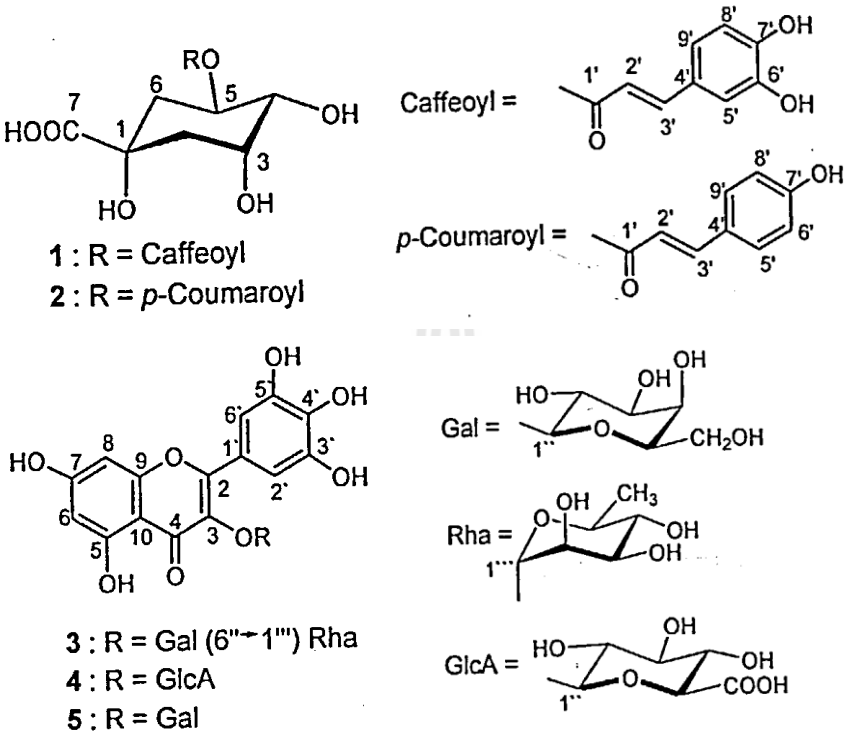


Figure 2.19 Chemical structures of some compounds in *S. terebinthifolius*. 5-O-caffeoylquinic acid (0.0019%) (1); 5-O-coumaroylquinic acid (0.0014%) (2); myricetin 3-O-α-L-rhamnopyranosyl β-D-galactopyranoside (myricetin 3-robinoside) (0.0004%) (3); myricetin 3-O-β-D-glucuronide (0.0009%) (4); myricetin 3-O-β-D-galactopyranoside (0.0004%) (5) (Farag, 2008).

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CHAPTER III

PHYTOCHEMICALS AND ANTIOXIDATIVE PROPERTIES OF SOME LOCAL THAI VEGETABLE LEAF EXTRACTS

3.1 Abstract

Plant-derived food has been of interested as source of active antioxidants as well as cancer protective agent. The leaf extracts of six local Thai vegetables, *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) were investigated for total phenolic contents (TPC), total flavonoid contents (TFC) and phytochemical availability by thin layer chromatography (TLC) and specific reagents. Antioxidant capacities were evaluated *in vitro* by ferric reducing antioxidant power (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, inhibition of microsome lipid peroxidation, and reactive oxygen species (ROS) production. The results showed that BaLE, SgLE, and StLE contained high TFC which were 377.28 ± 11.57, 303.70 ± 19.18, and 438.73 ± 14.06 µg GAE/mg, respectively. Whereas, CfLE, LaLE, and PoLE rich in TFC which were 251.36 ± 1.83, 112.35 ± 0.50, and 117.30 ± 0.68 µg CAE/mg, respectively. The antioxidant properties of BaLE, SgLE, and StLE, rich in tannins but low in flavonoids, were similar to catechin, trolox, and ascorbic acid and were significantly ($p < 0.05$) higher than those of CfLE, LaLE, and PoLE. BaLE, SgLE, and StLE expressed high FRAP activity with 8.76 ± 1.52, 7.74 ± 0.66, and 8.09 ± 0.44 µmolFe^{2+}/mg dried extract, respectively. Furthermore, these three vegetable extracts exhibited high DPPH scavenging with EC_{50} values of 6.67 ± 0.10, 8.85 ± 0.09, and 5.54 ± 0.13 µg/mL and high capacity to inhibit lipid

peroxidation with EC_{50} values of 3.92 ± 0.01, 3.54 ± 0.21, and 6.50 ± 0.29 µg/mL, respectively. However, it was more complex in cell-base assay, all of the extracts exhibited some antioxidative effects at low concentrations but produced somewhat cytotoxic effects at high concentrations. BaLE and StLE of 20 µg/mL and SgLE of 25 µg/mL presented significant ($p < 0.05$) reduction of ROS with the inhibited ROS of 26.40%, 12.34%, and 17.99%, respectively, while LaLE, PoLE, and CfLE of 100 µg/mL gave a reduction of 35.60%, 29.00%, and 28.30%, respectively. Since the extracts were crude mixture of diverse compounds, it elaborated diverse in antioxidant capacities. However, BaLE, SgLE, and StLE exhibited potent antioxidant equivalently to catechin, trolox and ascorbic acid which could be applicable in food processing to retard oxidation and in biological system by reducing ROS levels. These studies obviously provided informative data of some local Thai vegetables as source of effective antioxidant for regular consumption.

3.2 Introduction

Free radicals, reactive oxygen species (ROS), are essential intermediates in oxidative metabolism. An imbalance between ROS and defense antioxidant mechanism can cause oxidative stress leading to protein, carbohydrate and DNA damages, and initiating the peroxidation of polyunsaturated fatty acids in biological membranes (Halliwell, 1997). These events could consequently lead to the occurrence of various diseases such as diabetes, atherosclerosis, ischemic injury, inflammation and cancer (Waris and Ahsan, 2006).

Antioxidants are substances which capable for neutralizing free radicals by donating one of their own electrons, terminating the reaction of unstable molecules. They play importance role for preventing the formation of oxidative stresses by (1) inhibition of enzymes or chelating trace elements; (2) directly scavenging reactive oxygen species; and (3) upregulating the antioxidant defense systems (Halliwell and Gutteridge, 1999).

Increasing the intake of antioxidant-rich foods may potentially promote the health benefits and decrease the risk of chronic diseases. A number of studies have reported the relationship between antioxidant-rich foods consumption such as fruits and vegetables and lower risk of diseases (Steinmetz and Potter, 1996). Fruits and vegetables containing vitamins, minerals including polyphenols were claimed as potent antioxidants for counteracting with the free radicals (Kaur and Kapoor, 2001).

Recently, phytochemicals in food have been increasingly received attention as sources of many effective antioxidants because of safety and compatible with human normal physiology. Many of dietary plants are consumed in Southeast Asian countries and claimed for health promotion and treatment of various symptoms, such as inflammation, asthma, hypertensive, indigestion, laxative, peptic ulcer, diarrhea, and antibacterial infection (Panthong, Kanjanapothi, Taesotikul, and Taylor, 1991). Local Thai vegetables, *Barringtonia acutangula, Cratoxylum formosum, Limnophila aromatica, Polygonum odoratum, Syzygium gratum,* and *Schinus terebinthifolius* are widely grown in the Northeast of Thailand. They are of our interest to investigate their biological activities since their young leaves have been commonly consumed as vegetables in traditional daily diet. The leave extracts of these plants have been reported to possess antioxidant (Rahman et al., 2010; Bureemas, Kukongviriyapan, Kukongviriyapan, Senggunprai, and Jetsrisuparb, 2007; Maisuthisakul, Suttajit, and Pongsawatmanit, 2007), antimicrobials (Lokaewmanee, Chandang, Khumpeerawat, and Jantajaroonpong, 2009; Phongmanee and Sanampol, 2007; Martínez, Bentancourt, Alonso-González, and Jauregui, 1996), antifungal (Johann, Pizzolatti, Donnici, and Resende, 2007) and antimutagenic activities (Nakahara et al., 2002). Crude extracts from the leaves of *C. formosum*, *S. gratum* and *L. aromatica* were reported to contain antioxidant and vascular protective activities *in vitro* and *in vivo* (Kukongviriyapan, Luangaram, Leekhaosoong, Kukongviriyapan, and Preeprame, 2007). The ethanolic extract of *C. formosum* leaves contains chlorogenic acid which contributes to

the antioxidant activity (Maisuthisakul, Pongsawatmanit, and Gordon, 2007). The aerial part of *L. aromatica* contains several flavonoids, such as nevadensin, nevadensin-7-*O*-β glycopyranoside, gardenin B and other flavones, which are polyphenolic compounds with antioxidant activity (Bui et al., 2004). However, there are few studies about the phytochemicals and antioxidant activities of these plants.

The *in vitro* methods for quantifying antioxidant capacity in foods and biological systems could be classified into two groups: the electron transfer (ET)-based assays including FRAP and DPPH which measure the reducing properties, and the hydrogen atom transfer (HAT)-based assays, lipid peroxidation which antioxidants can compete in queching peroxyl radicals (Somogyi, Rosta, Pusztai, Tulassay, and Nagy, 2007). In addition, the cellular antioxidant activity (CAA) assay in cell culture was used to quantify the antioxidant activity which might be able to predict the *in vivo* activity. This method serves a more biologically relevant protocol for determining the antioxidative properties. There is evidence that many different methods are necessary to evaluate different antioxidant effects. Therefore, it is essential to use at least two assays to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals (Chanda and Dave, 2009).

Therefore, the aim of this study was to investigate the phytochemicals and antioxidative activities of the six pre-screened local Thai vegetable leaf extracts; *Barringtonia acutangula*, *Cratoxylum formosum*, *Limnophila aromatica*, *Polygonum odoratum*, *Syzygium gratum* and *Schinus terebinthifolius* in chemical and cellular systems.

3.3 Materials and methods

3.3.1 Reagents

Catechin, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), bismuth (III) subnitrate, potassium iodide, ρ-anisaldehyde, vanillin, 3,5-

dinitrobenzoic acid, aluminium trichloride ($AlCl_3$), sodium nitrite ($NaNO_2$), 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), thiobarbituric acid (TBA), malonaldehyde (MDA) and 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) were obtained from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Gallic acid was obtained from Fluka Co. (Buchs, Switzerland). 2-4-6-Tripyridyl-s-triazine, potassium hydroxide and *tert*-butyl hydroperoxide were obtained from Acros Organics (Geel, Belgium). Methanol, ethanol, hydrochloric acid, sulfuric acid, di-sodium hydrogen phosphate anhydrous, sodium carbonate (Na_2CO_3) and Folin-Ciocalteu's reagent were purchased from Carlo Erba Reagents (Milano, Italy). Trichloroacetic acid (TCA) and bovine serum albumin (BSA) were purchased from BDH laboratory supplier (Poole, UK). Dimethylsulfoxide (DMSO) and sodium bicarbonate were obtained from Amresco (Ohio, USA). All chemicals were analytical grade.

3.3.2 Plant materials

Fresh young leaves of six local Thai vegetables were purchased from the local market in the Nakhon Ratchasima Province, Thailand during September 2008 to February 2009 and identified by a plant taxonomist, Professer Dr. Pranom Chantaranothai of Khon Kaen University.

3.3.3 Sample preparation

The leaves were freeze-dried and grounded into powder. The leave ethanolic extracts of *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) were prepared. The leaf powder of 60 g was macerated in 70% ethanol (v/v) for 24 h (3 times). The extracts were filtered and concentrated in rotary evaporator (Buchi instruments, Switzerland) and lyophilized into powder (Freeze-zone 12 plus, Labconco Corporation, Missouri, USA). The extracts were stored at -20ºC until

analysis. The extract was dissolved in absolute DMSO and diluted to 0.2% DMSO in the desired concentrations used in cell culture assays.

3.3.4 Determination of phytochemical compounds

3.3.4.1 Total phenolic contents (TPC)

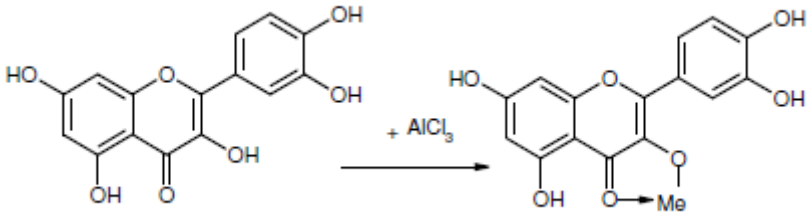
The total phenolic contents of local Thai vegetables were determined by Folin-Ciocalteu method (Waterhouse, 2002). This method is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. Briefly, 0.1 mL of the extract in DMSO, 1.5 mL of dH_2O and 0.1 mL Folin-Ciocalteu's reagent were mixed and incubated for 5 min. Then, 0.3 mL of 20% Na_2CO_3 was added, incubated for 45 min at room temperature (RT), and measured the absorbance at 750 nm by a spectrophotometer (CE1011, Cecil Instruments, Cambridge, England). The TPC of sample was expressed as µg of gallic acid equivalent (GAE) per mg dried extract.

3.3.4.2 Total Flavonoid Contents (TFC)

Total flavonoid content was measured by the aluminium trichloride colorimetric assay (Zhishen, Mengcheng, and Jianming, 1999). The reaction bases on aluminium chloride forming acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of favones and favonols. Flavonoids form internal complexes, chelate type, with Al^{3+} (Figure 3.1). The intensity of yellow color of the kelate formed by the flavonoids, when treated with $AlCl_3$ in acetate buffer, was spectrophotometrically determined (Constanta and Rodica, 2010).

An aliquot of 0.5 mL extract in DMSO or catechin standard solution was mixed with 2 mL dH_2O and 0.15 mL of 5% $NaNO_2$. The mixture was incubated for 6 min and then 0.15 mL of 10% $AlCl_3$ was added. After allowing to stand for 6 min, 1 mL of 1M NaOH was added and made up the total volume of 5 mL with dH_2O and measured the

absorbance at 510 nm. Total flavonoid content was expressed as µg catechin equivalents (CAE) per mg of dried extract.



Flavonoid Kelate form (yellow)

Figure 3.1 The chemical reaction of flavonoid determination (Constanta and Rodica, 2010).

3.3.4.3 Qualitative phytochemical assay

For qualitative screening of the phytochemical group of the extracts, thin layer chromatography (TLC) was performed and tested with the specific reagents as followed.

a) Thin layer chrmotography (TLC)

Precoated TLC plate of silica gel 60 F254 0.25 mm (Merck, Darmstadt, Germany) was used for analytical TLC. The extract was redissolved in 70% (v/v) ethanol. The volume of 0.5 µL of each extract (25 mg/mL) was spotted onto the TLC plate (5 times/spot). The TLC plate was conducted in mobile phases; hexane:acetone (70:30), ethyl acetate:methanol:water (81:11:8) and chloroform:acetic acid:methanol:water (64:32:12:8), depending on polarity of detected compounds. The plates were observed under UV lamps at 254 and 366 nm and sprayed with specific reagents to detect some major phytochemicals (Gibbons and Gray, 1998); (a) Dragendorff's reagent for an alkaloids group, (b) Potassium hydroxide (KOH) reagent for an anthraquinones (red), anthones (yellow, UV-366 nm) and coumarins group (blue-green, UV-366 nm), (c) Kedde reagent for cardic glycosides, (d) Iron (III) chloride reagent for phenolic compounds including tannins,

(e) Anisaldehyde/sulfuric acid (H_2SO_4) reagent and (f) Vanillin/sulfuric (H_2SO_4) reagent for essential oils, steroid, terpenoids and phenols. The detail protocols of sprayed reagents preparation were summarized in Appendix A.

b) Testing with specific reagents

The vegetable leaf extracts were screened for terpenoids, saponins, and tannins as following (Farnswarth, 1966; Silva, Lee, and Kinghorn, 1998):

1) Terpenoids (Salkowski test)

The extract of 25 mg/mL in 70% EtOH was diluted in absolute EtOH (1:5). The extract of 3 mL was mixed with 2 mL of chloroform in a test tube. Concentrate H_2SO_4 (3 mL) was carefully added to form a layer. A reddish brown color of the interface indicates the presence of terpenoids.

2) Saponins

The extract (0.25 g) was redissolved in 2.5 mL of water and boiled. The solution was shaken vigourously and observed for a stable persistent froth for approximately 10 min. To confirm the presence of saponin, NaOH was added to react with some proteins or acids such as fatty acid (interference), then salt were formed. The persistent of froth after shaking, represents the saponins. Another way, HCl was added and boiled, the hydrolysis of saponin glycoside to aglycone was occurred. The aglycone of saponin could not froth when vigourously shaking (Trease and Evans, 1983).

3) Tannins (ferric chloride test)

The extracts 25 mg/mL (70% EtOH) was diluted with DI water to 1:25 (3 mL), and then 20 µL of ferric chloride solution (0.1% w/v) was added. Observation of brownish green or blue-black coloration confirms the presence of condense tannins or hydrolysable tannins, respectively.

3.3.5 Determination of antioxidant properties

3.3.5.1 Ferric reducing antioxidant power (FRAP)

Measurement of reducing ability of the vegetable leaf extracts was investigated using FRAP method according to Benzie and Strain (1999). FRAP is a simple direct test of antioxidant capacity, based on the ability of phenolics to reduce Fe^{III} to Fe^{II}. This assay measures the formation of a blue colored at 593 nm of Fe^{II}-tripyridyltriazine compound from colorless oxidized Fe^{III} formed by the action of electron donating antioxidants. FRAP reagent was freshly prepared by mixing 10 volumes of 1.0 mol/L acetate buffer, pH 3.6, 1 volume of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L hydrochloric acid and 1 volume of 20 mmol/L ferric chloride. Sample of 50 μL was added into 1.5 mL of FRAP reagent and incubated for 40 min. The absorbance was measured at 595 nm. Standard curve of 100-1000 μmol $FeSO_4.7H_2O$ was prepared. Catechin, trolox, and ascorbic acid were used as standard antioxidant. The antioxidant power was expressed as μmol Fe^{2+}/mg dried extract and also mg CAE/mg, mg TRE/mg, and mg AAE/mg dried extract for $FeSO_4$, catechin, trolox, and ascorbic acid equivalent, respectively.

3.3.5.2 Free radical scavenging activity (DPPH assay)

This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH, which evaluates the absorbance decrease at 515-528 nm. The free radical DPPH (purple) is reduced to the corresponding hydrazine (no color) when it reacts with hydrogen donors from antioxidant compounds (Figure 3.2) (Sánchez-Moreno, Larrauri, and Saura-Calixto, 1998).

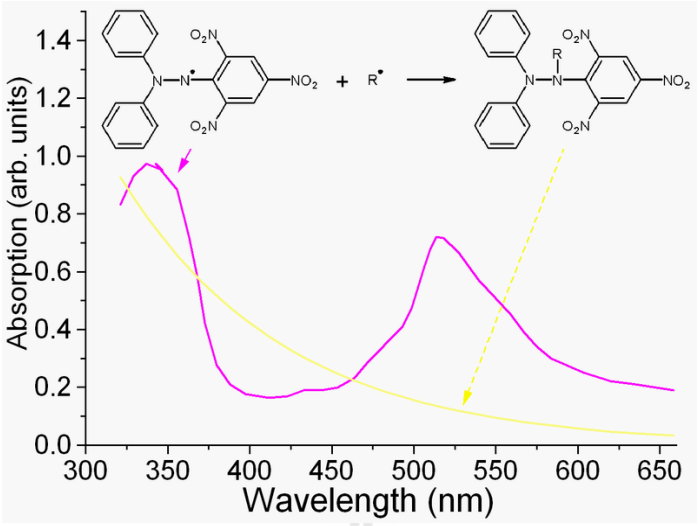


Figure 3.2 The mechanism of DPPH radical interact with antioxidant compound. R = antioxidant, DPPH = 2,2-diphenyl-1-picrylhydrazyl (Wikipedia, www, 2011).

The DPPH˙ scavenging activity was determined following the method of Sánchez-Moreno et al. (1998) with some modification. The sample solution of 0.1 mL at different concentration was added to 3.9 mL of 40 mg/L methanolic DPPH˙ solution, incubated for 45 min in the dark, and measured at 515 nm. Control was prepared by adding 0.1 mL of DMSO to DPPH˙ solution. The DPPH˙ remaining was calculated against DPPH standard curve. The percentage of DPPH˙ inhibition was calculated comparing with control as following:

$$DPPH^{\bullet}\,inhibition\ (\%) = \left(1 - \left(\frac{AB_{sample}}{AB_{control}}\right)\right) \times 100$$

Antioxidative activity of the sample was defined as the amount of antioxidant necessary to reduce the initial DPPH˙ concentration by 50% (Medium effective concentration, EC_{50}) and expressed as µg dried sample/mL of a reaction assay. EC_{50} values also reported as mg sample/mg DPPH˙ to elaborate the scavenging ratio of extract against

DPPH radical which can be further used to calculate antiradical activity (A_{AR}). A_{AR} defined as $1/EC_{50}$, the higher the antioxidant activity, the higher the value of the A_{AR}.

3.3.5.3 Inhibition of lipid peroxidation

Measurement of lipid peroxidation using rat liver or cardiac microsomes, ox-brain phospholipid liposomes, arachidonic acid, and other lipid model system (e.g. bulk oil and emulsified oil system) have been used for establish the potential antioxidant action of antioxidant compounds (Aruoma, 1999). Thiobarbituric acid reactive substances (TBARS) assay is one of the most popular methods for detecting the lipid peroxidation products such as malondialdehyde (MDA) because it is quite sensitive. The reaction of MDA with TBA under acidic condition at 90ºC-100ºC form a MDA-TBA product (Figure 3.3) which can be measured colorimetrically at 532 nm or by fluorescence using excitation wavelength of 530 nm and emission wavelength of 550 nm (Held, www, 2010).

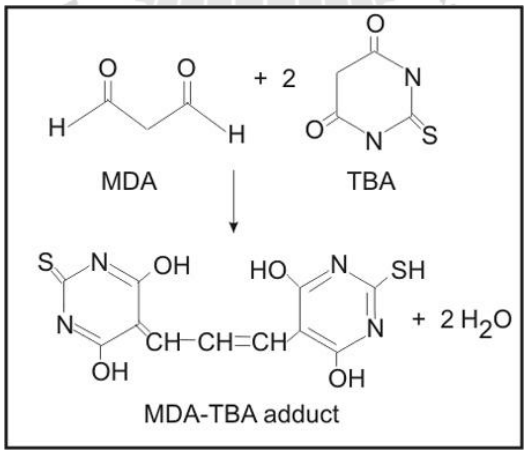


Figure 3.3 Reactions between MDA and TBA (Held, www, 2010).

Lipid peroxidation was elucidated in the Fe^{2+}-induced microsome lipid peroxidation system. The microsome was prepared according to the method of Van Acker et al. (1996). The liver of a female Wistar rat (from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand) was washed and homogenized in phosphate

buffer saline, pH 7.4, containing 50 mM phosphate and 0.15 M KCl, in a motor driven homogenizer (RW20digital, IKA, China) at 2000 rpm for 15 min. The homogenate was centrifuged (Universal 320R centrifuge, Hettich-Zentrifugen, Tuttlingen, Germany) at 9,000×g, 4ºC for 10 min. Subsequently, the supernatant was ultracentrifuged (ultracentrifuge, CP-WX series, Himac centrifuge, Hitachi, Hong Kong) at 100,000×g, 4ºC for 60 min. The microsome pellet was collected and washed in phosphate buffer and recentrifuged at 100,000×g, 4ºC for 60 min and resuspended in phosphate buffer and stored at -80ºC. The protein content of microsome suspension was quantified by Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951).

The inhibition of lipid peroxidation by the extracts was determined by the ferric-ascorbate induced microsome method (Joubert et al., 2008) with some modification. The extract of different concentrations in 50 µL and 200 µM $FeCl_2$ were added to 1 mg/mL microsomes. Sodium ascorbate at 100 µM, final concentration, was added and incubated at 37ºC for 1 h. The mixture of 0.5 mL of chilled mixture of 20% trichloroacetic acid, 0.25 N HCl, and 0.01% BHT and 0.5 mL of 0.8% TBA was added. The mixture was incubated at 90ºC for 60 min, allowed to cool in tap water and centrifuged at 4000×g for 10 min. The supernatant was transferred onto a 96-well microplate and measured the absorption at 532 nm. The amount of MDA production in the presence of the extract was calculated. LPO inhibition was calculated from the malonaldehyde (MDA) formed in the presence of the extract in relative to the control as following:

$$LPO\ inhibition\ (\%) = \left(1 - \left(\frac{AB_{sample} - AB_{blank}}{AB_{control} - AB_{blank}}\right)\right) \times 100$$

The Fe^{2+}-ascorbate induced microsome without sample was used as control while un-induced microsome was used as blank. LPO inhibition (%) against various concentrations of the extract was plotted and calculated for EC_{50} (µg dried extract/mL).

3.3.5.4 Cellular antioxidant activity (CAA)

Currently, there are many chemical assays used to quantify antioxidant activity, but their ability to predict *in vivo* activity is lacking. They do not address critical factors such as the uptake, distribution and metabolism of antioxidants in cells. The CAA assay has been introduced since it serves as a more biologically relevant protocol. 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) is a useful indicator of reactive oxygen species (ROS) and oxidative stress. The nonpolar and non-ionic DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterase to non-fluorescent 2′,7′-dichlorofluorescin (DCFH). In the presence of ROS, hydrogen peroxide (H_2O_2), lipid hydroperoxides and peroxynitrite, DCFH is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) (Wolfe and Liu, 2007) as illustrated in Figure 3.4.

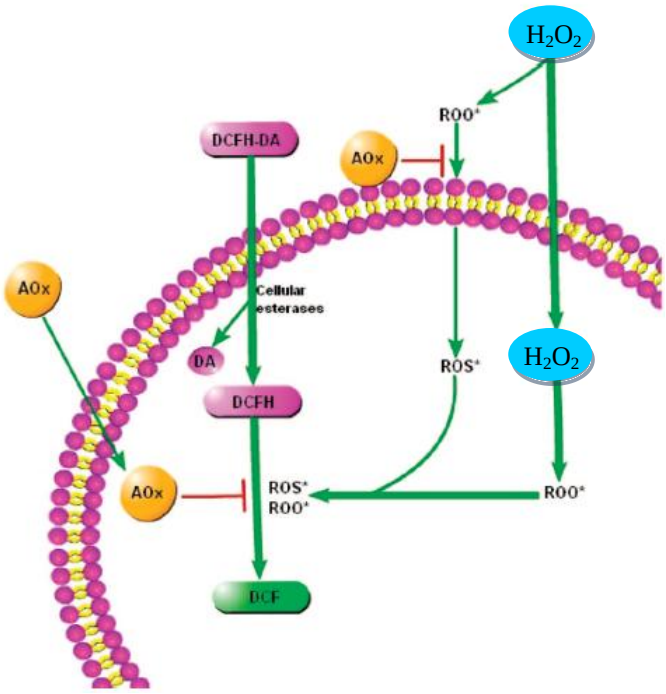


Figure 3.4 The proposed principle of cellular antioxidant activity (CAA) assay. AOx = antioxidant compounds or extracts. The oxidant initiator (H_2O_2) can generate peroxyl radicals ($ROO^{\cdot}$) which can oxidize the intracellular DCFH to the fluorescent DCF (adapted from Wolfe and Liu, 2007).

Intracellular reactive oxygen species (ROS) production was measured by modification of Girard-Lalancette, Pichette, and Legault (2009) method. Briefly, The HepG2 cells were plated in a 96-well microplates, black plate and clear bottom, (Corning Incorporated, New York, USA) at density of 40,000 cells/well and then incubated for 24 h at 37ºC, 5% CO_2. The cells were washed with 150 µL of phosphate buffer saline (PBS), pH 7.4 and treated for 1 h with 200 µL of treatment medium (without serum) containing various concentrations of extract. Then, 20 µM DCFH-DA was added and incubated at 37ºC for 30 min. After washing twice with PBS, the cells were incubated with 200 µM *tert*-butyl hydroperoxide (*t*-BuOOH) (as inducer for ROS production) in Hank's balance salt solution, and the amount of ROS production in cells were evaluated by spectrofluorometer (Spectra MAX Gemini EM, Molecular devices, California, USA) with an excited wavelength at 485 nm and emitting wavelength at 530 nm. The percentage of ROS inhibition of sample against positive control was calculated as following:

$$\%\ ROS\ inhibition = \left(1 - \left(\frac{AB_{sample} - AB_{blank}}{AB_{control} - AB_{blank}}\right)\right) \times 100$$

The cells treated with 0.2% DMSO, then induced with *t*-BuOOH were used as control, while the cells treated with 0.2% DMSO, un-induced were used as blank.

3.3.6 Statistical analysis

Experiments were performed in triplicate and repeated twice. Data were analyzed by ANOVA, followed by Duncan's multiple range tests. Results were presented as mean ± SD. Data analysis and correlations were analyzed using Statistical Package for Social Sciences (SPSS) program for Windows, v.17.

3.4 Results and discussion

3.4.1 Total phenolic and total flavonoid contents

The total phenolic content was analyzed by fitting the calibration curve of gallic acid (R^2 = 0.997). The total flavonoid content was calculated using the calibration curve of catechin (R^2 = 1). The total phenolic (TPC), total flavonoid content (TFC) including their recovery yield of extract were shown in Table 3.1. The % yields of these extracts were ranged from 21.04-48.62%. LaLE and PoLE showed poor yield, only 21.04% and 24.40% indicating that the components in their leaves could be less soluble in 70% ethanol.

Table 3.1 Percentage of yield, total phenolic and total flavonoid contents of the 6 vegetable leaf extracts.

Botanical name	% Yield	Total phenolic (μg GAE/mg)	Total flavonoid (μg CAE/mg)
B. acutangula (BaLE)	36.69 ± 0.48	377.28 ± 11.57^{b}	15.47 ± 4.11^{e}
C. formosum (CfLE)	48.62 ± 3.70	313.81 ± 12.96^{c}	251.36 ± 1.83^{a}
L. aromatica (LaLE)	21.04 ± 3.06	152.41 ± 14.20^{e}	112.35 ± 0.50^{c}
P. odoratum (PoLE)	24.40 ± 4.10	216.74 ± 15.33^{d}	117.30 ± 0.68^{b}
S. gratum (SgLE)	32.90 ± 2.69	303.70 ± 19.18^{c}	37.24 ± 0.86^{d}
S. terebinthifolius (StLE)	44.21 ± 1.78	438.73 ± 14.06^{a}	39.80 ± 1.18^{d}

Data are expressed as mean ± SD (n = 6), statistical analysis was performed by ANOVA, different letters within the same column were significantly different ($p < 0.05$).

TPC ranged from 152.41 ± 14.20 μg GAE/mg in LaLE to 438.73 ± 14.06 μgGAE/mg in StLE. TFC ranged from 15.47 ± 4.11 μg CAE/mg in BaLE to 251.36 ± 1.83 μg CAE/mg in CfLE. Flavonoids were the main components in CfLE, LaLE and PoLE which were 251.36 ± 1.83, 112.35 ± 0.50, and 117.30 ± 0.68 μg CAE/mg, respectively. It

was noticed that CfLE contained both high TPC and TFC while BaLE, SgLE, and StLE had high TPC but low TFC.

3.4.2 Phytochemical screening of the extracts

3.4.2.1 Thin layer chromatography (TLC) fingerprinting

TLC plate was used for determining of phytochemical availability in the extracts. After developing, the TLC plate was monitored as mentioned above. TLC fingerprints of the 6 vegetable leaf extracts, which were developed in EtOAc:MeOH:H_2O (81:11:8) and visualized under UV-254 nm and UV-366 nm light, and their Rf values are shown in Figure 3.5 and Table 3.2. Compounds with conjugated double bonds, such as anthraquinones, coumarins, flavonoids and polyphenols, that absorb light at UV-254 nm will appear as dark spots against a light background (Wagner and Bladt, 1995). TLC profile of the 6 vegetable leaf extracts under UV-254 nm (Figures 3.5A, 3.5B) illustrated that all extracts contained compound with conjugated double bonds, polyphenolic compounds. In addition, all extracts present red fluorescence under UV-366 nm (Figures 3.5C, 3.5D) with R_f of value 7.8-7.9 and 0.57-0.59, which could be chlorophyll and their derivatives. The flavonoid group in CfLE, LaLE, and PoLE showed blue fluorescence under UV-366 nm with R_f values of ≥ 0.57. Cardiac glycosides, triterpenoids, and saponins were not detectable when exposed to UV-366 nm (Wagner and Bladt, 1995).

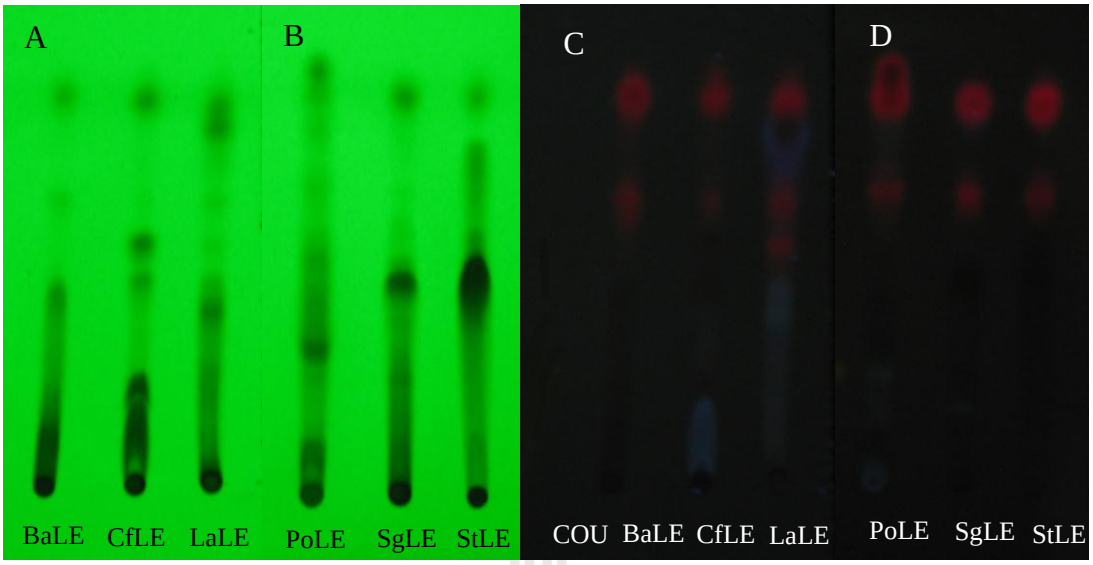


Figure 3.5 TLC of the 6 vegetable leaf extracts was developed in ethyl acetate: methanol:water (81:11:8) and exposed under UV light: A and B, 254 nm; C and D, 366 nm. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; COU, coumarin.

Table 3.2 R_f values of TLC fingerprints of the 6 vegetable leaf extracts visualized under UV-254 and UV-366 nm.

TLC Detection	Leaf extracts					
	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
UV 254 nm[a]	0.78 (D)	0.76 (D)	0.76 (D)	0.82 (D)	0.77 (D)	0.77 (D)
	0.58 (L)	0.48 (D)	0.71 (D)	0.77 (L)	0.54 (L)	0.67 (D)
	0.39 (D)	0.41 (D)	0.47 (L)	0.69 (L)	0.41 (D)	0.50 (L)
	0.10 (D)	0.20 (D)	0.40 (L)	0.60 (L)	0.22 (L)	0.42 (D)
		0.10 (D)	0.34 (D)	0.46 (L)		0.11 (L)
			0.18 (L)	0.37 (L)		
				0.28 (D)		
UV 366 nm[b]	0.79 (R)	0.78 (R)	0.78 (R)	0.83 (R)	0.78 (R)	0.78 (R)
	0.57 (R)	0.57 (LR)	0.66 (Bl)	0.78 (R)	0.57 (R)	0.59 (R)
		0.16 (Bl)	0.57 (R)	0.59 (R)	0.13 (Bl)	
		0.07 (Bl)	0.48 (R)	0.22 (Bl)		
			0.39 (Bl)	0.12 (Bl)		
			0.33 (Bl)	0.00 (Bl)		
			0.14 (Bl)			

[a](D), dark; (L), light. [b](R), red; (LR), light red; (Bl), blue. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extracts.

TLC plate of the extracts was sprayed with Dragendorff's reagent and KOH reagent as demonstrated in Figure 3.6 and the R_f values are shown in Table 3.3. No brown or orange-brown zone (vis) appeared when sprayed with Dragendorff's reagent (Figures 3.6A, 3.6B, and Table 3.3), meaning the absence of alkaloids. KOH reagent was used to detect the anthraquinones (red in visible and show more red fluorescence under UV-366 nm), anthrones (yellow in visible and more bright yellow fluorescence under UV-366 nm) and coumarins (bright green-blue fluorescence). The coumarin was used as standard (green) with R_f values of 0.72 (Figure 3.6C and Table 3.3). No green or yellow spot appeared in the TLC profile of the extracts (Figures 3.6C, 3.6D). These could be saying that all of the 6 vegetable extracts not have anthraquinones, anthrones, and coumarins.

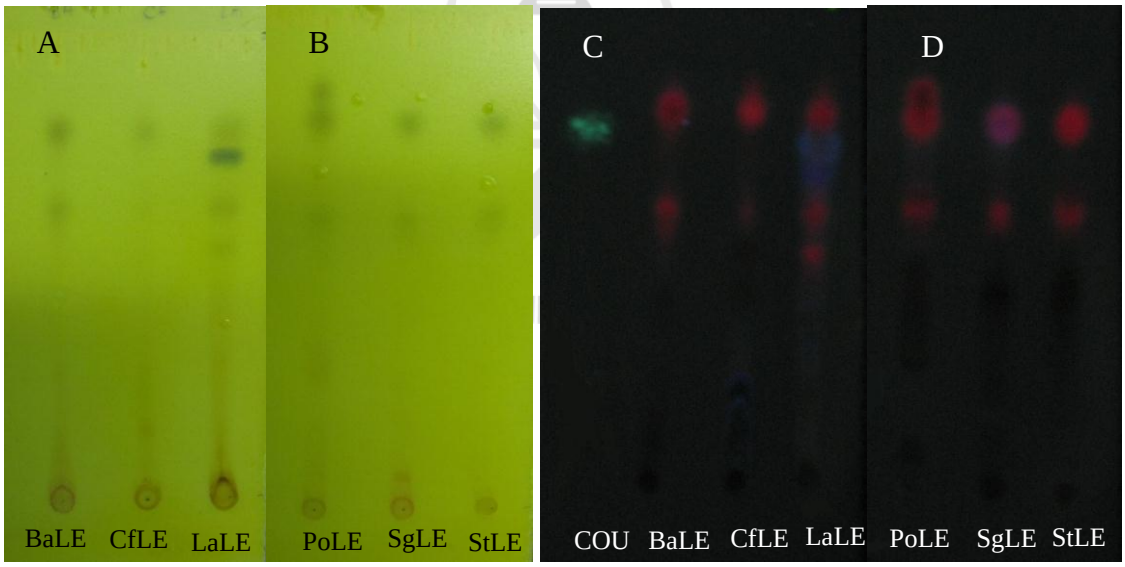


Figure 3.6 TLC plate of the 6 vegetable extracts was developed in ethyl acetate:methanol: water (81:11:8) and sprayed with specific reagents: A and B, Dragendorff's reagent; C and D, KOH reagent under UV-366 nm. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; COU, coumarins.

Table 3.3 R_f values of the 6 vegetable extracts by Dragendorff and KOH TLC detection.

TLC Detection	Leaf extracts					
	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
Dragendorff[a]	0.77 (D)	0.77 (D)	0.77 (D)	0.83 (D)	0.77 (D)	0.77 (D)
	0.60 (D)	0.19 (LBr)	0.71 (DBl)	0.77 (D)	0.57 (D)	0.57 (D)
			0.60 (LBr)	0.67 (D)	0.06 (Br)	
			0.52 (LBr)	0.58 (LBr)		
				0.28 (LBr)		
KOH[b]	0.75 (R)	0.75 (R)	0.75 (R)	0.82 (R)	0.77 (P)	0.78 (R)
	0.55 (R)	0.55 (R)	0.66 (Bl)	0.76 (R)	0.57 (R)	0.59 (R)
COU	0.51 (R)	0.18 (Bl)	0.61 (Bl)	0.57 (R)		
0.72 (G)		0.14 (Bl)	0.54 (R)			
			0.45 (R)			

[a](D), dark; (Br), brown; (LBr), light brown; (DBl), dark blue. [b](R), red; (Bl), blue; (P), pink; (G), green. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; COU, coumarins.

TLC plate of the 6 vegetable leaf extracts after spraying with Kedde reagent and $FeCl_3$ reagent are demonstrated in Figure 3.7 and the R_f values are shown in Table 3.4. Cardiac glycoside, appeared pink or red in visible light after spraying with Kedde reagent, was also not detected in all extracts (Figures 3.7A, 3.7B, and Table 3.4). For $FeCl_3$ reagent, catechin was used as standard reference for phenolic compound with R_f value of 0.64 (Figure 3.7C and Table 3.4). All extracts show dark spots when spraying with $FeCl_3$ reagent represent the phenolic compounds and tannins (Figures 3.7C, 3.7D). When developing in EtOAc:MeOH:H_2O (81:11:8) solvent, less polar compound in crude extract tended to soluble in less polar solvent (EtOAc and MeOH) and migrated well along with mobile phase while high polar compounds did not or less migrate. Almost all of the polyphenols found in all extracts seem to be more polar than catechin, assumed by the lower in R_f values (< 0.64). It is possible that flavonoid glycoside, phenolic acids as well as tannins which are more hydrophilic polyphenol compounds, could be the components in these extracts.

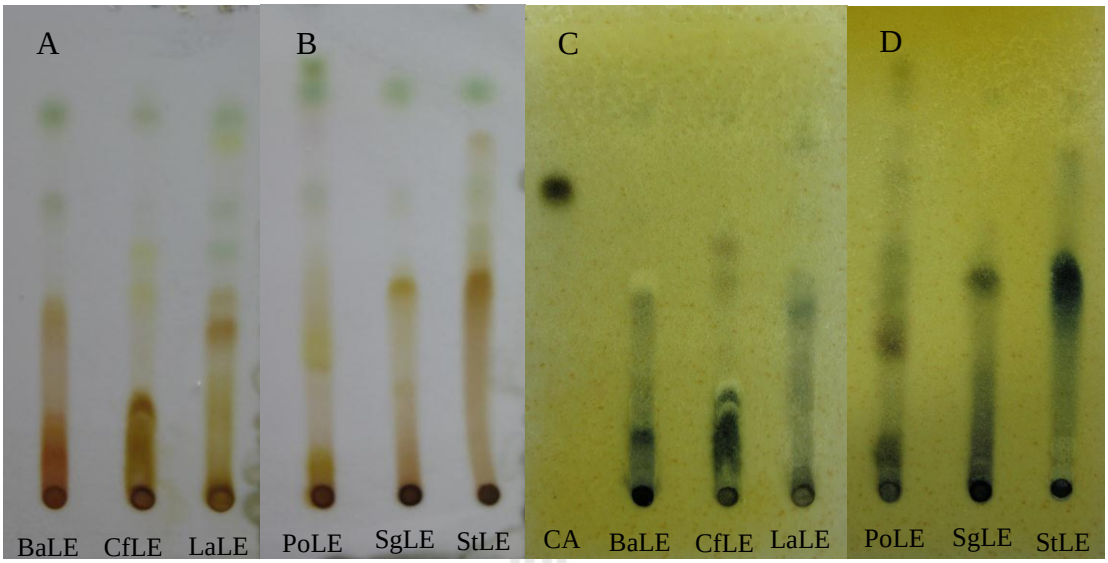


Figure 3.7 TLC plate of the 6 vegetable extracts was developed in ethyl acetate:methanol: water (81:11:8) and sprayed with specific reagents: A and B, Kedde reagent; C and D, $FeCl_3$ reagent. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; CA, catechin.

Table 3.4 Rf values of the 6 vegetable extracts by Kedde and $FeCl_3$ TLC detection.

TLC Detection	Leaf extracts					
	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
Kedde[a]	0.78 (G)	0.78 (G)	0.78 (G)	0.84 (G)	0.79 (G)	0.79 (G)
	0.60 (G)	0.50 (LY)	0.74 (LY)	0.79 (G)	0.41 (DY)	0.69 (LY)
	0.36 (LBr)	0.42 (LY)	0.59 (G)	0.59 (G)	0.21 (LBr)	0.60 (LY)
	0.14 (Br)	0.18 (DY)	0.51 (G)	0.42 (LY)	0.10 (LBr)	0.51 (LY)
	0.09 (Br)	0.11 (DY)	0.41 (LBr)	0.31 (LY)		0.46 (DY)
			0.34 (Br)	0.25 (Y)		
			0.18 (LBr)	0.06 (Y)		
$FeCl_2$[b]	0.78 (L)	0.78 (L)	0.74 (L)	0.84 (L)	0.80 (L)	0.80 (L)
	0.41 (L)	0.52 (L)	0.39 (L)	0.80 (L)	0.41 (D)	0.67 (L)
CA	0.13 (D)	0.45 (L)	0.21 (L)	0.62 (L)	0.22 (D)	0.42 (D)
0.64 (D)	0.01 (D)	0.21 (D)	0.04 (D)	0.47 (L)	0.08 (D)	0.00 (D)
		0.12 (D)		0.41 (L)	0.00 (D)	
		0.00 (D)		0.29 (D)		

[a](G), green; (Br), brown; (LBr), light brown; (Y), yellow; (LY), light yellow; (DY), dark yellow. [b](D), dark; (L), light. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; CA, catechin.

To analyze for high hydrophobic compounds such as essential oils and terpenoids, the system of hexane:acetone (70:30) was used as a mobile phase. TLC plate of the 6 vegetable leaf extracts after spraying with anisaldehyde/H_2SO_4 reagent and vanillin/H_2SO_4 reagent are demonstrated in Figure 3.8 and the R_f values are shown in Table 3.5. Essential oils, terpenoid and sterol form mainly blue, blue-violet, red zone (visible) when sprayed with these reagents (Figure 3.8). Eugenol and estradiol were used as the standards of phenyl essential oils and sterol triterpenoid which were detected at R_f value of 0.48 and 0.23, respectively. Essential oils were found in all extracts with different levels, mostly detected in CfLE, PoLE, and StLE. Interestingly, SgLE was the only one found as intense blue spot with R_f of 0.15 similar to estradiol (Figures 3.8B, 3.8D, and Table 3.5). These could be triterpenoids, being one of the crucial compounds found in SgLE.

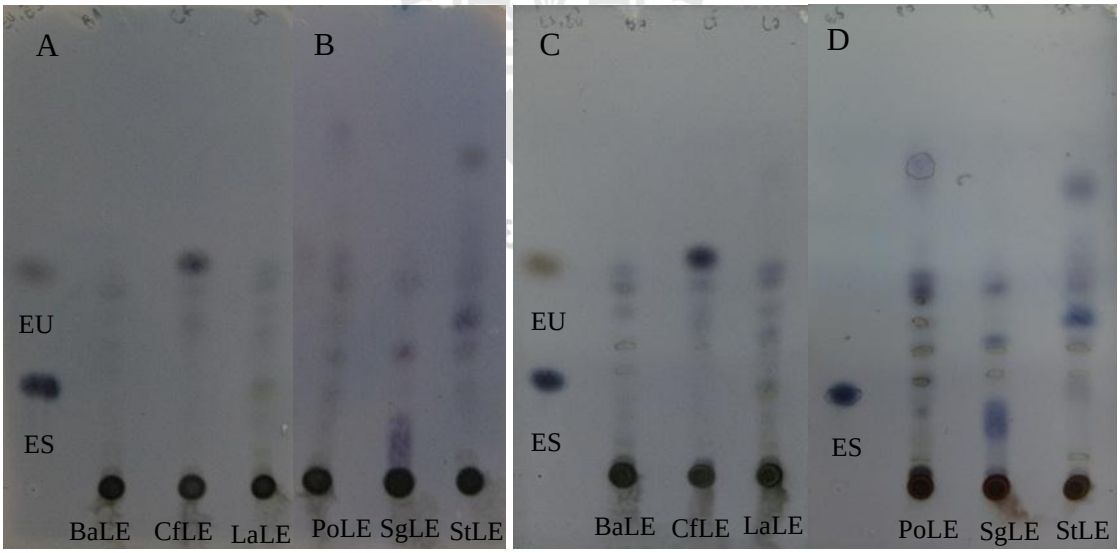


Figure 3.8 TLC plate of the 6 vegetable extracts was developed in hexane:acetone (70:30) and sprayed with specific reagents: A and B, anisaldehyde/H_2SO_4 reagent; C and D, vanillin/H_2SO_4 reagent. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; EU, eugenol; ES, estradiol.

Table 3.5 R_f values of the 6 vegetable leaf extracts for essential oils and terpenoids by anisaldehyde/H_2SO_4 and vanillin/H_2SO_4 TLC detection.

TLC Detection	Leaf extracts					
	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
Anis aldehyde[a]	0.43 (LV)	0.50 (BV)	0.46 (LV)	0.72 (LBl)	0.44 (Bl)	0.67 (Bl)
	0.40 (LV)	0.43 (LV)	0.40 (LBl)	0.48 (Bl)	0.33 (Bl)	0.45 (Bl)
	0.32 (LV)	0.38 (LV)	0.34 (LBl)	0.44 (Bl)	0.15 (BBl)	0.39 (BBl)
EU 0.48 (RV)			0.30 (Bl)			
			0.23 (YBl)			
ES 0.23 (BBl)						
Vanilin/ H_2SO_4[b]	0.47 (LBl)	0.45 (BV)	0.46 (LBl)	0.72 (LBl)	0.44 (Bl)	0.67 (Bl)
	0.43 (LBl)	0.43 (LBl)	0.38 (LBl)	0.48 (Bl)	0.33 (Bl)	0.45 (Bl)
	0.38 (LBl)	0.36 (LBl)	0.33 (LBl)	0.44 (Bl)	0.15 (BBl)	0.39 (BBl)
ES 0.23 (BBl)			0.21 (LBl)			

[a, b](LV), light violet; (BV), bright violet; (Bl), blue; (LBl), light blue; (BBl), bright blue; yellow blue; (YBl), red violet (RV). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; EU, eugenol; ES, estradiol.

For a precise differentiation of saponins and high hydrophilic compounds, chromatography must be performed in the more hydrophilic solvent system of chloroform:glacial acetic acid:methanol:water (64:32:12:8). TLC plate of the 6 vegetable leaf extracts after spraying with anisaldehyde/H_2SO_4 and vanillin/H_2SO_4 reagent for detecting saponins are demonstrated in Figure 3.9 and the R_f values are shown in Table 3.6. Saponins were mainly found in BaLE and StLE which indicated by the intense blue-violet zone and did not absorb 254 nm and 366 nm UV light at R_f values of 0.38 and 0.40-0.43, respectively.

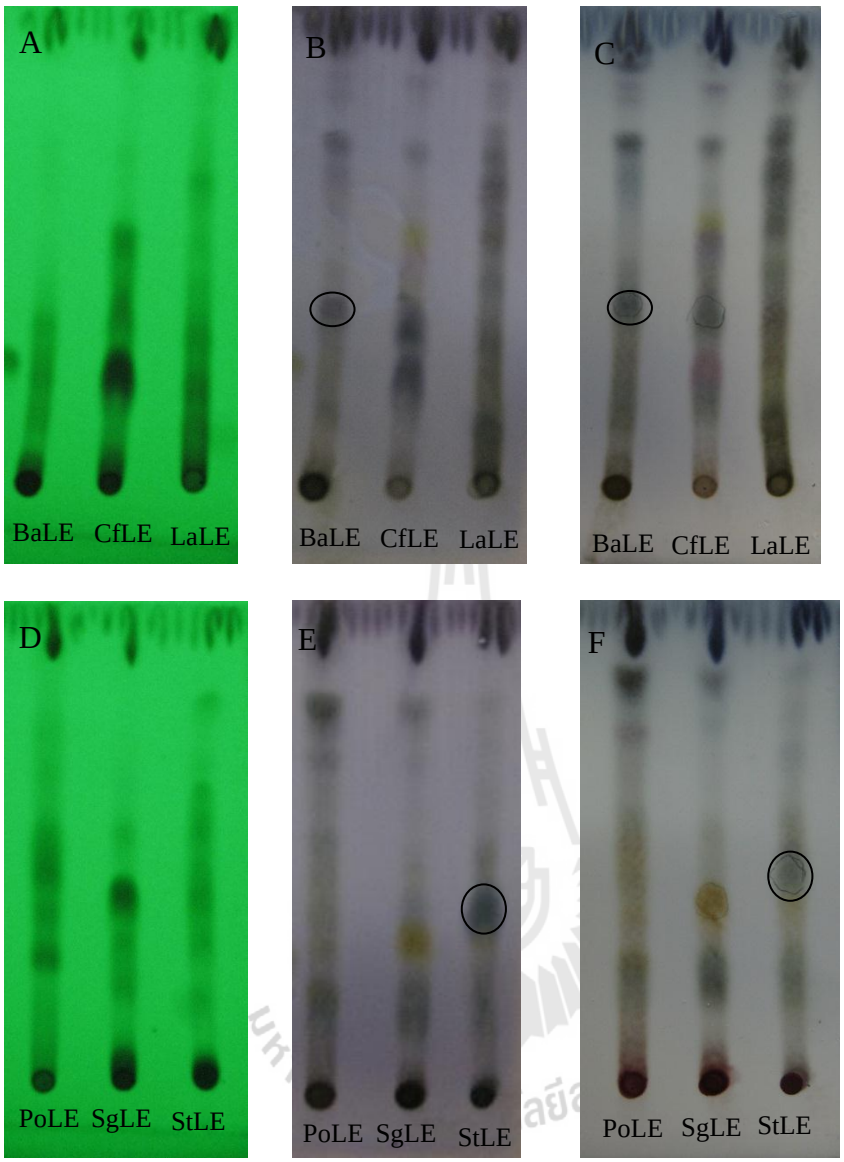


Figure 3.9 TLC plate of the 6 vegetable extracts was developed in chloroform:glacial acetic acid:methanol:water (64:32:12:8) under UV-254 light (A and D) and sprayed with specific reagents: anisaldehyde/H_2SO_4 reagent (B and E); vanillin/H_2SO_4 reagent (C and F). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

Table 3.6 R_f values of the 6 vegetable leaf extracts for saponins by anisaldehyde/H_2SO_4 and vanillin/H_2SO_4 TLC detection.

TLC Detection	Leaf extracts					
	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
Anis aldehyde[a]	0.72 (DBl)	0.71 (Bl)	0.68 (DBl)	0.83 (DBl)	0.83 (DBl)	0.53 (LBl)
	0.38 (Bl)	0.51 (YBr)	0.62 (DBl)	0.72 (Bl)	0.40 (LBl)	0.40 (BBl)
	0.14 (Bl)	0.33 (Bl)	0.56 (DBl)	0.54 (Bl)	0.33 (YBr)	0.35 (LBr)
		0.19 (Bl)	0.29 (DBl)	0.30 (Br)	0.22 (DBl)	0.19 (DBl)
			0.12 (DBl)	0.19 (DBl)	0.17 (DBl)	
Vanillin[b]	0.72 (DBl)	0.71 (DBl)	0.72 (DBl)	0.83 (DBl)	0.83 (Bl)	0.83 (LBl)
	0.58 (LBl)	0.54 (YBr)	0.65 (DBl)	0.72 (LBl)	0.70 (Bl)	0.54 (Bl)
	0.44 (LBl)	0.50 (Bl)	0.54 (DBl)	0.48 (YBr)	0.37 (YBr)	0.43 (Bl)
	0.38 (DBl)	0.37 (Bl)	0.34 (DBl)	0.34 (YBr)	0.24 (Bl)	0.38 (Br)
		0.25 (BlV)	0.15 (DBl)	0.24 (YBr)	0.19 (Bl)	0.24 (Bl)
				0.19 (Bl)		

[a, b] (Bl), blue; (DBl), dark blue; (LBl), light blue; (BBl), bright blue; (BlV), blue violet; (Br), brown; (YBr), yellow brown. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

TLC technique is widely used for the rapid analysis and qualitative screening of characteristic constituents in drugs and extracts. In addition, TLC also provides semi-quantitative information on major active constituents of drugs and extracts (Wagner and Bladt, 1995). TLC technique used in this study provided an important data on major constituents in the vegetable leaf extracts which would be useful for further identification.

3.4.2.2 Specific reagent testing of the 6 vegetable leaf extracts

1) Terpenoids (Salkowski test)

Salkowski test was used for qualitative detection of terpenoids in crude extract. Formation of yellow color ring at the interface that turned reddish brown color after 2 min was detected in SgLE and LaLE, representing the terpenoids (Figure 3.10).

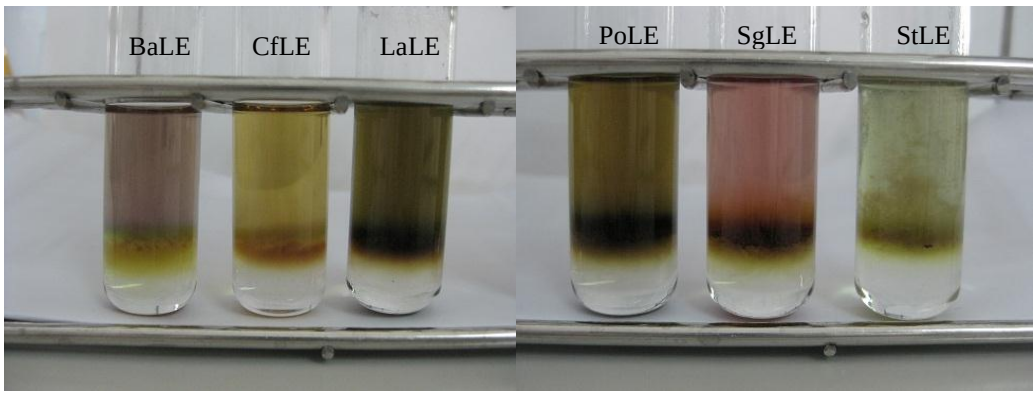


Figure 3.10 Salkowski test (for terpenoids) of the 6 vegetable leaf extracts. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

2) Saponins (Frothting test)

Saponins are classified as amphiphatic glycosides because of their surface-active properties to form a soap-like foaming when shaken in aqueous solution, and the form persists after 10 min (Silva, Lee, and Kinghorn, 1998). Their ability to form honeycomb froth is caused by the combination of the non-polar sapogenin and the water soluble side chains. The presence of saponins was confirmed by adding NaOH to basify the extract and eliminate the interference of some proteins and fatty acids which able to form froths. The formation of stable and dense froth indicates the presence of fatty acids. Furthermore, saponins were also confirmed by adding HCl, saponin glycoside can hydrolyze into aglycone sapogenin which cannot or is less ability to form a stable frothing when shaking (Trease and Evans, 1983). After shaking, BaLE showed honeycomb froth which greater than 2 cm height from the surface and stable for 10 min. The ability to foam was also detected in SgLE and StLE but low level in honeycomb froth approximately 1 cm height (Figure 3.11A). After adding NaOH (Figure 3.11B), stable and dense froth was detected in BaLE, while froth was unchanged or higher in LaLE, SgLE, and StLE. After

adding HCl (Figure 3.11C), less and no froth was observed in BaLE, LaLE, SgLE, and StLE indicated that these four extracts might contain different levels of saponins.

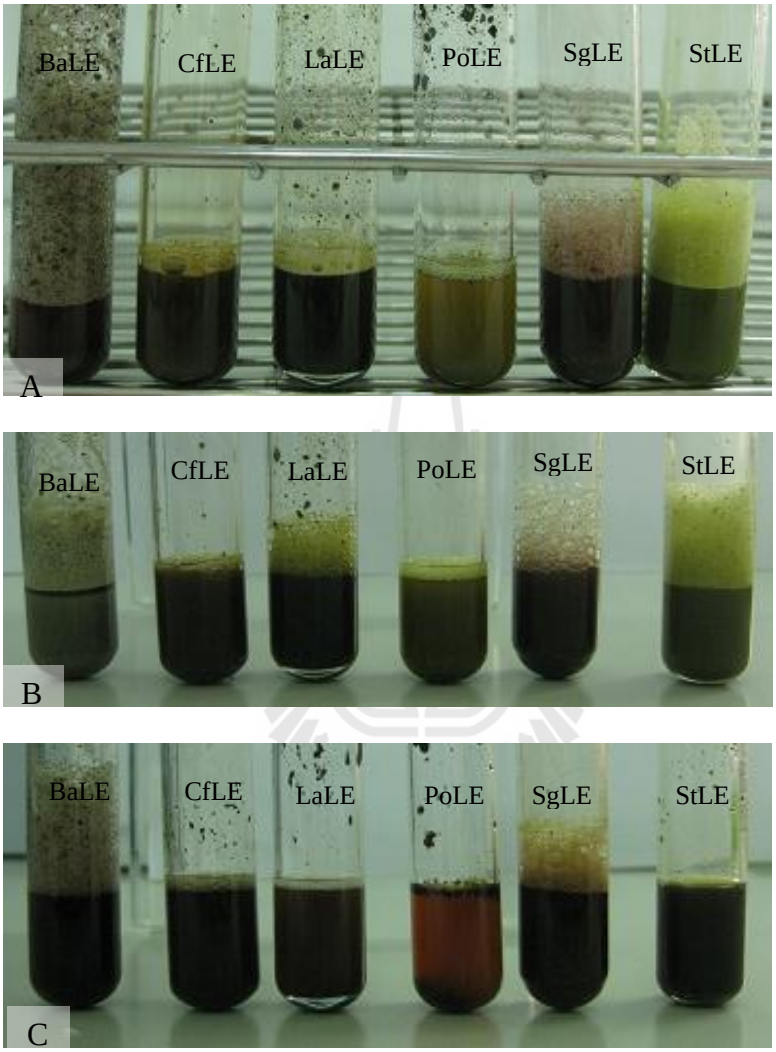


Figure 3.11 Frothing test (for saponins) of the 6 vegetable leaf extracts: A, after shaking, B, after adding NaOH and shaking, C, after adding HCl, boiling 10 min and shaking. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

3) Tannins (Ferric chloride test)

When adding 0.1% ferric chloride to the extracts, blue-black coloration was observed in BaLE, SgLE, and StLE while brownish green was in CfLE, LaLE, and PoLE with different levels indicating the hydrolysable tannins and condensed tannins (Figure 3.12).

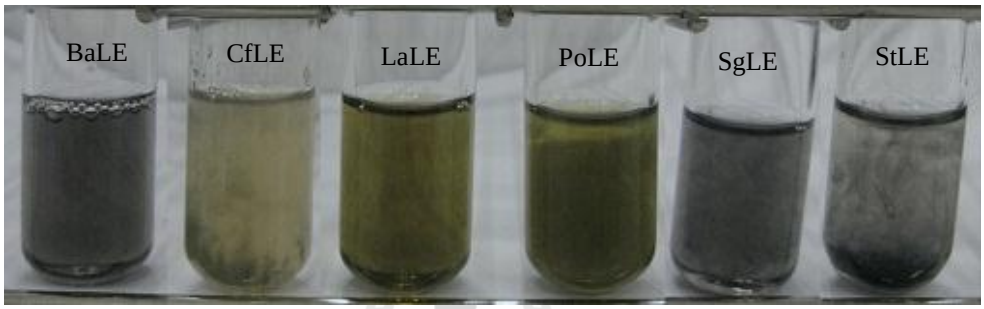


Figure 3.12 Ferric chloride test (for tannins) of the 6 vegetable leaf extracts. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

The phytochemical screening of the 6 vegetable leaf extracts by TLC and specific reagents are summerized in Table 3.7. Essential oils and tannins existed in all extracts with different levels, but alkaloids, anthraquinones, quinones, coumarins, and cardiac glycosides were not detected. Terpenoids were found only in LaLE and SgLE. Saponins were present in BaLE, LaLE, SgLE, and StLE.

These results indicated that BaLE possessed essential oils, saponins, tannins, and high TPC, which was similar to the study of Narayan, Row, and Sastry (1976). CfLE showed high TFC comprising mostly flavonoid phenolics, which was in agreement with the study of Maisuthisakul, Pongsawatmanit et al. (2007). The flavonoids in *C. formosum* leaf extract reported were chlorogenic, dicaffeoylquinic and ferulic acids. Furthermore, tannins

and the high amounts of essential oils were detected in CfLE. The aromatic plants, LaLE and PoLE, possessed high TFC and essential oils but low level in tannins.

Table 3.7 Summary of phytochemicals availability in the 6 vegetable leaf extracts detected by TLC fingerprinting and specific test.

Phytochemicals	Extract					
	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
Alkaloids[a]	-	-	-	-	-	-
Anthraquinones[a]	-	-	-	-	-	-
Quinones[a]	-	-	-	-	-	-
Coumarins[a]	-	-	-	-	-	-
Cardiac glycosides[a]	-	-	-	-	-	-
Essential oils[a]	+	++++	++	+++	+	+++
Terpenoids[b]	-	-	+	-	+++	-
Saponins[b]	+++	-	+	-	+	++++
Tannins[c]	+++	+	+	++	++++	+++++

[a] detected by TLC, [b]detected by TLC and testing with specific reagent, and [c]detected with specific reagent. The degrees of phytochemical content were arbitrarily assigned: +, very low; ++, low; +++, medium; ++++, high; +++++, very high; -, absent. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

LaLE could contain several flavonoids, such as nevadensin, nevadensin-7-o-β glycopyranoside, gardenin B and other flavones which are polyphenolic compounds with antioxidant activity as reported by Bui et al. (2004). The major essential oils in LaLE could be limonene, *trans*-isolimonene and α-humulene (Sribusarakum, Bunyapraphatsara, Vajragupta, and Watanabe, 2004). Furthermore, the low amounts of terpenoids and saponins were detected in LaLE. PoLE could contain flavonoids such as rutin, quercetin, catechin, isorhamnetin and kaempferal (Nanasombat and Teckchuen, 2009). In the meantime, essential oils in PoLE might be the aromatic organic compounds of (Z)-3-

hexenal, (Z)-3-hexenol, decanal, undecanal and dodecanal as well as sulfur compounds, 3-sulfanylhexanal and 3-sulfanyl-hexan-1-ol, which suspected to be responsible for the strong tropical fruit odors (Starkenmann, Luca, Niclass, Praz, and Roguet, 2006). Our study found that StLE contained high essential oils, saponins and tannins, which were in agreement with other reports (Johann et al., 2007; De Lima et al., 2006; Farag, 2008). In addition, the phenolic compounds such as caffeic acid, syringic acid, coumaric acid, ellagic acid, and gallic acid and terpenes were identified in *S. terebinthifolius* leaf extract and essential oil respectively (El-Massry, El-Ghorab, Shaaban, and Shibamoto, 2009). It was noted that this was the first study showing that terpenoids and tannins were the major compounds in SgLE with some essential oils and saponins.

3.4.3 Antioxidative properties (chemical assay) of the 6 vegetable leaf extracts

3.4.3.1 Ferric reducing antioxidant power

The FRAP assay is widely used for determination of antioxidants with reducing capacity. The reducing ability of the 6 vegetable leaf extracts was calculated by the formation of Fe^{II}-tripyridyltriazine compound (blue) when fitting with the calibration curve of ferrous sulfate. The more Fe^{II}-tripyridyltriazine is produced, the more reducing power ability. The 6 vegetable leaf extracts were determined for their reducing ability along with three standard antioxidants, the results of FRAP values in term of ferrous (Fe^{2+}), catechin, trolox and ascorbic acid equivalents are shown in Table 3.8. FRAP values of all extracts, catechin and ascorbic acid increased with incubating time up to 40 min (the observed time point) while trolox was unchanged after 10 min incubation. Regarding their antioxidant activities, all leaf extracts were obviously divided into 2 groups. Group I with high antioxidant activity were BaLE, SgLE, and StLE, and group II with low antioxidant activity were CfLE, LaLE, and PoLE.

Table 3.8 Ferric reducing antioxidant power (FRAP) of the 6 vegetable leaf extracts.

Sample	FRAP value (μmol Fe^{2+}/L)				FRAP value*			
	Incubation time (min)				μmol Fe^{2+}/mg	mg CAE/mg	mg TRE/mg	mg AAE/mg
	10	20	30	40				
BaLE	582.90	719.13	800.13	876.04	8.76 ± 1.52^{c}	0.71 ± 0.02	1.44 ± 0.34	0.58 ± 0.04
CfLE	346.32	376.34	402.04	426.34	4.26 ± 0.25^{e}	0.38 ± 0.02	0.71 ± 0.06	0.30 ± 0.02
LaLE	167.24	194.24	221.81	229.76	2.30 ± 0.12^{f}	0.15 ± 0.05	0.34 ± 0.15	0.14 ± 0.02
PoLE	281.41	317.86	342.17	369.05	3.69 ± 0.55^{e}	0.27 ± 0.02	0.57 ± 0.17	0.23 ± 0.01
SgLE	561.62	651.87	716.65	774.18	7.74 ± 0.66^{cd}	0.56 ± 0.05	0.93 ± 0.09	0.48 ± 0.04
StLE	481.03	621.33	716.26	808.93	8.09 ± 0.44^{c}	0.66 ± 0.07	1.36 ± 0.49	0.54 ± 0.03
CA	678.68	842.60	1037.15	1197.62	11.98 ± 1.52^{b}			
Trolox	651.19	661.03	661.99	665.10	6.65 ± 2.91^{d}			
AA	1220.34	1296.61	1394.09	1480.65	14.81 ± 1.46^{a}			

Presented value are means of determination (n = 6). *FRAP values are expressed as mean ± SD, (n = 6). Statistical analysis was performed by SPSS program ANOVA $p < 0.05$, alphabets: a-f. CAE, catechin equivalent; TRE, trolox equivalent; and AAE, ascorbic acid equivalent (mg/mg dried extract). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; CA, catechin; AA, ascorbic acid.

The reducing powers of group I (BaLE, SgLE, and StLE) were 8.76 ± 1.52, 7.74 ± 0.66, and 8.09 ± 0.44 µmol Fe^{2+}/mg extract respectively and showed higher than that of group II (CfLE, LaLE, and PoLE) which were 4.26 ± 0.24, 2.30 ± 0.12 and 3.69 ± 0.55 µmol Fe^{2+}/mg extract, respectively ($p < 0.05$) (Table 3.8 and Figure 3.13). The group I activity, BaLE, SgLE, and StLE were 1.44 ± 0.34, 0.93 ± 0.09, and 1.36 ± 0.49 mg TRE/mg sample which were similar to that of trolox but about 1.3-1.9 fold lower than CA with 0.71 ± 0.02, 0.56 ± 0.05, and 0.66 ± 0.07 mg CAE/mg sample, respectively. The reducing ability of the extracts was in order of BaLE > StLE > SgLE > CfLE > PoLE > LaLE, respectively.

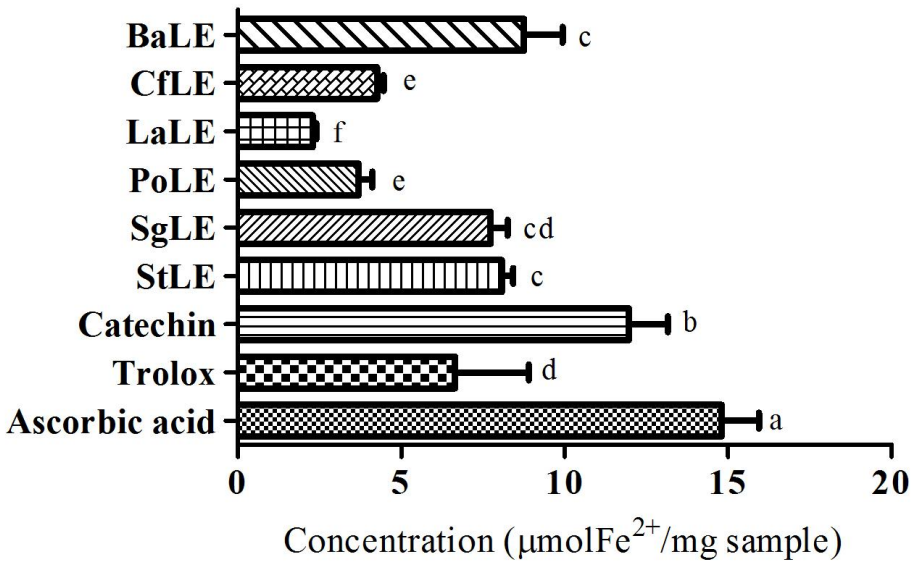


Figure 3.13 FRAP values of the 6 vegetable leaf extracts. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extracts. Numbers with different letters were statistically different ($p < 0.05$).

3.4.3.2 DPPH free radical scavenging activity

The amount of DPPH˙ remaining from initial of 40 mg DPPH˙/mL was calculated by fitting a linear regression of DPPH (2.5-40 mg DPPH˙/L), the results are shown in Table 3.9. The percentage of DPPH˙ inhibition was gradually increased with dose-dependent manner. The radical scavenging activity of group I (BaLE, SgLE, and StLE) expressed as EC_{50} values of 6.67 ± 0.10, 8.85 ± 0.09 and 5.54 ± 0.13 µg/ml, respectively, which were similar to CA, Trolox, and AA ($p < 0.05$). The radical scavenging activity of group II (CfLE, LaLE, and PoLE) showed EC_{50} values of 17.96 ± 0.03, 24.44 ± 0.30, and 16.19 ± 0.06 µg/ml, respectively. These results were quite different from Kukongviriyapan et al. (2007) studies which reporting that the aqueous extract of *C. formosum*, *S. gratum*, and *L. aromatica* had strong anti-oxidative property for DPPH assay which EC_{50} values of 3.63, 4.08, and 10.78 µg/mL, respectively. Moreover, as compared with the amount of DPPH˙, group I expressed EC_{50} value of 0.17, 0.22, and 0.14, respectively and group II were 0.45, 0.61, and 0.40 mg sample/mg DPPH˙, respectively. These results were different from Maisuthisakul et al. (2007) which reported that the EC_{50} values of 95% ethanolic extract of *C. formosum* and *S. gratum* were 0.23 and 0.55 µg/µg DPPH˙. The differences in sample preparations and extraction solvents could result different phytochemicals extracted into the solution and also gave different antioxidant activity.

Table 3.9 DPPH radical scavenging activity of the 6 vegetable leaf extracts.

Sample	$DPPH^{\cdot}$ remaining (mg/L)					$DPPH^{\cdot}$ scavenging activity (%)					EC_{50} (µg sample/ mL)	EC_{50} (mg sample/ mg $DPPH^{\cdot}$)	A_{AR} ($1/EC_{50}$)
	Concentration (µg/mL)					Concentration (µg/mL)							
	2	6	10	15	20	2	6	10	15	20			
BaLE	32.75	22.59	12.20	0.62		18.12	43.52	69.49	98.45	-	6.67 ± 0.10^{e}	0.17	5.85
CfLE	35.86	31.92	27.86	23.19	18.07	10.36	20.20	30.04	42.02	54.82	17.96 ± 0.03^{b}	0.45	2.23
LaLE	37.44	34.32	31.25	27.39	23.38	6.41	14.19	22.07	31.52	41.53	24.44 ± 0.30^{a}	0.61	1.64
PoLE	36.66	31.54	27.41	21.47	15.83	8.36	21.14	31.61	46.33	60.42	16.19 ± 0.06^{c}	0.40	2.47
SgLE	35.28	26.32	17.77	8.07	1.22	11.80	34.30	55.56	79.83	96.95	8.85 ± 0.09^{d}	0.22	4.52
StLE	31.30	19.00	7.11	0.84	-	21.74	52.49	82.22	97.89	-	5.54 ± 0.13^{ef}	0.14	7.22
CA	26.76	11.43	0.60	-		33.11	71.47	98.67	-	-	3.73 ± 0.07^{g}	0.09	10.73
Trolox	34.67	21.67	9.19	0.30	-	13.33	45.83	77.02	99.30	-	6.66 ± 0.02^{e}	0.17	6.01
AA	31.36	12.87	0.41	-	-	21.60	67.83	98.98	-	-	4.49 ± 0.13^{fg}	0.11	8.93

Presented value are means of determination (n = 6). EC_{50} values are expressed as mean ± SD, n = 6. Statistical analysis was performed by SPSS program ANOVA $p < 0.05$, alphabets: a-g. A_{AR}, Antiradical activity defined as $1/EC_{50}$. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; CA, catechin; AA, Ascorbic acid.

3.4.3.3 Microsomal lipid peroxidation

The product from lipid peroxidation system was calculated from calibration MDA curve and expressed as the percent lipid peroxidation (LPO) inhibition as shown in Table 3.10. The LPO inhibition capacity (EC_{50}), on rat liver microsomal model and induced by ferric-ascorbate, of the group I (BaLE, SgLE, and StLE) also showed significantly higher capacity ($p < 0.05$) than group II with 3.92 ± 0.01, 3.54 ± 0.21, and 6.50 ± 0.29 µg/mL, respectively. It was similar to the capacity of CA and Trolox. The inhibition capacity (EC_{50}) of the group II (CfLE, LaLE, and PoLE) was 23.13 ± 1.34, 17.58 ± 1.04, and 11.44 ± 2.61 µg/mL, respectively.

The EC_{50} values of the 6 vegetable extracts and standard antioxidants by DPPH and LPO inhibition assay were plotted as shown in Figure 3.14. The lower in EC_{50} values, the higher in antioxidant capacity. The antioxidative capacity were in the order of StLE > BaLE > SgLE > PoLE > CfLE > LaLE on DPPH radical scavenging and SgLE > BaLE > StLE > PoLE > LaLE > CfLE on LPO inhibition.

Because of the antioxidant properties of phytochemicals are very complex and different between the tested systems, therefore *in-vitro* study for quantifying antioxidant capacity of the extracts was conducted comparing with the standard compounds. The electron transfer (ET)-based assays including FRAP and DPPH is excellent for measuring the reducing capacity of antioxidants (Somogyi et al., 2007). The high sensitivity, reproducibility and speed of these assays are the reasons for the popular evaluating assay. The inhibition of ferric/ascorbate-induced lipid peroxidation is classified as hydrogen atom transfer (HAT)-based assays. The present of metals such as Fe^{3+} can results in an acceleration of hydroperoxide decomposition to form the peroxyl radical and alkoxyl radical: $Fe^{3+} + ROOH \rightarrow Fe^{2+} + ROO^{\cdot} + H^{+}$. Peroxyl radicals are good oxidizing agents, having more than 1000 mV of standard reduction (Decker, 1998).

Table 3.10 The amount of MDA, LPO inhibition and EC_{50} values of the 6 vegetable leaf extracts against lipid microsome peroxidation.

Sample	Amount of MDA (µg/L)								LPO Inhibition (%)							LPO inhibition
	Concentration (µg/mL)								Concentration (µg/mL)							EC_{50}
	0	1	5	10	15	20	30	40	1	5	10	15	20	30	40	(µg sample/ mL)
Negative control	0.53															
Positive control	6.47															
BaLE		6.03	2.36	0.49	-	-	-	-	6.77	62.35	92.33	-	-	-	-	3.92 ± 0.01^{e}
CfLE		-	-	5.59	-	3.84	2.09	1.28	-	-	13.62	-	40.70	61.69	80.13	23.13 ± 1.34^{a}
LaLE		-	-	4.65	-	2.38	1.41	0.65	-	-	28.05	-	63.23	78.17	89.91	17.58 ± 1.04^{b}
PoLE		-	4.10	3.87	2.65	1.63	-	0.49	-	36.68	40.15	59.07	74.75	-	92.48	11.44 ± 2.61^{c}
SgLE		6.12	2.14	1.05	0.45	-	-	-	5.39	66.98	83.81	93.06	-	-	-	3.54 ± 0.21^{e}
StLE		4.85	4.15	0.92	0.50	-	-	-	25.01	35.80	85.69	92.23	-	-	-	6.50 ± 0.29^{d}
CA		6.13	4.15	1.54	0.25	-	-	-	5.24	35.80	76.23	96.02	-	-	-	7.24 ± 0.14^{d}
Trolox		4.60	1.81	1.27	0.33	-	-	-	28.97	72.04	80.38	94.86	-	-	-	2.33 ± 0.17^{e}

Presented value are means of determination (n = 6). EC_{50} values are expressed as mean ± SD, (n = 6). Statistical analysis was performed by SPSS program ANOVA $p < 0.05$, alphabets: a-e. LPO inhibition, lipid peroxidation. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; CA, catechin; AA, ascorbic acid.

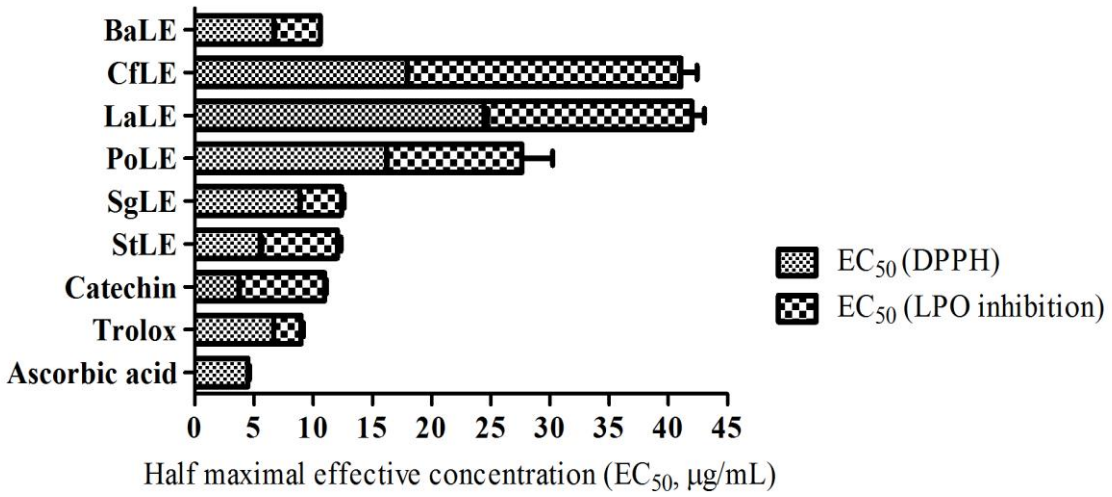


Figure 3.14 Half maximal effective concentration (EC_{50}) of the 6 vegetable leaf extracts by DPPH and LPO inhibition assay. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extracts.

Furthermore, Fe^{3+} is reduced to Fe^{2+} by ascorbic acid: ascorbic acid + Fe^{3+} → dehydroascorbic acid + Fe^{2+}. In the presence of Fe^{2+}, O_2 produces O_2^- or H_2O_2, and H_2O_2 and LOOH (lipid hydroperoxide) are converted to $OH^{\cdot}$ (hydroxyl radical) and $LO^{\cdot}$ (Wakabayashi et al., 1999). The chain reactions of free radical occur where a free radical (such as $OH^{\cdot}$) capture a hydrogen atom from an unsaturated fatty acid. This causes an unpaired electron on the fatty acid that is then capable of capturing oxygen, forming a peroxy radical. Lipid peroxides are unstable and decompose to form a complex series of compounds, including malondialdehyde (MDA) thereafter. Our study used the above mentioned three systems to evaluate these 6 vegetable extracts, similar patterns for each plant were observed in each measuring system.

The phytochemical composition in the vegetable leaf extracts contributed to the antioxidant capacities. The high amount of tannin phenolics in BaLE, SgLE, and StLE (group I) could contribute to the high potency of antioxidant activity.

Polyphenolic tannins from plants such as condensed and hydrolysable tannins have been shown to be powerful antioxidants as supported by Yokozawa et al. (1998). They demonstrated that tannins have more potential DPPH free radical scavenging property than flavonoids. It could be a reason that CfLE, LaLE, and PoLE (group II), containing much higher flavonoids and lesser tannins than group I, possessed low antioxidant potency. This study suggested that BaLE, SgLE, and StLE (group I), containing very high polyphenolics and very low flavonoids, were highly potent as antioxidants. The CfLE, LaLE, and PoLE (group II), containing very high flavonoids, were less potent as antioxidants.

3.4.4 Relationships of TPC, TFC and antioxidant activities (chemical assay)

The correlations of TPC and TFC with antioxidant activities of the 6 vegetable leaf extracts are demonstrated in Figure 3.15. Our study showed that the extracts containing high TPC were well correlated with high FRAP value of the extracts (R = 0.855), but the ones with low TFC were correlated with high FRAP values (R = -0.678) (Figures 3.15A, 3.15B). Furthermore, high TPC demonstrated well correlated with low value of EC_{50} in DPPH scavenging activity (R = -0.882), but high TFC were not correlated (R = 0.676) (Figure 3.15 C, D). These results were in agreement with other reports (Wojdylo, Oszmianski, and Czemerys, 2007; Katalinic, Milos, Kulisic, and Jukic, 2006; Capecka, Mareczek, and Leja, 2005). TPC showed low correlation with the EC_{50} values of lipid peroxidation (LPO) inhibition (R = -0.469) while TFC showed high correlation (R = 0.948) (Figures 3.15E, 3.15F). The complex of polyphenols which accounted for their contribution between hydrophobic and hydrophilic phases and their interface in LPO system could be the reason for the poor correlation (Joubert et al., 2008). Furthermore, the pro-oxidant effects of some flavonoids on the metal-induced LPO system (Sugihara, Arakawa, Ohnishi, and Furuno, 1999) could be the reason for high EC_{50} values in the extracts with high TFC. It could be summarized that polyphenolic components in these extracts were potent as

antioxidants in free radical scavenging and reducing power while small amount of flavonoid components were potent as antioxidants in lipid peroxidation.

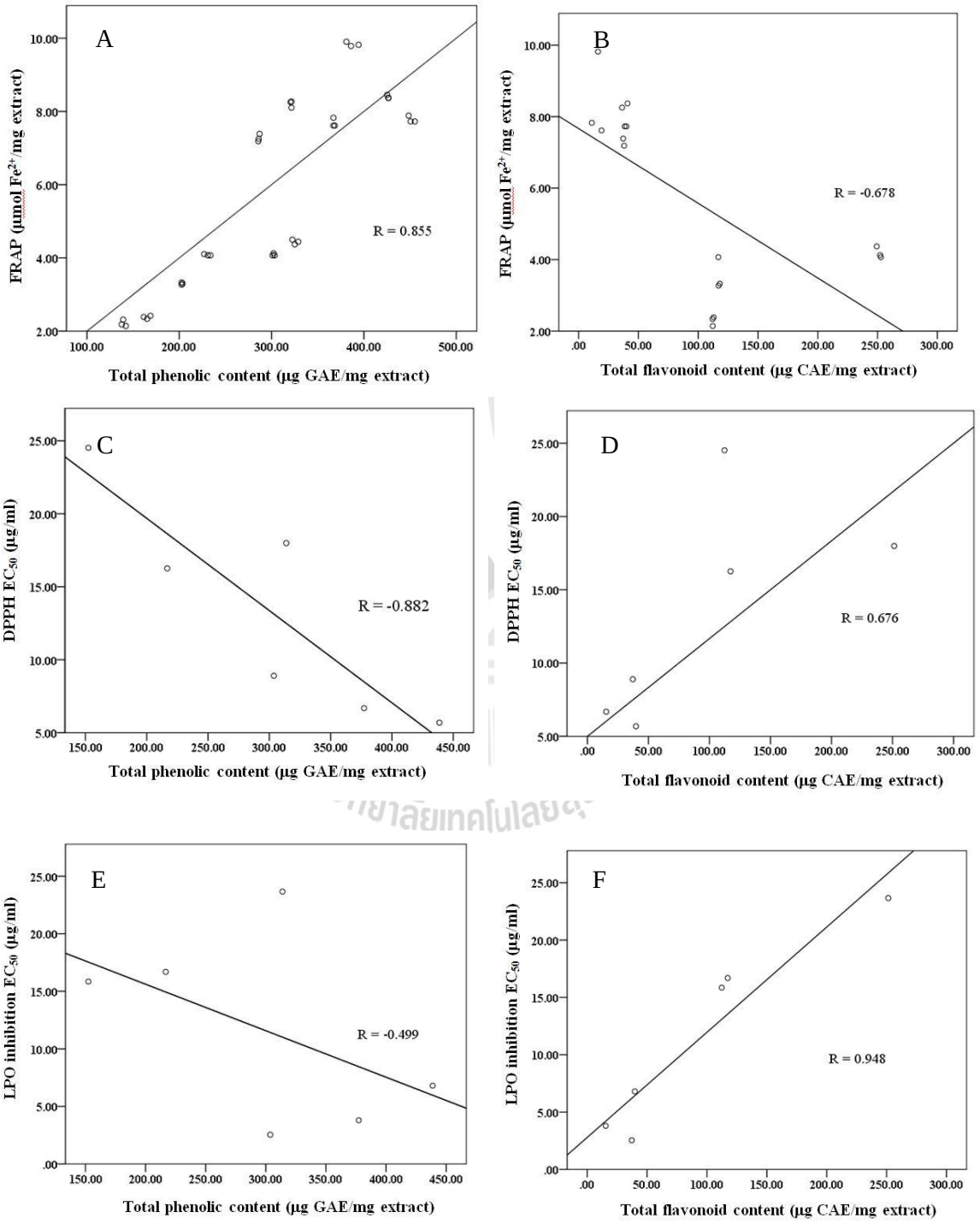


Figure 3.15 Relationships of TPC, TFC and antioxidant activity. (A) TPC and FRAP; (B) TFC and FRAP; (C) TPC and DPPH, EC_{50}; (D) TFC and DPPH, EC_{50}; (E) TPC and lipid peroxidation (LPO) inhibition, EC_{50}; (F) TFC and LPO inhibition, EC_{50}.

3.4.5 Cellular antioxidant activity (ROS inhibition)

The concentrations of 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) used to detect intracellular ROS were ranged 5-100 µM with the variety of oxidizing agents used. *Tert*-butylhydroperoxide (*t*-BuOOH) was chosen as an oxidizing agent in this experiment. The concentrations of DCFH-DA used to detect intracellular ROS in HepG2 cell line, induced by 200 µM *t*-BuOOH, are illustrated in Figure 3.16.

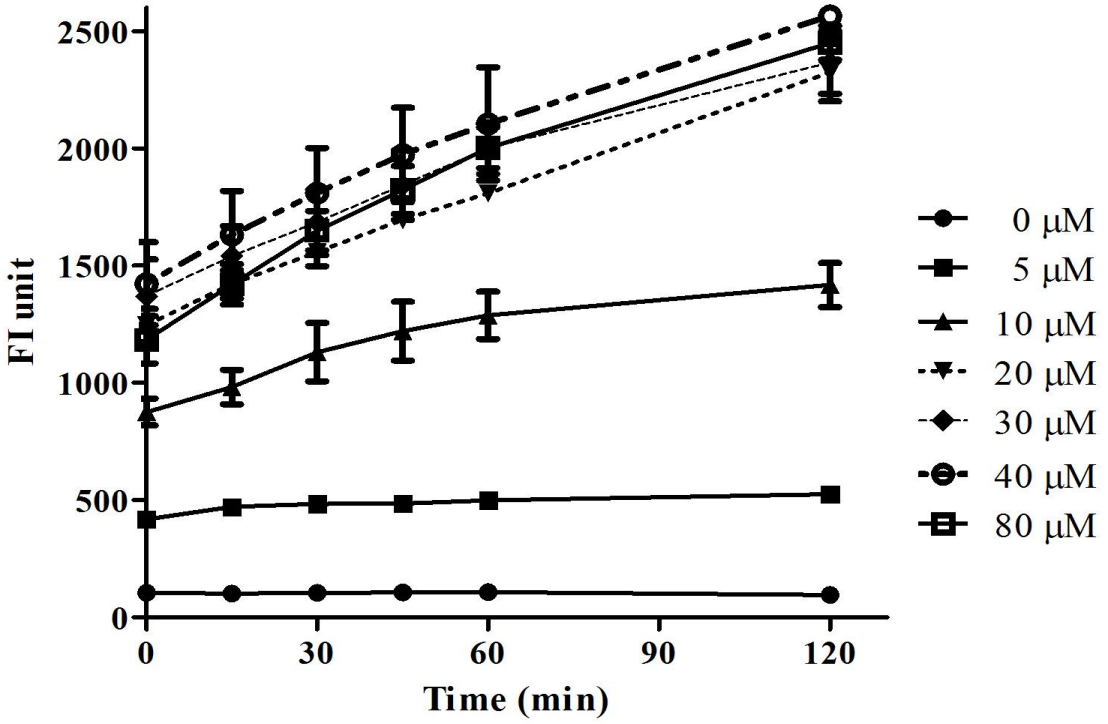


Figure 3.16 Measurement, using concentrations of DCFH-DA (0-80 µM), of ROS production in HepG2 cells line during incubation with *t*-BuOOH (200 µM).

The results showed that 20 µM DCFH-DA was the optimum concentration for detecting ROS production. At this concentration, relative fluorescence was not significantly different from that was observed with 30 or 40 µM DCFH-DA. Therefore, the concentration of 20 µM DCFH-DA was used for this experiment.

Unlike the chemical antioxidant activity measurements, the cellular antioxidants demonstrated different results, in term of the concentration used. The

concentrations used for monitoring their ability to inhibit ROS production were significantly higher than that used in chemical assay even with catechin and trolox. Furthermore, there was a limit on the concentration use; the cytotoxic was occurred when using the high concentration. The highest concentration used for each extract that did not showed the cytotoxicity for cells, were presented in Figures 3.17A, 3.17B. The fluorescence intensity of cells decreased as compare with control when treated with various extracts. In this experiment, we could not calculate for the 50% inhibition, nevertheless, the dose-dependence manner of each extract could be demonstrated as Figure 3.18. BaLE and StLE of 20 µg/mL, and SgLE of 25 µg/mL presented significant reduction ($p < 0.05$) with the percentage of ROS inhibition of 26.43% and 12.34%, and 17.98%, respectively whereas LaLE, PoLE, and CfLE did not show significant ROS reduction ($p > 0.05$) when compared to induced control. LaLE, PoLE and CfLE of 100 µg/mL significantly reduced ROS productions ($p < 0.05$) which were 35.59%, 28.99%, and 28.30% ROS inhibition, respectively. Catechin and trolox demonstrated dramatically decrease in the ROS production with EC_{50} value of 76.80 and 95.90 µg/mL, respectively (data not show). This study had similar result to Wolfe and Liu (2007) study which reported the EC_{50} value of 292 µM for catechin.

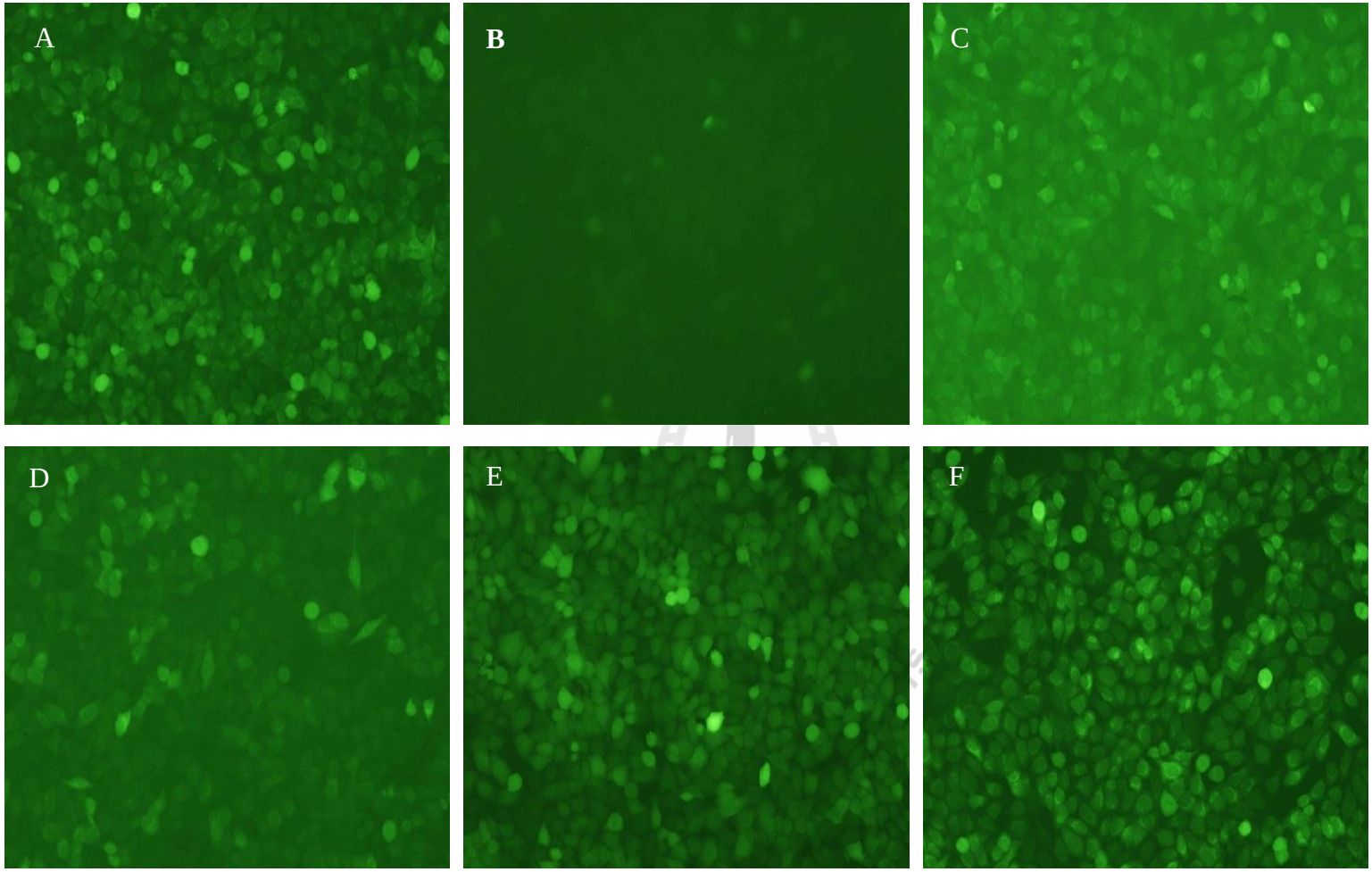


Figure 3.17A Photomicrographs of HepG2 cells pre-treated with effective concentration of BaLE, CfLE, LaLE and PoLE, then DCFH-DA uptake and induced the oxidative stress with *t*-BuOOH for 60 min. The induced control (A), un-induced control (B), and pre-treated with BaLE 20 µg/mL (C), CfLE, LaLE and PoLE 100 µg/mL (D-F) (Photomicrograph, magnification × 100).

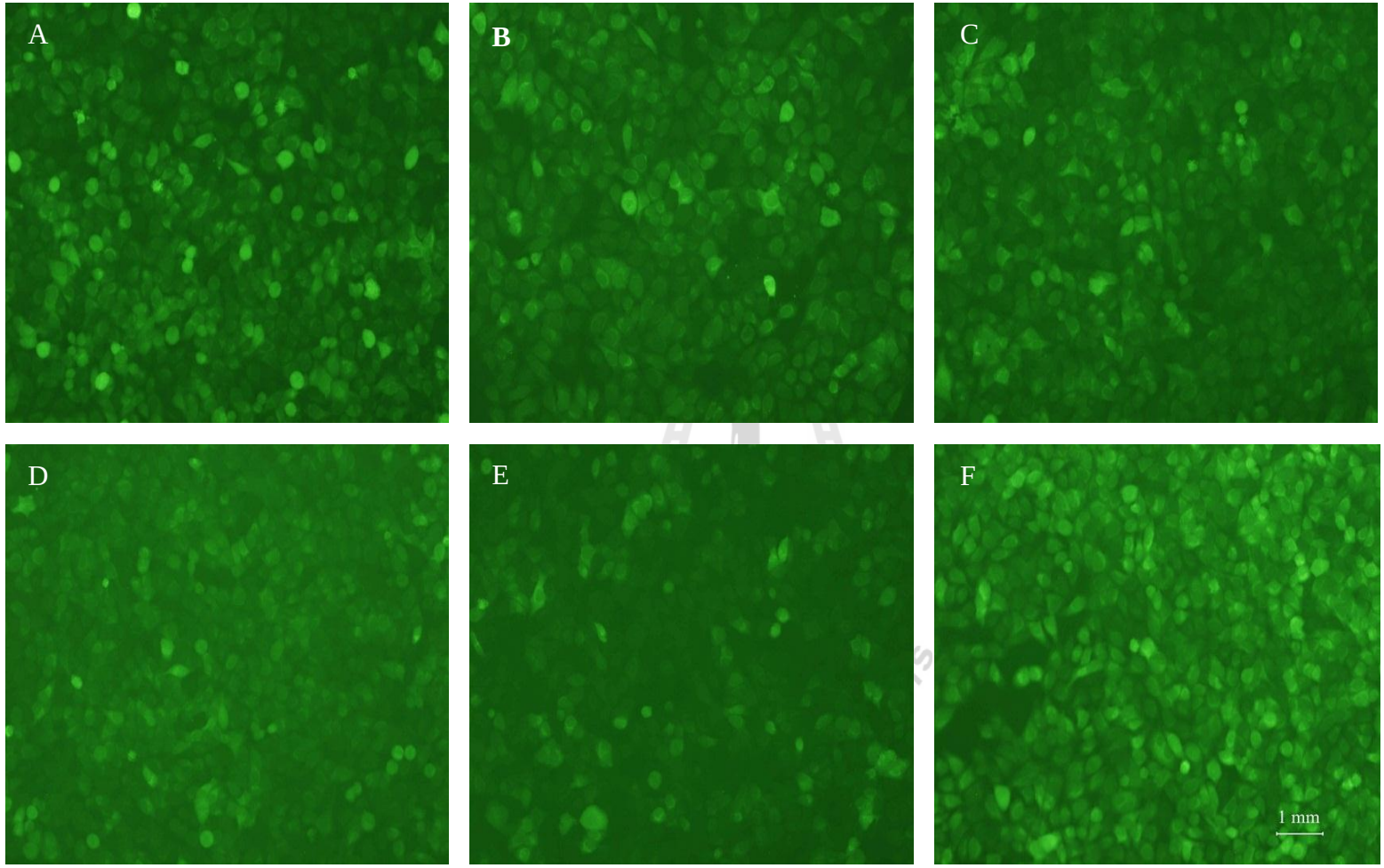


Figure 3.17B Photomicrographs of HepG2 cells pre-treated with effective concentration of SgLE, StLE, and standard antioxidants, then DCFH-DA uptake and induced the oxidative stress with *t*-BuOOH for 60 min. The induced control (A), pre-treated with SgLE 50 µg/mL, StLE 40 µg/mL (B-C) and catechin, trolox, and ascorbic acid 100 µg/mL (D-F) (Photomicrograph, magnification×100).

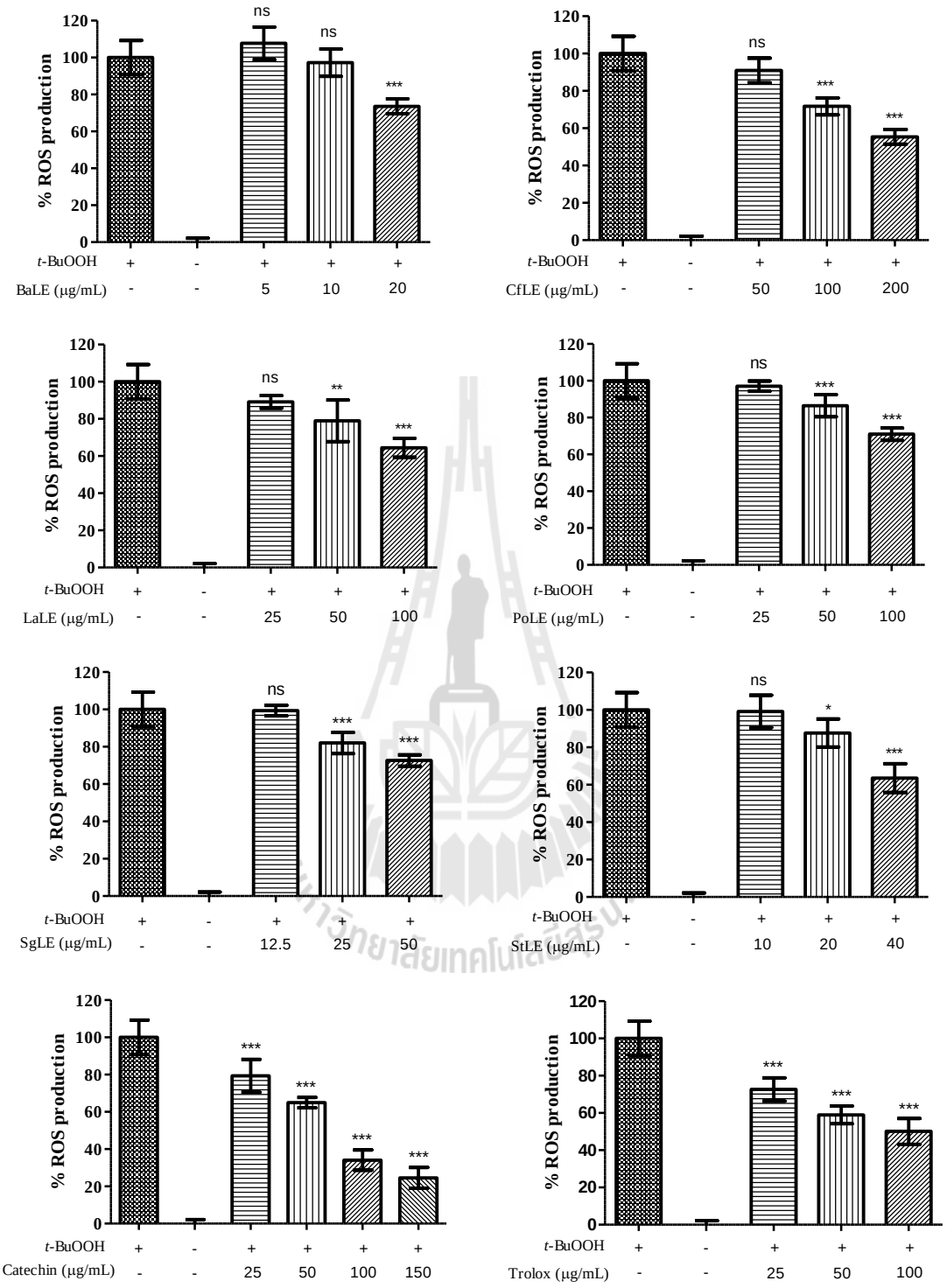


Figure 3.18 The percentage of ROS production in HepG2 cells treated with the 6 vegetable extracts and pure standard compounds. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extracts. Statistical analysis was performed by ANOVA and compared to induced control by Dunnett's comparison test: ns, no significant different; *, 0.05; **, 0.01; ***, 0.001.

Cellular antioxidant activity (CAA) assay was developed for a more biologically representative method but this assay is not consistent in the protocols used. Differences exist in the cell lines, types of oxidants, media, concentrations of reagents, treatment orders and times, and oxidative stress quantification methods has been reported. The results should be standardized and compared among laboratories (Wolfe and Liu, 2007). In this study, HepG2 cells were treated with extracts and then uptaked the DCFH-DA, washed and generated ROS by *t*-BuOOH. According to the study of Wolfe et al. (2008), it was mentioned that some phenolics highly absorbed by the HepG2 cells or tightly bound to the cell membrane were more likely to exert their radical scavenging activities after the cells are washed in the PBS wash protocol than those poorly absorbed or only loosely associated with the cell membrane and easily washed away. These could be the reason for low antioxidant activity detecting in the tested vegetable extracts. In addition, the serum-free medium used in the experiment could somehow starve the cells, these could be the reason for the cytotoxicity on HepG2.

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3.5 Conclusions

The six local Thai vegetable leaf extracts, *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) contained similar phytochemicals with different levels. BaLE contained high TPC, saponin, and tannin, but low in TFC and essential oils. CfLE was high in TFC and essential oils with some tannins. LaLE was high in TFC with some essential oils, terpenoids, and tannins. PoLE was high in TFC and essential oils. SgLE contained most tannins and terpenoids with some essential oils and saponins. StLE contained essential oils, saponins and tannins in high levels.

The phytochemicals composition in the vegetable leaf extracts contributed to the antioxidant properties. These tested extracts could be classified into 2 groups. Group I were BaLE, SgLE, and StLE contained high amount of tannin phenolics and processed high antioxidant activities in three chemical assays: FRAP, DPPH, and lipid peroxidation assay. Group II were CfLE, LaLE, and PoLE, contained lower tannins and higher flavonoids than Group I, and exhibited lower antioxidant activities than Group I and standard antioxidants; catechin, trolox, and ascorbic acid ($p < 0.05$).

Group I also showed higher cellular antioxidant capacity than Group II. Group I, BaLE and StLE of 20 µg/mL, and SgLE of 25 µg/mL significantly reduced ROS generation ($p < 0.05$) with 26.40%, 12.34%, and 17.99% ROS inhibition, respectively. Whereas, Group II, LaLE, PoLE and CfLE) exhibited significant difference from induced control ($p < 0.05$) when treated at 100 µg/mL with 35.59%, 28.99%, and 28.30% ROS inhibition, respectively.

These studies provided the valuable information for supporting the health benefit of ethanolic extracts of *Barringtonia acutangula*, *Cratoxylum formosum*, *Limnophila aromatica*, *Polygonum odoratum*, *Syzygium gratum*, and *Schinus terebinthifolius* which could be recommend for regular consumption as sources of effective antioxidants.

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CHAPTER IV

CYTOTOXIC EFFECT OF SOME LOCAL THAI VEGETABLE LEAF EXTRACTS

4.1 Abstract

The leaf extracts of six local Thai vegetables, *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) were screened for cytotoxic effect by brine shrimp lethality test (BSLT). The cytotoxic activity of the extracts was also tested on human normal lymphocyte and three cell lines by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and on the cell membrane instability of human erythrocyte cells by hemolytic assay. BaLE demonstrated cytotoxic against *Artemia salina* nauplii, normal lymphocyte cells, and also on MCF7 human breast cancer and HepG2 human liver cancer cells with similar in IC_{50} values ranging from 112.70-196.40 µg/mL. However, BaLE and StLE seems to be more selective on Jurkat human leukemic cells with IC_{50} values of 66.91 and 75.36 µg/mL, respectively. Moreover, it was found that cytotoxic of BaLE and StLE might be related to cytolytic ability on erythrocytes which showed the EC_{50} values of 6.69 and 134.60 µg/mL. CfLE showed high cytotoxic effect only on *A. salina* with IC_{50} values of 37.0 µg/mL. LaLE and PoLE were selectively toxic to Jurkat cells with IC_{50} values of 128.50 and 146.80 µg/mL, whereas less toxic on normal lymphocytes with IC_{50} value of > 400 and 332.90 µg/mL, respectively. SgLE was toxic to MCF7 breast cancer cells with IC_{50} values of 66.71 µg/mL and slightly effect on erythrocyte membrane at high concentration (1200 µg/mL). Crude

extract often contain a mixture of diverse cytotoxic compounds so it elicits different diverse activities. However, the screening data provided important information on cytotoxicity which could be extrapolate for further *in vivo* study.

4.2 Introduction

The universal property of tumor cells is uncontrollable cell proliferation. In order to prevent tumor development by suppressing mechanism, the effective chemopreventive agents might target on the cell growth control mechanism including regulation of cell cycle, apoptosis or cellular senescence (Ramos, 2007). Thus, cytotoxicity screening models provide important preliminary data to help for selection of plant extracts with potential antitumor properties for further studies.

The methods for detection of biological activity of plant extracts can be divided into two groups for screening purposes: primary screening bioassays and specialized screening bioassays. Primary and specialized screenings are ideal in the first step in testing a specific hypothesis about the potential of agonist or antagonist of a particular molecular target to demonstrate pharmacological activity (Ghisalberti, 1993).

Primary screening bioassay by brine shrimp lethality test (BSLT) is one of the general bioassay that is capable of detecting a broad spectrum of bioactivity present in crude extracts. (Meyer et al., 1982). This technique is an *in vivo* lethality test using a tiny crustacean *Artemia salina*. It is easily mastered, low cost, and utilizes small amounts of test material. The aim of the method is to provide a front-line screen that can be backed up by more specific and more expensive bioassays once the active compounds have been isolated. It appears that BSLT is predictive of cytotoxicity and pesticidal activity (Anderson, Goetz, McLaughlin, and Suffness, 1991).

The specialized screening assays are the *in vitro* tests which are more sophisticated than primary screening bioassays. Cellular assays utilizing tumor cell line derived from

human or animal origin have been adopted by National Cancer Institute (NCI) for more than half a century to screen for cytotoxic agents from natural sources. Furthermore, a large number of cytotoxicity based assays on the human tumor cell line has been used as prescreen for further studies by *in vivo* testing on antitumor and antineoplastic activity (Ghisalberti, 1993). The assays dealing with cell cultures have one major advantage i.e. all potential mechanisms concerning cellular proliferation are simultaneously monitored.

Cytotoxicity, or toxicity to cells in culture, can be subdivided into cytostatic activity i.e., stopping cell growth (often reversible) and cytocidal activity i.e. killing cells. The cytotoxic effect can be assessed by different methods, e.g. cell counting, determination of proliferation rates, synthesis of different cell products or determination of enzyme activities. The vital stain trypan blue, which differentiate live cells from dying cell, has been used but this is time consuming and inconvenient for screening. The use of tetrazolium salts, MTT dye, to assay cell proliferation, cell viability, and cytotoxicity is now used instead and a widespread and establish practice for their sensitive and scalable approach. The reaction is attributed mainly to mitochondrial enzymes and electron carries in living cells.

Plant-derived foods offer a source of natural compounds with interesting biological and pharmacological properties (Lampe, 1999). The consumption of diets rich in plant foods e.g. fruits and vegetables has been associated with reduced risk of a variety of tumors (Steinmetz and Potter, 1996). Therefore, many studies of tumor cells have been continuously emphasize on the cytotoxic effect of dietary phytochemicals which could contribute to the prevention of cancer development (Yáñez et al., 2004; Chang et al., 2008). Local Thai vegetables, *Barringtonia acutangula*, *Cratoxylum formosum*, *Limnophila aromatica*, *Polygonum odoratum*, *Syzygium gratum*, and *Schinus terebinthifolius* are mostly grown in the Northeast of Thailand and their young leaves are commonly consumed in daily diet as fresh or cooked vegetables or ingredients in Thai cuisine. Some of these plants have been investigated on cytotoxic and antitumor activity. Crude extract from *C. formosum*

showed cytotoxic effect on HepG2 liver cancer cell line (Prayong, Barusrux, and Weerapreeyakul, 2008). The methanolic extract of *L. aromatica* and *P. odoratum* leaves had potent antitumor-promoting activity *in vitro* determined by Epstein-Barr virus activation assay (Murakami, Jiwajinda, Koshimizu, and Ohigashi, 1995). Moreover, Nanasombat and Teckchuen (2009) demonstrated that *P. odoratum* leave extract had an active cytotoxic effect against MCF7 breast cancer cells proliferation. The polyphenols from *S. terebinthifolius* leaves extract inhibits cell proliferation and induces cell cycle arrest and apoptosis in the androgen-insensitive DU145 prostate carcinoma cells (Queires et al., 2006). There is little information available at the present regarding to the cytotoxic and cytolytic effects of these selected plants.

Therefore, the aims of this study is to evaluate the cytotoxic potential of six Thai local vegetable extracts on *A. salina* nauplii, normal lymphocytes, three cancerous cell lines and the cytolytic property on human erythrocytes.

4.3 Materials and methods

4.3.1 Cell lines, Reagents

MCF7 (human breast cancer) was a gift from R.P. Shiu, Dubik, and Shiu, 1992 and HepG2 (human liver cancer) was from American Type Culture Collection (ATCC) and Jurkat E6.1 (human leukemic T) cell line was purchased from Cell Line Services (CLS), Germany. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco (New York, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) was purchased from Invitrogen (California, USA). Catechin, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), HISTOPAQUE®-1077 were purchased from Sigma-Aldrich Company Ltd. (St. Louis MO, USA). Dimethylsulfoxide (DMSO) was obtained from Amresco (Ohio, USA).

4.3.2 Plant materials

Fresh young leaves of six local Thai vegetables were purchased in the local market at Nakhon Ratchasima province, Thailand during September 2008 to February 2009.

4.3.3 Sample preparation

The young leave ethanolic extracts of *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) were prepared as previously described in Chapter III : Sample preparation. The dried crude extracts were dissolved in DMSO, and then diluted to 0.2% (v/v) DMSO in cell culture medium when preparing the indicated concentrations.

4.3.4 Brine shrimp lethality assay

4.3.4.1 Preparation for artificial sea water and samples

Artificial sea water was prepared using sea salt 38 g/L and adjusted to pH 8.5. Dried crude extracts were dissolved in DMSO before diluted with sea water to desired concentrations with 0.4% (v/v) DMSO in final. Artificial sea water with 0.4% DMSO was used as vehicle control.

4.3.4.2 Brine shrimp hatching

Brine shrimp (*Artemia salina*) cysts were obtained from a local pet shop. The cysts were hatched in a shallow rectangular dish container, divided into two unequal compartments with a multi-holes plastic plate. The dried cysts were allowed to hatch in artificial sea water in the large compartment and covered with a lid to make dark environment. The small compartment was constantly illuminated at room temperature (25ºC) (Figure 4.1). After hatching, the nauplii (hatching larvae) migrated to the lighted

small compartment. After 24 h, the phototropic first instar nauplii (Figure 4.2) were collected by pipette and counted against a light background. Ten nauplii were transferred into a 24-well plate (SPL Life Sciences, Gyeonggi-do, Korea) for treatment.

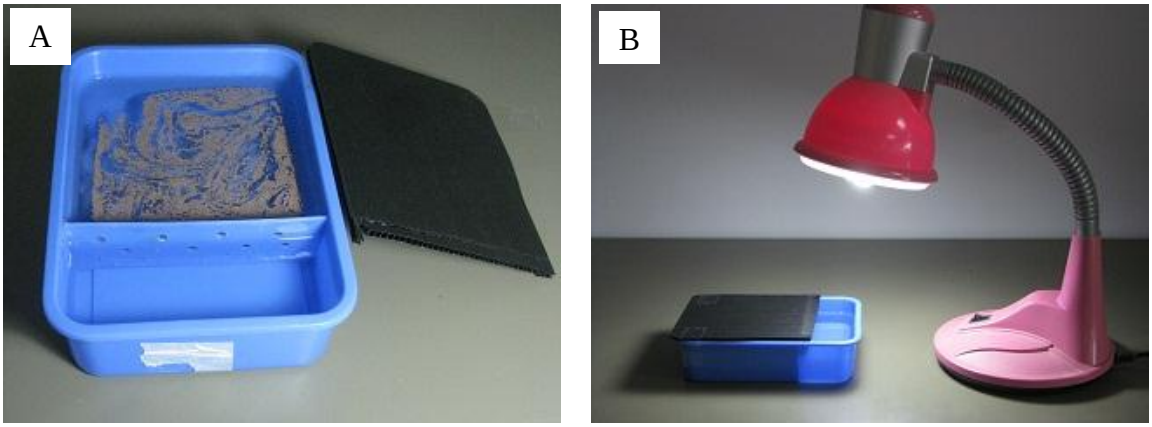

Figure 4.1 BSLT assay: *Artemia salina* cysts hatched in the large compartment (A) and the light was applied to the small compartment (B).

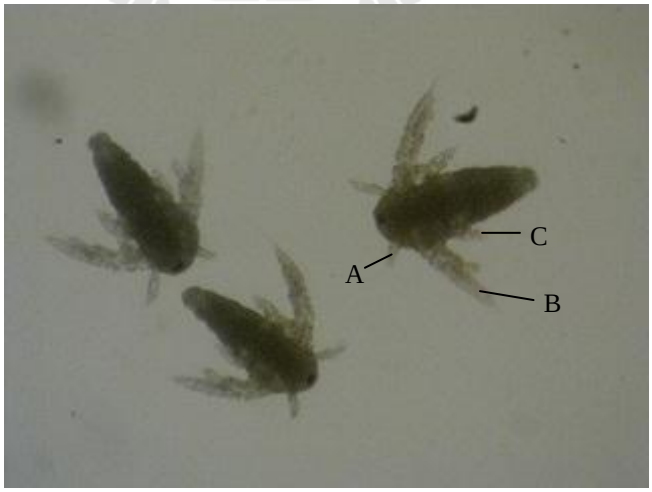

Figure 4.2 Freshly hatched 1st instar nauplii, under stereomicroscope, showing antennule (A), antennae (B), and mandibles (C).

4.3.4.3 Brine shrimp lethality test (BSLT)

BSLT was carried out to investigate the cytotoxicity of the extracts according to Meyer et al. (1982) with slight modification. Ten nauplii in 200 µL were

transferred to a 24-well plate. Different concentrations of the extracts in sea water of 800 µL were added into each well to obtain the final concentrations 10-1000 µg/mL. The plates were covered and maintained at 25ºC with constant illumination. After 24 h, dead larvae that failed to show any movement were counted under stereomicroscope (Olympus SZ61, Olympus Corporation, Tokyo, Japan). Experiments were conducted along with control (vehicle treated). Six replicates and repeated twice of each treatment were performed and calculated as following:

$$\%\,Mortality = \frac{Observed\ dead\ treatment\ nauplii}{Total\ nauplii\ used} \times 100$$

The lethal concentration to kill 50% of the test organisms (LC_{50}) and 95% confidence intervals (CI 95%) were determined using the probit analysis as described by Finney (1971).

4.3.5 Cytotoxic effect against normal human lymphocyte

4.3.5.1 Preparation of normal lymphocyte

Blood with EDTA from healthy donors was kindly given from Blood Bank (Red Cross, Nakhon Ratchasima, Thailand). The whole blood was layered onto the HISTOPAQUE®-1077, and then centrifuged at 400×g for exactly 30 min at room temperature. The opaque interface was carefully transferred into a clean conical centrifuge tube. The mononuclear cells were washed with isotonic phosphate buffered saline (PBS), pH7.4 and centrifuged at 250×g for 10 min and repeated 3 times. The cells were cultured in complete RPMI medium overnight to allow the monocytes and platelets attach to the culture flask. The floating lymphocytes were collected for assay.

4.3.5.2 Cytoxicity test (MTT assay)

The cytotoxic effect of the 6 local Thai vegetable leaf extracts upon cell proliferation were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). MTT assay is widely used for determination of cell proliferation rate. This method is based on the reduction of MTT, yellow tetrazole to purple formazan insoluble product by the mitochondrial succinate dehydrogenase or succinate coenzyme Q reductase in living cells (Figure 4.3). Therefore, the color conversion is often used as indirect method for measuring the number of viable cells. The formazan crytals can dissolve in dimethyl sulfoxide (DMSO) and can be quantified by measuring the absorbance of 540 nm.

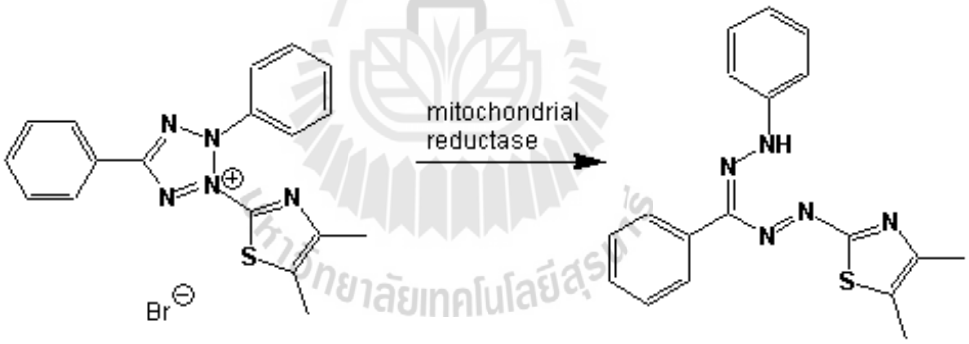

Figure 4.3 Reaction of (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. A yellow tetrazole is reduced to purple formazan product by mitochondrial reductase in living cells (Wikipedia, www, 2011).

The suspension of normal lymphocyte cells in 100 µL were plated at 100,000 cells/well in a 96-well plate (Costar, Corning Incorporated, NY, USA). The cells were treated with 100 µL of various concentrations of the extracts to obtain the final concentration of 100, 200, 300, and 400 µg/mL. After 48 h incubation at 37ºC, the medium were removed and the volume of 100 µL MTT solution (0.5 mg/mL) in Hank's Balanced Salt Solutions (HBSS) was added. After incubation for 4 h, the formazan crytal formed

were dissolved by adding 150 µL of absolute DMSO. The absorbance was measured at 540 nm using a microplate spectrophotometer (Benchmark Pluz, Bio-Rad, Japan).

4.3.6 Cytotoxic effect on cancer cell lines

4.3.6.1 Cell culture condition

The MCF7 human breast cancer cells and HepG2 liver cancer cells were cultured in Dulbecco's modified eagle medium (DMEM) with high glucose (Cat No. 12430) and low glucose (Cat No. 31600), respectively, supplemented with 100 IU/mL penicillin, 100 g/mL streptomycin and 10% fetal bovine serum (FBS). The Jurkat human leukemia cells were cultured in RPMI 1640 medium (Cat No. 31800) supplemented with 1 mM sodium pyruvate, 100 IU/mL penicillin, 100 g/mL streptomycin and 10% fetal bovine serum (FBS). All media and reagents were purchased from Gibco. The cells were incubated at 37ºC in 5% CO_2 and 95% humidity.

4.3.6.2 Cytotoxicity test (MTT assay)

MCF7 and HepG2 cells were grown in the 96-well plate at 25,000 and 30,000 cells/well in 100 µL media, respectively. The cells were allowed to attach for 24 h., then treated with various concentrations of the extracts in 200 µL of fresh media for 48 h. For Jurkat cells, the cells suspension of 100 µL were plated at 25,000 cells/well and incubated with 100 µL of various concentrations of the extracts, for 48 h. the cultured medium was removed and MTT solution was added and incubated for 3-4 h at 37ºC. After removing the medium, the formazan crytal formed was dissolved in DMSO and measured the absorbance at 540 nm. The standard antioxidants, catechin, trolox, and ascorbic acid, were also evaluated for their cytotoxicity on cancer cells. Vehicle control, 0.2% (v/v) DMSO in medium, was used as a negative control. Blank was set using well without cells

and subtracted as background from each sample. Experiments were carried out in triplicate and repeated twice. The percentage of cell viability was calculated as following:

$$\%\,Cell\ viability = \frac{Absorbance\ of\ sample - Absorbance\ of\ blank}{Absorbance\ of\ control - Absorbance\ of\ blank} \times 100$$

The percentage of cell viability was plotted against various concentrations of extracts. The concentration of extracts effectively inhibits 50% of cell viability (IC_{50}) and 95% confidence intervals were calculated using PRISM program. Selective index (SI) value was calculated as following:

$$SI\,value = \frac{IC50\ value\ of\ extract\ on\ normal\ cells}{IC50\ value\ of\ extract\ on\ cancer\ cells}$$

4.3.7 Hemolysis assay

In order to verify whether the observed cytotoxicity is related to membrane disruption, the ability of plant extracts to induce lysis of human erythrocytes was evaluated. The hemolytic effect was carried out by the disruption of erythrocytes and the release of the hemoglobin into the surrounding. The intensity of released hemoglobin can be monitored by measuring the absorbance of 540 nm.

The red blood cells (RBC), obtained from healthy donors, were washed with isotonic phosphate buffered saline (PBS), pH 7.4 and centrifuged at 900×g, 3 times. The packed red blood cells were diluted 1:25 (v/v) in PBS. The hemolysis assay was performed followed the method of Malagoli (2007) with minor modification. The volume of 480 µL of RBC suspension (4%) was incubated with 1,320 µL of various concentrations of the extracts at 37ºC for 3 h in PBS and then centrifuged at 900×g for 10 min. The supernatant

was carefully collected and measured the absorbance at 540 nm. The negative control contained only the vehicle, 0.2% (v/v) DMSO in PBS. The total hemolysis was obtained with 50 µL of 1% TritonX-100 and incubation for 3 h, at 37ºC. Experiments were performed in triplicate, repeated twice. The percentage of cell viability was calculated as following:

$$\%\ Hemolysis = \frac{Absorbance\ of\ sample - Absorbance\ of\ control}{Absorbance\ of\ total\ hemolysis - Absorbance\ of\ control} \times 100$$

4.3.8 Statistical analysis

The percentage of lethality was determined by comparing the 50% lethality of the test compounds and controls. The LC_{50} (the lethal concentration for 50% mortality) within 95% confidence intervals was determined using Probit analysis. The cytotoxicity was determined by comparing the mean cell viability of test compounds with controls. The IC_{50} (concentration required to inhibit cell growth by 50%) values within 95% confidence intervals was obtained from the best-fit regression when plotted concentration verses percentage viability using PRISM program.

4.4 Results and discussion

4.4.1 Cytotoxic effects on *Artemia salina*

The cytotoxic effects on *A. salina* nauplii by various concentrations of extracts for 24 h, and the IC_{50} values are shown in Table 4.1A. The cytotoxicity values on *A.salina* of the extract in descending order are as followes: CfLE > StLE > SgLE > BaLE > PoLE > LaLE. The highest of lethality on brine shrimp was observed in CfLE which presenting the LC_{50} value of 37.00 µg/mL. BaLE, SgLE, and StLE showed the similar cytotoxicity which LC_{50} ranged from 116.20-196.40 µg/mL. The percentage of *A. salina* mortality dramatically increased when treated with SgLE and StLE at the concentration of 100 and 150 µg/mL,

while they steadily increased when treated with BaLE. BaLE, SgLE, and StLE has a similar profile in phytochemical contents, as previously described in Chapter III, these might be the reason for their resemble in brine shrimp cytotoxicity. LaLE and PoLE, which are an aromatic plant, contain flavonoids and volatile terpenene, exhibited low cytotoxic effect on *A. salina* nuplii with LC_{50} values of 785.40 and 594.00 µg/mL, respectively.

According to Meyer et al. (1982), the criteria that crude extract giving the LC_{50} values of less than 250 µg/mL or pure compound with LC_{50} values of less than 40 µg/mL were considered significantly active and had potential for further investigation. From the results, BaLE, CfLE, SgLE, and StLE presented significant cytotoxic effect implying for the pharmacological potential of these four plants. The standard antioxidant compounds, trolox and ascorbic acid showed the LC_{50} value of 23.10 and 14.40 µg/mL, respectively, which were less than 40 µg/mL. While catechin gave high LC_{50} value of 2,654.90 µg/mL on *A. salina* nauplii as shown in Table 4.1B. These could be said that trolox and ascorbic acid might induce some toxic whereas catechin is less toxic for living organisms.

BSLT was employed as a screening technique for antitumor compounds, following the premise that pharmacology is simply toxicology at a lower dose, and recognizing that most antitumor compounds are cytotoxic (Meyer et al., 1982). It can also be extrapolated for cell-line toxicity and antitumor activity. As evidences have shown that the brine shrimp assay demonstrated good correlation with the human nasopharynx cancer 9KB cell cytotoxicity assay ($p = 0.036$) (McLaughlin, Rogers, and Anderson, 1998) and the *in vivo* P-388 murine leukemia assay ($p = 0.033$) (Anderson et al., 1991).

Table 4.1A Cytotoxic effect of the 6 vegetable leaf extracts on *A. salina* at 24 h.

Sample	Conc. (µg/mL)	% Mortality*	LC_{50} (µg/mL)**
BaLE	100	8.33 ± 6.69	196.40 (185.70-207.80)
	150	21.67 ± 9.83	
	200	41.11 ± 13.44	
	250	90.00 ± 8.94	
CfLE	10	5.16 ± 3.33	37.00 (29.20-48.00)
	25	8.16 ± 3.33	
	50	90.00 ± 12.65	
	100	100.00 ± 0.00	
LaLE	250	4.08 ± 1.67	785.40 (709.80-873.40)
	500	8.16 ± 6.67	
	750	18.33 ± 7.53	
	1000	91.83 ± 9.83	
PoLE	250	4.08 ± 1.67	594.00 (484.50-715.40)
	500	13.33 ± 8.16	
	750	85.00 ± 10.49	
	1000	100.00 ± 0.00	
SgLE	100	13.33 ± 8.16	119.40 (113.90-125.40)
	125	58.33 ± 11.69	
	150	95.00 ± 5.48	
	200	100.00 ± 0.00	
StLE	100	10.00 ± 8.94	116.20 (110.70-122.20)
	125	80.33 ± 8.85	
	150	95.00 ± 5.48	
	200	100.00 ± 0.00	

*presented as mean ± SD (n = 6), **presented as mean (95% confidence interval). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

Table 4.1B Cytotoxic effect of standard antioxidants on *A. salina* at 24 h.

Sample	Conc. (µg/mL)	% Mortality*	LC_{50} (µg/mL)**
Catechin	500	5.48 ± 5.00	2,654.90 (2,277.00-3,086.30)
	1000	11.67 ± 7.53	
	2000	39.08 ± 12.69	
	4000	63.33 ± 16.33	
	6000	86.67 ± 8.16	
Trolox	10	8.33 ± 7.53	23.10 (19.50-27.00)
	20	15.00 ± 8.37	
	30	78.33 ± 14.72	
	40	100.00 ± 0.00	
Ascorbic acid	10	10.95 ± 7.69	14.40 (12.10-17.20)
	20	81.67 ± 13.29	
	30	96.67 ± 5.16	
	40	100.00 ± 0.00	

*presented as mean ± SD (n = 6), **presented as mean (95% confidence interval).

Moreover, Solis, Wright, Anderson, Gupta, and Phillipson (1993) also supported that this assay may be a useful testing for pharmacologic activities, since brine shrimp responds similar to the corresponding mammalian system. For example, *A. salina* has the DNA-dependent-RNA-polymerase which is similar to the mammalian type (Bagshaw, 1976), and also has an ouabain sensitive Na^+ and K^+ dependent ATPase. Therefore compounds or extracts acting on these systems might be established for their cytotoxicity and antitumor property against higher organisms.

4.4.2 Cytotoxic effect on human lymphocytes cells

To elucidate whether these plant extracts induced cytotoxic cell death in normal cell, the human normal lymphocytes were used in this assay according to Rao, Geethangili, Fang, and Tzeng (2007). All the extracts were able to induce the cytotoxic

effects on normal lymphocytes which different concentrations used (Table 4.2). The highest toxicity on normal lymphocyte was BaLE with IC_{50} values of 158.85 µg/mL, while the lowest was LaLE with more than 400 µg/mL. SgLE and StLE had similar in cytotoxicity with IC_{50} values of 202.50 and 260.60 µg/mL, respectively. The cytotoxicity of CfLE resembled to PoLE with IC_{50} values of 358.92 and 332.90 µg/mL, respectively. All standard antioxidant compounds exhibited less cytotoxic effect on normal lymphocyte, they demonstrated 78.92-86.28 % of cell viability when treated with 400 µg/mL. It is noticed that CfLE was the most cytotoxicity to an arthropod, *A. salina*, was not toxic to normal human lymphocytes.

4.4.3 Cytotoxic effect on human cancer cell lines

Jurkat cells are an immortal line of T lymphocyte cells which produce interleukin 2 and useful for study human acute T lymphocyte leukemia. The percentage of Jurkat human leukemia cells viability when treated with various extracts, the IC_{50} values, and the selectivity of extract on each cancerous cell line were calculated and shown in Table 4.3. SI value higher than 3 is considered to be high selectivity. All extracts were able to evoke the cytotoxic effect of Jurkat cells. BaLE and StLE were most toxic to the cells with IC_{50} values of 66.91 and 75.36 µg/mL, respectively. Furthermore, BaLE and StLE seemed to be selective to Jurkat cells with SI value of 2.37 and 4.00, respectively. CfLE was more toxic to normal cells than to Jurkat cells with SI value less than 0.90. LaLE, PoLE, and SgLE exhibited moderate activity on inducing Jurkat cells death with IC_{50} values ranged 128.50-146.80 µg/mL. Nevertheless, these three vegetables showed a higher sensitivity to Jurkat cells than to normal cells with SI values of 3.11, 2.27, and 1.43, respectively. Catechin, trolox, and ascorbic acid had low cytotoxicity, they exhibited 57.11, 80.63, and 67.53% cell viability at 400 µg/mL, respectively.

Table 4.2 Cytotoxic effect of the 6 vegetable leaf extracts on normal human lymphocyte cells at 48 h.

Sample	Cell Viability (%)*				
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	IC_{50} (µg/mL)**
BaLE	66.12 ± 2.13	41.34 ± 2.47	NP	10.81 ± 1.77	158.85 (150.66-166.72)
CfLE	NP	74.92 ± 1.64	53.72 ± 4.63	46.53 ± 4.52	358.92 (336.51-386.37)
LaLE	NP	78.62 ± 3.18	67.59 ± 1.91	58.61 ± 2.31	> 400
PoLE	NP	71.94 ± 0.70	56.61 ± 2.50	37.89 ± 6.81	332.90 (318.00-348.80)
SgLE	97.42 ± 3.25	55.10 ± 14.12	NP	11.33 ± 3.52	202.50 (175.10-234.10)
StLE	86.92 ± 4.34	66.62 ± 6.61	22.51 ± 2.04	10.26 ± 3.24	260.60 (252.00-269.30)
Catechin	NP	84.42 ± 11.87	NP	78.92 ± 4.41	> 400
Trolox	NP	92.43 ± 1.27	NP	86.28 ± 2.43	> 400
Ascorbic acid	NP	89.26 ± 10.21	NP	84.11 ± 1.99	> 400

*presented as mean ± SD (n = 6); **presented as mean (95% confidence interval) which obtained by non-linear regression, PRISM; NP, Not performed. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

Table 4.3 Cytotoxic effect of the 6 vegetable leaf extracts on Jurkat human leukemic cell line at 48 h and their selective indexes.

Sample	Cell Viability (%)*			IC_{50}**	Selective
	100 µg/mL	200 µg/mL	400 µg/mL	(µg/mL)	Index (SI)
BaLE	29.12 ± 10.03	11.02 ± 1.48	8.80 ± 0.42	66.91 (61.94-72.27)	2.37
CfLE	86.27 ± 4.42	67.54 ± 3.92	66.21 ± 3.67	> 400	< 0.90
LaLE	73.05 ± 4.08	59.20 ± 7.82	25.91 ± 3.42	128.50 (109.30-151.10)	> 3.11
PoLE	71.41 ± 4.66	57.39 ± 5.04	27.03 ± 1.59	146.80 (103.40-208.40)	2.27
SgLE	74.87 ± 7.11	39.91 ± 5.03	22.02 ± 4.15	141.30 (117.50-170.00)	1.43
StLE	46.53 ± 5.17	31.81 ± 4.01	26.78 ± 4.62	75.36 (40.95-104.30)	4.00
Catechin	75.52 ± 4.90	67.81 ± 4.10	57.11 ± 3.31	> 400	
Trolox	91.40 ± 5.62	85.79 ± 12.21	80.63 ± 12.45	> 400	
Ascorbic acid	84.44 ± 9.81	65.20 ± 6.30	67.53 ± 4.72	> 400	

*presented as mean ± SD (n = 6); **presented as mean (95% confidence interval) which obtained by non-linear regression, PRISM. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

มหาวิทยาลัยเทคโนโลยีสุรนารี

MCF7 breast cancer cell line, malignant adenocarcinoma in a pleural effusion, is one of the most commonly used *in vitro* breast cancer models. The majority of this cell line is derived from more aggressive and metastatic tumors, rather than the primary lesion. MCF7 cells are useful for *in vitro* studies because the cell line retained several ideal characteristic particular to the mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors. The cytotoxicity of the vegetable leaf extracts on MCF7 human breast cancer cell line are shown in Table 4.4. SgLE was the highest effective ability to induce cytotoxic of the cells with IC_{50} and SI values of 66.71 µg/mL and 3.03, respectively. All extracts at concentration of 400 µg/mL demonstrated less than 50% cell viability. Moreover, all of them were more toxic to MCF7 breast cancer cells than to normal cells with SI values of 1.09-3.03. PoLE and LaLE had moderate inhibition of MCF7 cell with IC_{50} of 205.20 and 291.70 µg/mL, whereas they showed the strong anti-proliferation property in Nanasombat and Teckchuen (2009) study with IC_{50} of 6.01 and more than 20 µg/mL for 72 h incubation, respectively. Catechin and trolox were again less cytotoxicity, whereas ascorbic acid was a strong sensitive compound for inducing the death of MCF7 cells with IC_{50} values of 78.64 µg/mL. The cytotoxicity of ascorbic acid on MCF7 cells also supported by Kurbacher et al., 1996 which reported 51.30% reduction of survival at concentration of 100 µM (17.6 µg/mL). The excessive production of hydrogen peroxide might be partly responsible for their inhibitory effect of MCF7 cells (Noto et al., 1989).

Table 4.4 Cytotoxic effect of the 6 vegetable leaf extracts on MCF7 human breast cancer cell line at 48 h and their selective indexes.

Sample	Cell Viability (%)*			IC_{50}**	Selective
	100 µg/mL	200 µg/mL	400 µg/mL	(µg/mL)	Index (SI)
BaLE	79.42 ± 3.55	22.91 ± 2.63	16.18 ± 2.24	124.80 (113.00-137.80)	1.27
CfLE	94.43 ± 5.69	47.37 ± 8.75	19.73 ± 3.35	176.50 (154.20-202.00)	2.03
LaLE	98.44 ± 1.81	84.40 ± 1.91	16.51 ± 1.53	291.70 (270.70-315.70)	> 1.37
PoLE	94.04 ± 9.18	64.03 ± 9.21	31.03 ± 0.43	205.20 (163.70-257.10)	1.62
SgLE	38.17 ± 3.42	19.43 ± 5.62	11.80 ± 2.54	66.71 (47.37-93.93)	3.03
StLE	80.10 ± 4.42	55.14 ± 5.19	17.39 ± 3.47	238.20 (223.10-254.60)	1.09
Catechin	94.24 ± 11.88	82.71 ± 9.14	71.13 ± 9.09	> 400	
Trolox	87.02 ± 5.41	84.35 ± 10.92	51.01 ± 5.14	> 400	
Ascorbic acid	23.11 ± 2.31	18.33 ± 2.26	20.63 ± 2.04	78.64 (74.91-82.56)	

*presented as mean ± SD (n = 6); **presented as mean (95% confidence interval) which obtained by non-linear regression, PRISM. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

HepG2, a differentiated human hepatocellular carcinoma, was evaluated for the susceptibility to the vegetable leaf extracts. The effect of the extracts on HepG2 cell viability (%), their IC_{50} values, and SI are shown in Table 4.5. HepG2 cell was less sensitive to plant extract than Jurkat and MCF7 cell lines with SI values less than 1.41. BaLE showed the highest toxicity to HepG2. There are only three extracts, BaLE, LaLE, and StLE, demonstrated the IC_{50} value less than 400 µg/mL which were 112.70, 349.94, and 231.20 µg/mL, respectively. CfLE and PoLE did not show the cytotoxic effect on this type of cell, the number of cell viability was more than 98% even treated with the 400 µg/mL. Furthermore, SgLE could inhibit HepG2 proliferation only 27.88% at 400 µg/mL. All standard compounds showed less cytotoxic effect with IC_{50} more than 400 µg/mL.

The phytochemical composition of plants might influence their cytotoxic properties. Tannins, terpene essential oils and saponins in BaLE and StLE could be the major compounds that inhibited the growth of Jurkat cells. There were reports that barringtonic acid and acutangulic acid in *B.acutangula* produced inflammatory effect (Aggarwal et al., 2006). Stigmasterol, *β*-sitosterol and gallic acid also found in *B. acutangula* and *S. terebinthifolius*, these compounds could contribute to the inhibition of cancer cell proliferation and induction of apoptosis (Awad and Fink, 2000; Ji et al., 2009). CfLE, containing high flavonoids and essential oils, were selectively high toxic to *A. salina*, moderately inhibited the MCF7 cell proliferation, but not other cell types. This contrast to Prayong et al. (2008)'s study which reported that *C. formosum* leaf extract selectively and moderately toxic to HepG2 cells. LaLE and PoLE possesses similar cytotoxicity, were potent against the proliferation of Jurkat and MCF7 cell lines, which were in agreement with Nanasombat and Teckchuen (2009). Tannins and terpenoids the main component in SgLE, could be responsible for the selective cytotoxicity on MCF7 cells, but less toxic to n-lymphocytes and erythrocytes.

Table 4.5 Cytotoxic effect of the 6 vegetable leaf extracts on HepG2 human liver cancer cell line at 48 h and their selective indexes.

Sample	Cell Viability (%)*			IC_{50}**	Selective
	100 µg/mL	200 µg/mL	400 µg/mL	(µg/mL)	Index (SI)
BaLE	70.55 ± 6.02	24.43 ± 2.98	NP	112.70 (103.00-123.30)	1.41
CfLE	107.42 ± 5.81	101.19 ± 3.54	103.64 ± 4.81	> 400	< 0.90
LaLE	76.55 ± 3.93	61.32 ± 5.02	47.42 ± 3.09	349.94 (316.23-393.67)	> 1.14
PoLE	97.11 ± 3.08	98.95 ± 3.79	98.03 ± 1.55	> 400	< 0.83
SgLE	85.15 ± 2.78	87.03 ± 2.81	72.12 ± 6.41	> 400	< 0.50
StLE	79.50 ± 5.04	58.12 ± 5.25	31.67 ± 3.91	231.20 (146.30-365.50)	1.13
Catechin	102.01 ± 6.99	102.04 ± 7.57	104.10 ± 4.07	> 400	
Trolox	104.13 ± 5.15	96.31 ± 1.10	90.42 ± 5.76	> 400	
Ascorbic acid	97.31 ± 2.61	95.91 ± 4.72	85.21 ± 3.42	> 400	

*presented as mean ± SD (n = 6); **presented as mean (95% confidence interval) which obtained by non-linear regression, PRISM; NP, not performed. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

4.4.4 Hemolytic effects on human erythrocytes cells

The hemolytic activities indicate the cytolytic activity of extracts on the biological membrane disruption. The results are presented in Figure 4.6. BaLE was the most active in disrupting the membrane of erythrocyte cells with EC_{50} values of 6.69 µg/mL. It was followed by StLE which showed EC_{50} values of 134.6 µg/mL. The CfLE, LaLE, PoLE, and SgLE demonstrated less cytolytic effects on human erythrocytes with 33.28%, 22.78%, 9.64%, 15.64% hemolysis at concentration of 1200 µg/mL, respectively. Catechin and trolox had less effect with 0.63% and 4.22% hemolysis at concentration of 1200 µg/mL. Red blood cells (RBCs) contain high lipid content, rich oxygen supply and transition metals such as iron and copper, leading to their susceptible to oxidation. The oxidative damage of RBCs lipid membrane may cause its malfunctioning of membrane fluidity and membrane-bound enzyme and receptor functions which has been proposed as a general mechanism leading to RBC hemolysis (Alvarez-Suarez et al., 2012). BaLE and StLE, contained high saponins (Chapter III) (Daniel and Robin, 2011; Johann, Pizzolatti, Donnici, and Resende, 2007) and there are many reports demonstrated that triterpenoid and steroidal saponins are capable for hemolysis of RBCs (Lacaille-Dubois and Wagner, 2000). These could make an assumption that the saponins content or the pro-oxidation property of BaLE and StLE might affect the plasma membrane instability and relate to their cytotoxicity.

Table 4.6. Hemolytic activity of the 6 vegetable leaf extracts on human erythrocytes (1%).

Sample	Percentage of hemolysis (%)*				EC_{50} (µg/mL)**
	200 µg/mL	400 µg/mL	600 µg/mL	1200 µg/mL	
BaLE	100	NP	NP	NP	6.69 (6.66- 6.72)
CfLE	0.23 ± 0.15	2.34 ± 0.17	6.63 ± 0.78	33.28 ± 2.26	> 1200
LaLE	0.90 ± 0.11	1.50 ± 0.41	6.08 ± 0.99	22.78 ± 5.93	> 1200
PoLE	0.11 ± 0.04	0.17 ± 0.06	3.06 ± 0.70	9.64 ± 0.50	> 1200
SgLE	3.20 ± 0.85	5.20 ± 1.21	7.38 ± 1.08	15.64 ± 4.62	> 1200
StLE	70.72 ± 2.76	79.50 ± 0.78	100	NP	134.60 (125.30-144.60)
CA	0.17 ± 0.15	0.13 ± 0.06	0.35 ± 0.15	0.63 ± 0.46	> 1200
Trolox	0.04 ± 0.01	0.08 ± 0.01	0.63 ± 0.47	4.22 ± 3.79	> 1200
AA	0.09 ± 0.01	0.23 ± 0.10	0.58 ± 0.32	63.61 ± 1.45	960.70 (951.50-970.10)

*presented as mean ± SD (n = 6); **presented as mean (95% confidence interval) which obtained by non-linear regression, PRISM; NP, not performed. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

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4.5 Conclusions

The six Thai local vegetable leaf extracts possessed cytotoxicity to normal cells/ organisms and cell lines at different degrees. Moreover, their toxicity could depend upon the different phytochemical components. BaLE showed high cytotoxic activity against *A. salina,* normal lymphocyte, Jurkat, MCF7, and HepG2, indicating the presence of cytotoxic compounds in this extract. The hemolytic data suggest that the cytotoxic property of BaLE, was related to the lytic properties or the induction of cell membrane instability. StLE also exhibited cytotoxicity on cells but less than BaLE. The cytotoxic effect of BaLE and StLE, could due to the high saponin content.

CfLE possess high toxic activity against only *A. salina* nauplii, whereas, it showed less effect on human normal lymphocytes and the three cancer cell line, Jurkat, MCF7, and HepG2. The cytotoxicity to *A. salina* was not correlated to the three types of cancer cells, indicated that the mode of toxic action of this extract prominently acted on small organisms, but not on human cells.

LaLE and PoLE, with similar levels of flavonoid content (Chapter III), exhibit similar cytotoxicity. Eventhrough, PoLE showed somewhat higher toxicity than LaLE on *A. salina*, normal lymphocytes and MCF7 breast cancer cells. However, the cytotoxicity of PoLE on HepG2 cells was approximately two fold higher than that of LaLE. The differences in the amount and the constituents of flavonoids and essential oils could contribute different cytotoxicity.

SgLE was the most cytotoxic extract on MCF7 cell line, but less cytolytic to normal cell membrane. The high amount of tannin and terpenoid found in SgLE might render for its selectivity or high cytotoxicity.

It is concluded that the six Thai local vegetable leaves, *Barringtonia acutangula*, *Cratoxylum formosum*, *Limnophila aromatica*, *Polygonum odoratum*, *Syzygium gratum*, and *Schinus terebinthifolius*, possessed cytotoxicity differently, dependent upon the

experimental models. Their prominent cytotoxic actions are the following: *Cratoxylum formosum* was prominent on *Artemia salina*, *Barringtonia acutangula* was prominent on normal human lymphocytes, and two cancer cell line, Jurkat leukemia cells and HepG2 liver cancer cells, whereas *Syzygium gratum* was prominent on MCF7 breast cancer cells.

This preliminary study could provide important data on cytotoxicity of these six Thai local vegetables for further study on anti-tumor activity at molecular levels which would be applicable for cancer prevention.

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CHAPTER V

EFFECTS OF SIX LOCAL THAI VEGETABLE LEAF EXTRACTS ON ANTIPROLIFERATION AND DEATH OF MCF7 BREAST CANCER CELLS

5.1 Abstract

As previously screening the cytotoxicity of the 6 vegetable leaf extracts on three cancer cells, MCF7 breast cancer cells was more susceptible to all extracts. Therefore, the cytotoxicity or antiproliferative properties of the leaf extracts of *Barringtonia acutangula* (BaLE), *Cratoxylum formosum* (CfLE), *Limnophila aromatica* (LaLE), *Polygonum odoratum* (PoLE), *Syzygium gratum* (SgLE), and *Schinus terebinthifolius* (StLE) were examined more inclusive at different concentrations and times by the *in vitro* MTT assay. The hallmarks of apoptotic cell death, nuclear morphological change and DNA ladder formation were observed by Hoechst staining and agarose gel electrophoresis. The induction of subG$_0$ and cell cycle progression of the 6 extracts were investigated by flow cytometer. The alteration on proteins involved in apoptosis was monitored by Western blotting. The results demonstrated that the 6 vegetable leaf extracts inhibited the growth of MCF7 breast cancer cells in dose- and time-dependent manner. SgLE was the highest activity with IC$_{50}$ values of 120.70 and 90.12 µg/mL at 24 and 48 h, respectively. SgLE, the most active, induced the fragmented nuclei (Hoechst) with EC$_{50}$ value of 136.60, 118.20, and 112.80 µg/mL at 12 h, 24 h, and 48 h, respectively and also DNA ladder formation. BaLE could induce apoptosis and concomitant with necrosis in high concentration (400 µg/mL) as observed from the release of nucleic acid from nucleus (Hoechst staining) and

the diffuse smear on DNA electrophoresis. CfLE, LaLE, and PoLE induced the large fragmented nuclei with high molecular weight DNA fragments, whereas StLE showed DNA ladder at 600-800 µg/mL, 24 h. All extracts, except BaLE, induced the remarkable increase in subG_0 fraction (apoptotic cell) at 400 µg/mL. StLE demonstrated the induction of cell death mediated by G_0/G_1 cell growth arrest. All extracts could induce the release of cytochrome c from mitochondria with different levels, the highest was observed in SgLE 200 µg/mL with 39.29% and 71.94% at 12 h and 24 h, respectively. The mechanisms on MCF7 cell growth inhibitory effects of the 6-extracts was prominently caspase-independent cell death, as observed by the atypical cleaved of PARP (~ 60 kDa) which might be the action of calpain or cathepsin enzyme. These observations on cytotoxicity and mode of cell death provided the informative data of the six local Thai vegetables, especially *Syzygium gratum*, which might be investigated further for the possible used to suppressing cancer development.

5.2 Introduction

Carcinogenesis is a multistage process consisting of at least three phases: initiation, promotion, and progression, in which a normal cell is transformed into a malignant cell. This sequence of events presents multiple intervention strategies. Chemoprevention which involves the use of pharmacological, phytochemicals and even whole plant extracts has been proposed to prevent, arrest or reverse the cellular and molecular processes of carcinogenesis. Furthermore, accumulated evidence suggests that dietary phytochemicals can interfere with each stage of carcinogenesis by blocking or/and suppressing mechanisms to prevent cancer development (Manson, 2003). Apoptosis, one of the key suppressing mechanisms, provides a number of clues with respect to effective anticancer therapy.

` Apoptosis or programmed cell death is a cell suicide mechanism with regulated and controlled fashions to remove unwanted cells: redundant, damaged, or infected cells. The

characteristic morphological hallmarks of apoptosis include cell shrinkage, chromatin condensation, nuclear DNA fragmentation, membrane blebbing and the formation of apoptotic bodies (Ziegler and Groscurth, 2004). The process of apoptosis can be initiated by two pathways: extrinsic and intrinsic apoptotic signaling pathway. The extrinsic pathway is triggering *via* the death receptor (tumor necrosis factor (INF) and Fas receptor) resulting in activation of caspase-8 and initiation of apoptosis. The intrinsic or mitochondrial pathway is activated by several of stimuli including DNA-damaging agents or cytotoxic stresses. Active phytochemicals and anticancer drugs almost induce apoptosis in cancer cells *via* mitochondria pathway. These stress signals modulate member of Bcl-2 family such as anti-apoptotic protein Bcl-2, Bcl-xL and pro-apoptotic proteins such as Bax, Bak resulting in the disruption of the mitochondrial membrane and subsequent leakage of inner proteins such as cytochrome c into the cytosol. An association of cytochrome C, Apaf-1 and procaspase-9 forms an apoptosome, which activates the caspase-9 and then downstream effector caspases: caspase-3, -6, and -7.

The classical apoptotic cell death or caspase-dependent signaling pathway processes on a specific family of cysteine protease, caspase, activity that lead to the typical morphological change. The caspases play an important role in hallmarks of apoptosis, the cleavage of chromosomal DNA into nucleosomal units and membrane blebing by activating DNases, inhibiting DNA repair enzymes, and breaking down structural proteins in nucleus (Degterev, Boyce, and Yuan, 2003).

However, cell death can triggering *via* caspase-independent cell death pathways include autophagy, parapoptosis, mitotic catastrophe, and the descriptive model of apoptosis-like and necrosis-like PCD. Caspase-independent cell death pathways are important safeguard mechanisms to protect the organism against unwanted and potential harmful cells when caspase-mediated routes fail but can also be triggered in response to cytotoxic agents or other death stimuli (Bröker, Kruyt, and Giaccone, 2005). Mitochondria,

lysosomes and endoplasmic reticulum (ER) may play a prominent role in certain types of PCD. The mitochondrial potentially contributes to caspase-independent cell death by releasing apoptosis inducing factor (AIF) (Cregan, Dawson, and Slack, 2004) and endonuclease G (Endo G) (Li, Luo, and Wang, 2001) into the cytosol. In recent years, it has become evident that the proteolytic enzymes, cathepsin B and cathepsin L in partial lysosomal permeabilization can triggers apoptotic-like PCD (Tardy, Codogno, Autefage, Levade, and Andrieu-Abadie, 2006). In addition, the stress on ER can initiate PCD through the release of calcium into the cytoplasm. This can induce permeabilization of the mitochondrial membrane to release the apoptogenic proteins such as cytochrome c, AIF and Endo G or induce the activation of cytosolic protease, the calpains. Calpains have been shown to act downstream of caspase activation and also induce the release of lysosomal cathepsins leading to cell death (Bröker et al., 2005).

To investigate the mechanism of cell death of the 6 vegetable extracts, the nuclear morphology, DNA fragmentation were examined as a hallmark of apoptosis. The occurring of hypoploidy DNA content and changing of apoptotic protein level was also evaluated to confirm the mode of cell death.

5.3 Materials and methods

5.3.1 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, penicillin-streptomycin and fetal bovine serum (FBS), trypsin/EDTA, TYPLE were purchased from Gibco (New York, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Hoechst 33258, propidium iodide were purchased from Invitrogen (California, USA). Resveratrol, ribonuclease A from bovine pancreas, acrylamide, bis-acylamide, sodium citrate were purchased from Sigma-Aldrich Company Ltd. (St. Louis MO, USA). Dimethylsulfoxide (DMSO), *p*-formaldehyde and agarose were obtained from Amresco

(Ohio, USA). Phenol:chloroform:isoamyl (25:24:1) solution and Super Signal West Pico chemilluminesence substrate were from Thermoscientific (Lillinos, USA). Proteinase K and Lambda DNA/ Hind III marker were purchased from Promega (Wisconsin, USA). Precision Plus protein standard and ethidium bromide were purchased from Bio-rad (California, USA). Bovine serum albumin (BSA) fraction V and TEMED were from BDH chemicals (Yorkshire, England). Protease inhibitor cocktail was from Roche (Mannheim, Germany). All of antibody was obtained from Santa-Cruz Biotechnology (California, USA).

5.3.2 Cell line and the cultured condition

MCF7 human breast cancer cells was a gift from R. P. Shiu, Dubik and Shiu (1992), cultured in 4.5 g/L glucose DMEM medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS). The cells were seeded at 7.5×10^5 cells/mL in a 75 cm^2 cell culture flask with 20 mL fresh medium and were cultured at 37ºC under a humidified, 5% CO_2 atmosphere. The cells in log-phase growth, 2-3 days incubation, were used in the experiment. The number and viability of cells were counted by trypan blue exclusion staining before seeding. Trysinization was carried out with 1 mL 0.5% trypsin/EDTA or 2X TYPLE in PBS in 75 cm^2 cultured flask for 5 min.

5.3.3 Cell anti-proliferation assay (MTT assay)

The effect of the 6 vegetable leaf extracts upon proliferation of MCF7 breast adenocarcinoma cell line was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). MCF7 cells were grown in a 96-well plate for 24 h at 2.5×10^4 cells/well in 100 µL media. The cells were allowed to attach for 24 h, then treated with different concentrations of the extracts (200 µL/well) for 24 h and 48 h. The cultured medium was removed and MTT solution (0.5 mg/mL in PBS, pH 7.4) was added and incubated for 3-4 h. The formazan crytal formed was dissolved by adding 150 µL

of absolute DMSO. The absorbance was measured at 540 nm by a microplate spectrophotometer (Benchmark Plus, Bio-Rad, Japan). The decreasing in absorbance indicated a reduction in cell viability and anti-proliferative effect. DMEM medium with 0.2% DMSO was served as the negative control and resveratrol was as a positive control. Experiments were carried out in triplicate and repeated twice. The results were expressed as the percentage of cell viability with respect to the control. The cell viability (%) was plotted against the various concentrations of samples. The concentration of sample efficiency to inhibit 50% cell viability (IC_{50}) and 95% confidence intervals were obtained from best-fit nonlinear regression (PRISM program).

5.3.4 Observation of cell morphological changes (Hoechst 33258)

Light microscopy is the classical technique for the study of apoptosis, which can also be enhanced by the use of fluorescence dye such as Hoechst 33258. Hoechst dyes are cell permeable nucleic acid stains that have multiple applications, including detecting the DNA conformation and chromatin state in cells. These bisbenzimidazole derivatives are supravital minor groove-binding DNA stains with AT selectivity.

Nuclear morphological change as a late marker of apoptosis was observed by staining of DNA with Hoechst 33258. The cells (2.5×10^4 cells/well) were allowed to grow overnight in a 96-well plate in 100 µL media. Cultured cells were treated with various concentrations of extracts (200 µL/well) and incubated for 12 h, 24 h, and 48 h. After treatment, the medium was removed and washed twice with cold PBS, pH 7.4. The cells were fixed with 200 µL of ρ-formaldehyde (4%, v/v) for 20 min and then washed with PBS. The cells were stained with 100 µL of Hoechst 33258 (10 µg/mL) in PBS containing 0.2% (v/v) tritonX-100 solution for further 30 min at room temperature. Cell morphology was visualized under the inverted fluorescence microscope (Olympus IX51 with Digital Camera DP50 + View Finderlite Program, Olympus corporation, Japan). The number of condensed

and fragmented nuclei of sample treaments were counted versus the normal nuclei of negative control (0.2% DMSO in DMEM medium), approximately 300 nuclei/field. Resveratrol was used as positive control. Experiments were carried out in triplicate and repeated twice. The results were expressed as the percentage of condensed and fragmented nuclei. Effective concentration of extract to induce 50% cell death with fragmented nuclei (EC_{50}) was calculated using nonlinear regression.

5.3.5 DNA fragmentation

One of the lately recognizable changes denoting an irreversible commitment to cell death is the activation of nuclear DNA fragmentation. The cultured cells, 0.76×10^6 cells/well (6-well plate), were incubated overnight. The cells were treated with the extracts for 12 and 24 h. After the desired time, the cells in cultured medium were collected and the attached cells were washed twice with PBS. The 200 µL of 0.5% trypsin/EDTA was added and incubated for 5 min for trypsinization, the fresh medium was added thereafter and collected. The harvested cells were centrifuged at 1,400×g (Sorvall RT-7, Kendro Laboratory Products, Connecticut, USA) for 5 min, lysed in 360 µL of lysis buffer solution (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.1 mM NaCl, and 0.5% (v/v) tritonX-100) for 30 min. The cell lysate was centrifuged at 2,600×g for 10 min and transferred the supernatant to another tube. Protinase K stock solution (40 µL) was added into the supernatant to give the final concentration of 100 µg/mL and incubated in water bath at 56ºC for 6-8 h. The phenol:chloroform:isoamyl (25:24:1) solution (200 µL) was added twice into the supernatant (to purify the DNA), the upper layer was collected. To precipitate the DNA, 3M sodium acetate (pH 5.8) was added at the ratio of 1/10 following with cold absoluted ethanol 2.5 volumes and kept in -20ºC overnight. Centrifugation was carried out at 12,000×g, 4ºC for an hour to obtain the DNA sediment. The DNA was washed 2 times with cold 70% ethanol and then dried. The DNA was resuspended in Tris-EDTA (TE) buffer.

DNA absorbance was measured at 230 and 260 nm and calculated by the Nanodrop ND-1000 spectrophotometer with ND-1000 V3.5.2 software. The amount of 10 µg DNA in TE buffer was mixed with ribonuclease A (RNase A) stock solution to give the final concentration of 100 µg/mL and incubated at 37ºC for 30 min. The DNA fragments were separated on a 1.5% agarose gel with applying the voltage of 70 V for 90 min and then stained with ethidium bromide. The DNA pattern was visualized under UV light. Three individual experiments were performed.

5.3.6 Detection of subG$_0$ phase in cell cycle by flow cytometer

Flow cytometric techniques have been used as an analytical tool for measuring the DNA ploidy as well as the alterations in cell cycle profiles. Measuring of DNA content through the cell cycle can distinguish cell cycle phase. The fluorescence signal intensity of the propidium iodide (PI) is directly proportional to the amount of DNA. The cells in G_0/G_1 phase of the cell cycle possess a normal diploid chromosomal and hence DNA content of (2n) whereas cells in G_2/M contain exactly twice in amount (4n). DNA is synthesized with a DNA content ranging between 2n and 4n defined as S phase. The hypoploidy can be classified as subG$_0$ phase which is the characteristic of apoptosis (Krysko, Vanden Berghe, D'Herde, and Vandenabeele, 2008).

MCF7 cells were plated at 0.76×10^6 cells/well (6-well plate) and incubated for 24 h. The various concentrations of extracts were added into the cultured cells. After incubating for 12 and 24 h, the medium was collected and washed with PBS, then trypsinized using 200 µL of 2X TRYPLE. The fresh medium was added to neutralize the trypsin activity. Collected the cells and centrifuged at 1,400×g, 4ºC. Cells were washed with 1% BSA in PBS buffer and recentrifuged at 1,400×g. Resuspensed the cells in 500 µL of 1% BSA in PBS buffer, then fixed by adding 2,500 µL of 80% ethanol drop wise under gently shaking and kept at -20ºC, overnight. The cells were washed twice with PBS and

resuspended in 3.8 mM sodium citrate in PBS buffer. The cells were filtered through the 40 µM nylon filter to eliminate clumping cells. The RNase stock solution (1 mg/mL) of 10 µL was added into 480 µL of 2×10^5 cells suspension and incubated at 37ºC for 1 h. After that, 10 µL of PI stock solution (1 mg/mL) was added and further incubated on ice for 30 min. The cells were determined by BD FACS Calibur flow cytometer and analyzed using Cell Quest Pro software (Becton Dickinson Biosciences, USA). Three individual experiments were performed.

5.3.7 Detection of cytochrome c release by flow cytometer

The release of key mitochondrial proteins such as cytochrome c is an important hallmark in apoptotic pathway and is considered a point of no return in the apoptosis process. The method for assessing of cytochrome c release by flow cytometry is based on the direct labeled of anti-cytochrome c-FITC antibody within the cells with permeabilized mitochondria membrane. Viable or live cells will demonstrate higher levels of cytochrome c staining while apoptotic cells which have released their cytochrome c from the mitochondria to the cytoplasm will demonstrate reduced staining intensity when probed with an anti-cytochrome c-FITC antibody. Stained cells can then be analyzed by flow cytometry.

Millipore's FlowCellect^{TM} Cytochrome c kit (Millipore, USA) was used to assess the loss of mitochondrial cytochrome c in cells during apoptotic induction by the 6 vegetable extracts. MCF7 cells were plated at 0.76×10^6 cells/well (6-well plate) and incubated for 24 h. The cultured cells were treated with the extracts for 12 and 24 h. After incubating, the medium was collected and washed with PBS, then trypsinized using 2X TRYPLE. The cells were collected and centrifuged at 1,400×g, 4ºC for 5 min. The cells suspension in fresh medium of 2×10^5 cells in 200 µL was added to each tube. Washed with 200 µL PBS and then recentrifuged at 300×g, 4ºC for 7 min. After aspirate off supernatant,

100 µL of permeabilization buffer working solution was added to each tube thoroughly. Caped each tube and incubated on ice for 10 min. Then, the 100 µL fixation buffer working solution was added, mixed thoroughly and incubated at room temperature (RT) for 20 min. After that, the cells were centrifuged at 600×g for 7 min, washed with 150 µL 1X blocking buffer, and recentrifuged. Aspirated off the supernatant, then added 100 µL of 1X blocking buffer to each sample, mixed, and incubated for 30 min, RT. The anti-cytochrome c-FITC antibody or anti-IgG_1-FITC isotype control 10 µL was added to each sample, mixed thoroughly and incubated at RT for 30 min in dark. The stained cells were washed and resuspended with 1X blocking buffer and then analyzed by flow cytometer (BD FACS Calibur with Cell Quest Pro software). Three individual experiments were performed.

5.3.8 Western blot analysis

During apoptotic induction, the levels of protein in apoptosis cascade are changes. Therefore, the increasing of pro-apoptotic protein; Bax, and apoptosis-associated protein; caspase-7, and also cleaved-PARP-1 (poly (ADP-ribose) polymerase-1) and the decreasing of anti-apoptotic protein; Bcl-2, procaspase-7, and PARP-1 are generally monitored. Western blotting is a classical technique used to identify the specific proteins in sample from cells or tissue based on their ability to bind to specific monoclonal antibodies.

5.3.8.1 Preparation for protein lysate

Cultured cells were seeded at 1.7×10^6 cells/well in 100 cm^2 dish and incubated for 24 h. Cultured cells were treated with the extracts for 12 and 24 h, then washed with PBS and lysed in plate with 200 µL of Tris-HCl lysis buffer on ice for 10 min. The cell lysate was harvested, vigorously mixed, and further incubated for another 10 min. Centrifugation was carried out at 12,000×g, 4ºC for 30 min. The lysate was kept in -80ºC for further analysis. The aliquot part was used for protein determination.

5.3.8.2 Determination of protein concentration

Protein contents were measured by Bradford assay (Bradford, 1976). The protein lysate was diluted in lysis buffer (1:5). Ten microliters of diluted protein lysate was mixed with 200 µL of dye reagent (1:5 dilution) in a 96-well plate. The reaction was incubated at room temperature for 10 min and measured the absorbance of 595 nm. Bovine serum albumin (BSA) was used as a standard curve for protein determination.

5.3.8.3 SDS-PAGE gel electrophoresis and Western blot

Denaturing polyacrylamide gel was performed according to the method of Laemmli (1970) with some modification. Protein lysate of 30 µg was mixed with 3 parts of 4X SDS-gel loading buffer, and boiled for 5 min. Protein lysate solution was separated in 12% SDS-PAGE using the constant voltage of 120 V for 2 h. Then, the proteins were electrotransferred onto nitrocellulose membrane using a constant 400 mA, 4ºC for 4 h. The gel was stained with Coomassie blue for checking the transfer. The blotted membrane was blocked for nonspecific binding with 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) containing 5% (w/v) bovine serum albumin (BSA), RT for 1 h. Then, the membrane was incubated with the primary antibodies against anti-mouse cleaved-PARP (poly(ADP-ribose) polymerase) (1:1000 dilution with 1% BSA in TBST, 3 h), procaspase-7 (1:1000 dilution with 3% BSA in TBST, 1 h), caspase-7 (p20) (1:1000 dilution with 3% BSA in TBST, 6 h), Bcl-2 (1:2000 dilution with 2% BSA in TBST, 1 h), Bax (1:1000 dilution with 2% BSA in TBST, 3 h), and actin (housekeeping protein) (1:1000 dilution with 2% BSA in TBST, 3 h) at room temperature. Then, the membrane was washed in TBST (5 min, 3 times). The membrane was further incubated with secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 or 1:2000 dilution with 3% BSA in TBST) for 1 h, then washed in TBST (5 min, 3 times) and TBS (5 min, 2 times).

The blotted membrane were soaked in chemiluminescent solution for 5-10 min and autoradiographed on X-ray films. Two individual experiments were performed.

5.3.9 Statistical analysis

Data were analyzed by ANOVA, followed by Duncan's multiple range tests were analyzed using SPSS program and results were presented as mean ± SD. The IC_{50} (concentration required to inhibit cell growth by 50%) values within 95% confidence intervals were obtained from the non-linear regression when plotted the log concentration verses percentage viability using PRISM program.

5.4 Results and discussion

5.4.1 Anti-proliferative effects on MCF7 breast cancer cells

Anti-proliferative effects of the 6 vegetable leaf extracts against MCF7 cells were dose- and time-dependent (Table 5.1). The 6 vegetable extracts exhibited different anti-proliferative patterns in term of concentrations used and times, and less activity than resveratrol 80 µg/mL. The percentage of cell viability when treated with BaLE ≥ 100 µg/mL showed significant difference from control ($p < 0.05$). They exhibited a dramatically decrease in % cell viability from 89.02% to 58.97%, 25.25%, and 13.09% and 85.97%, 29.84%, 14.95%, and 7.26% at 100, 200, 300, and 400 µg/mL with IC_{50} values of 223.00 and 152.20 µg/mL for 24 h and 48 h, respectively. BaLE 400 µg/mL seem to be high toxic to MCF7 cells which observed in the shrinkage and high eruption of cells at 24 h, as illustrated in Figure 5.1F. CfLE, LaLE, PoLE exhibited no toxic or cell proliferative effect when treated with 100 µg/mL which showed 100.00%, 108.26%, and 111.96% cell viability for 24 h and 103.70%, 97.95%, and 101.42% cell viability for 48 h, respectively but they showed the anti-proliferative effect when the concentrations increased with IC_{50} values of 350.90, 430.00, and 384.01 µg/mL for 24 h and 255.60, 383.70, and 332.50 µg/mL for 48 h,

respectively (Table 5.1 and Figures 5.2, 5.3, and 5.4). SgLE was the highest anti-proliferative effect on MCF7 cells with IC_{50} value of 120.70 and 90.12 µg/mL at 24 h and 48 h, respectively. SgLE 100 µg/mL exhibited a significant decreasing in % cell viability when compare ($p < 0.05$) to the control which were 65.40% and 45.61% at 24 and 48 h, respectively (Table 5.1). Moreover, the cells almost died when treated with SgLE 200 µg/mL which was 24.19% and 19.86% viability at 24 h and 48 h, respectively and observed from light microscope (Figure 5.5E). The cell viability (%) of StLE was significant different ($p < 0.05$) from control when treated $\geq$ 100 µg/mL with 87.88% and 79.38% at 24 h and 48 h, respectively. It produced a steady decreasing in MCF7 cell viability (Figure 5.6) with IC_{50} value of 382.50 and 215.77 µg/mL.

The IC_{50} values of the 6 vegetable leaf extracts ranged from 120.70 to 430.00 µg/mL and 90.12 to 383.70 µg/mL at 24 and 48 h, respectively as illustrated in Figure 5.7. Among the extracts, SgLE showed the highest ability on inhibiting of MCF7 cell proliferation with IC_{50} values of 120.70 and 90.12 µg/mL at 24 and 48 h, respectively, followed by BaLE. The other four extracts showed no significant different in IC_{50} values but it was more likely to follow this orders CfLE > StLE > PoLE > LaLE at 24 h and StLE > CfLE > PoLE > LaLE at 48 h.

Table 5.1 Percentage of MCF7 breast cancer cell viability treated with the 6 vegetable leaf extracts at 24 h and 48 h.

	Conc.	% Cell viability*					
	(µg/mL)	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
24 h	100	89.02 ± 5.61^{b}	100.00 ± 6.58^{a}	108.26 ± 5.60^{a}	111.96 ± 4.58^{a}	65.40 ± 2.36^{b}	87.88 ± 5.82^{b}
	200	58.97 ± 4.77^{c}	97.50 ± 4.94^{a}	92.56 ± 6.99^{c}	95.67 ± 6.16^{b}	24.19 ± 2.98^{d}	75.57 ± 4.87^{c}
	300	25.25 ± 5.75^{d}	75.67 ± 11.21^{b}	89.40 ± 4.07^{c}	66.67 ± 5.40^{c}	16.97 ± 3.24^{e}	60.67 ± 5.61^{d}
	400	13.09 ± 2.36^{e}	40.55 ± 6.90^{d}	62.87 ± 6.86^{d}	50.71 ± 5.14^{d}	8.33 ± 5.55^{f}	47.76 ± 6.11^{e}
	600	0.00^{f}	19.99 ± 1.21^{e}	24.17 ± 2.46^{f}	20.84 ± 4.59^{e}	0.00^{g}	27.66 ± 6.82^{f}
	800	0.00^{f}	11.66 ± 3.29^{e}	18.81 ± 2.39^{f}	3.90 ± 1.51^{f}	0.00^{g}	18.41 ± 1.41^{g}
	Resv	55.04 ± 2.06^{c}	55.04 ± 2.06^{c}	55.04 ± 2.06^{e}	55.04 ± 2.06^{d}	55.04 ± 2.06^{c}	55.04 ± 2.06^{d}
	Control	100.33 ± 2.31^{a}	100.33 ± 2.31^{a}	100.33 ± 2.31^{b}	100.33 ± 2.31^{b}	100.33 ± 2.31^{a}	100.33 ± 2.31^{a}
	IC_{50}**	223.00 (205.80-241.70)	350.90 (331.50-371.50)	430.00 (370.10-499.60)	384.01 (325.51-453.02)	120.70 (106.30-137.01)	382.50 (359.10-412.80)
48 h	100	85.97 ± 4.91^{b}	103.70 ± 6.68^{a}	97.95 ± 5.10^{a}	101.42 ± 4.06^{a}	45.61 ± 8.66^{b}	79.38 ± 5.41^{b}
	200	29.84 ± 5.37^{c}	77.77 ± 10.25^{b}	85.02 ± 3.94^{b}	76.42 ± 12.03^{b}	19.86 ± 3.98^{c}	52.92 ± 4.48^{c}
	300	14.95 ± 3.68^{d}	42.34 ± 14.86^{c}	61.86 ± 3.14^{c}	60.82 ± 3.18^{c}	11.53 ± 2.25^{d}	39.24 ± 7.98^{d}
	400	7.26 ± 1.70^{e}	17.63 ± 8.42^{d}	44.49 ± 6.30^{d}	32.79 ± 7.78^{d}	4.35 ± 3.35^{e}	24.59 ± 5.11^{e}
	600	0.00^{f}	10.34 ± 1.93^{d}	12.97 ± 3.56^{ef}	19.40 ± 2.11^{e}	0.00^{e}	14.32 ± 2.90^{f}
	800	0.00^{f}	8.33 ± 5.46^{d}	9.24 ± 8.49^{f}	2.68 ± 2.82^{f}	0.00^{e}	6.02 ± 1.70^{g}
	Resv	18.67 ± 1.70^{d}	18.67 ± 1.70^{d}	18.67 ± 1.70^{e}	18.67 ± 1.70^{e}	18.67 ± 1.70^{c}	18.67 ± 1.70^{ef}
	Control	100.01 ± 5.82^{a}	100.01 ± 5.82^{a}	100.01 ± 5.82^{a}	100.01 ± 5.82^{a}	100.01 ± 5.82^{a}	100.01 ± 5.82^{a}
	IC_{50}**	152.20 (142.90-162.12)	255.60 (230.50-283.30)	383.70 (352.81-417.33)	332.50 (267.00-414.10)	90.12 (78.42-103.60)	215.77 (202.30-230.14)

*presented as mean ± SD, n = 6, statistical analysis was performed by SPSS program, ANOVA ($p < 0.05$), alphabets: a-g in column. **presented as mean (95% confidence interval). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; Resv, Resveratrol 80 µg/mL; Control, 0.2% DMSO.

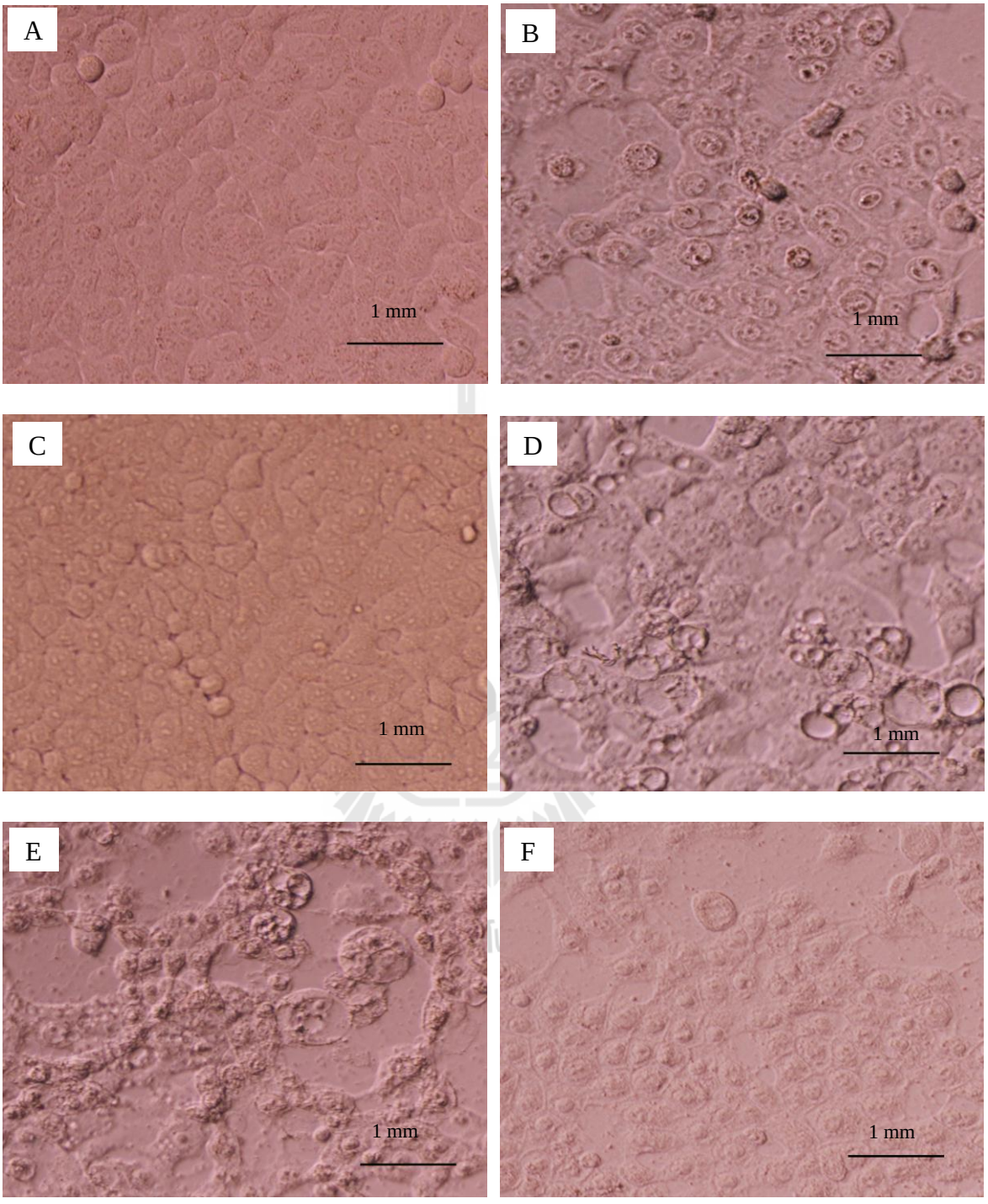

Figure 5.1 Morphology of MCF7 cells under bright field microscope when treated with BaLE (*B. acutangula*) for 24 h. A, 0.2% DMSO (control); B, resveratrol 80 µg/mL; C-F, BaLE 100, 200, 300, and 400 µg/mL, respectively (Magnified × 100).

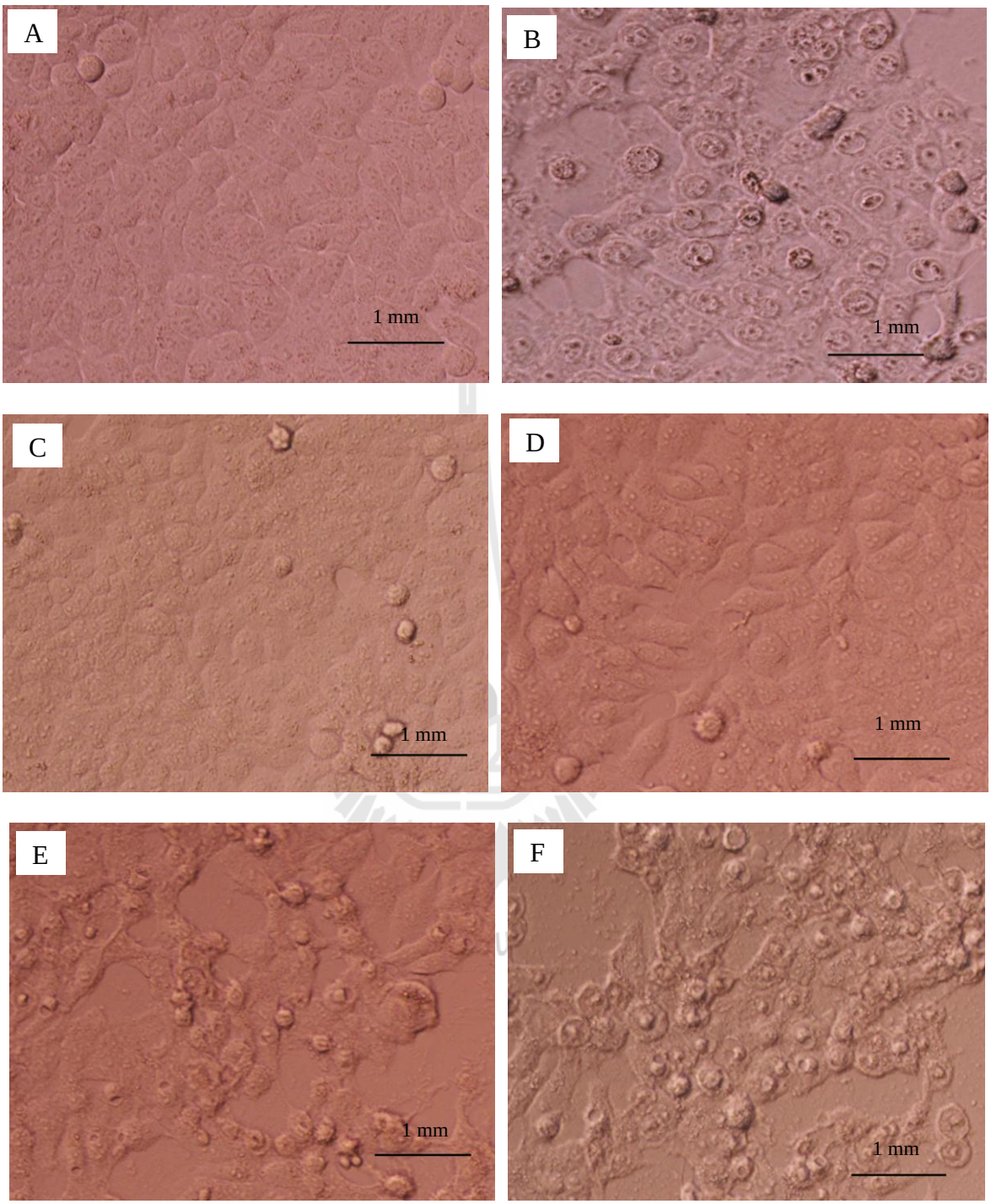

Figure 5.2 Morphology of MCF7 cells under bright field microscope when treated with CfLE (*C. formosum*) for 24 h. A, 0.2% DMSO (control); B, resveratrol 80 µg/mL; C-F, CfLE 100, 200, 300, and 400 µg/mL, respectively (Magnified × 100).

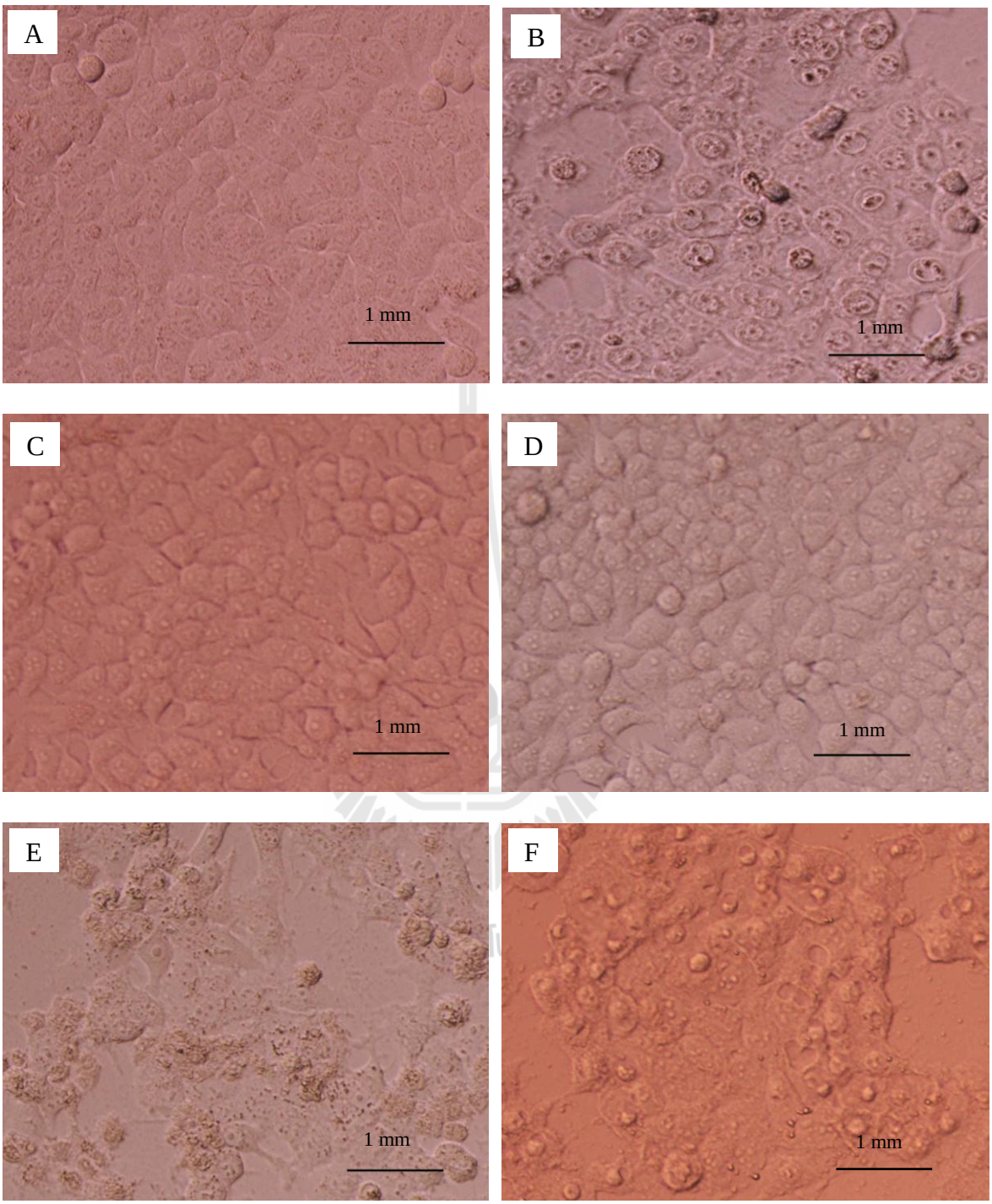

Figure 5.3 Morphology of MCF7 cells under bright field microscope when treated with LaLE (*L. aromatica*) for 24 h. A, 0.2% DMSO (control); B, resveratrol 80 µg/mL; C-F, LaLE 100, 200, 300, and 400 µg/mL, respectively (Magnified × 100).

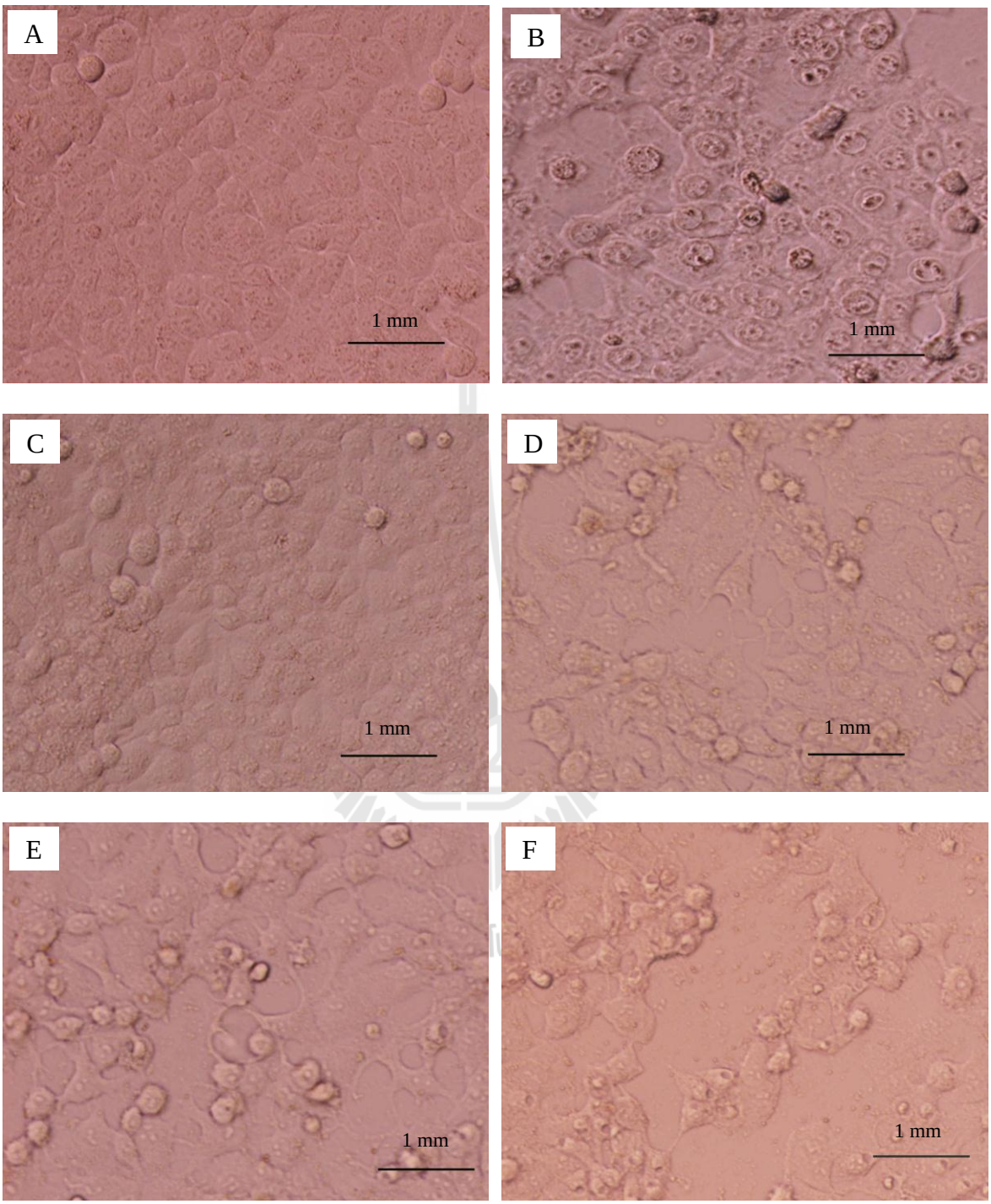

Figure 5.4 Morphology of MCF7 cells under bright field microscope when treated with PoLE (*P. odoratum*) for 24 h. A, 0.2% DMSO (control); B, resveratrol 80 µg/mL; C-F, PoLE: 100, 200, 300, and 400 µg/mL, respectively (Magnified × 100).

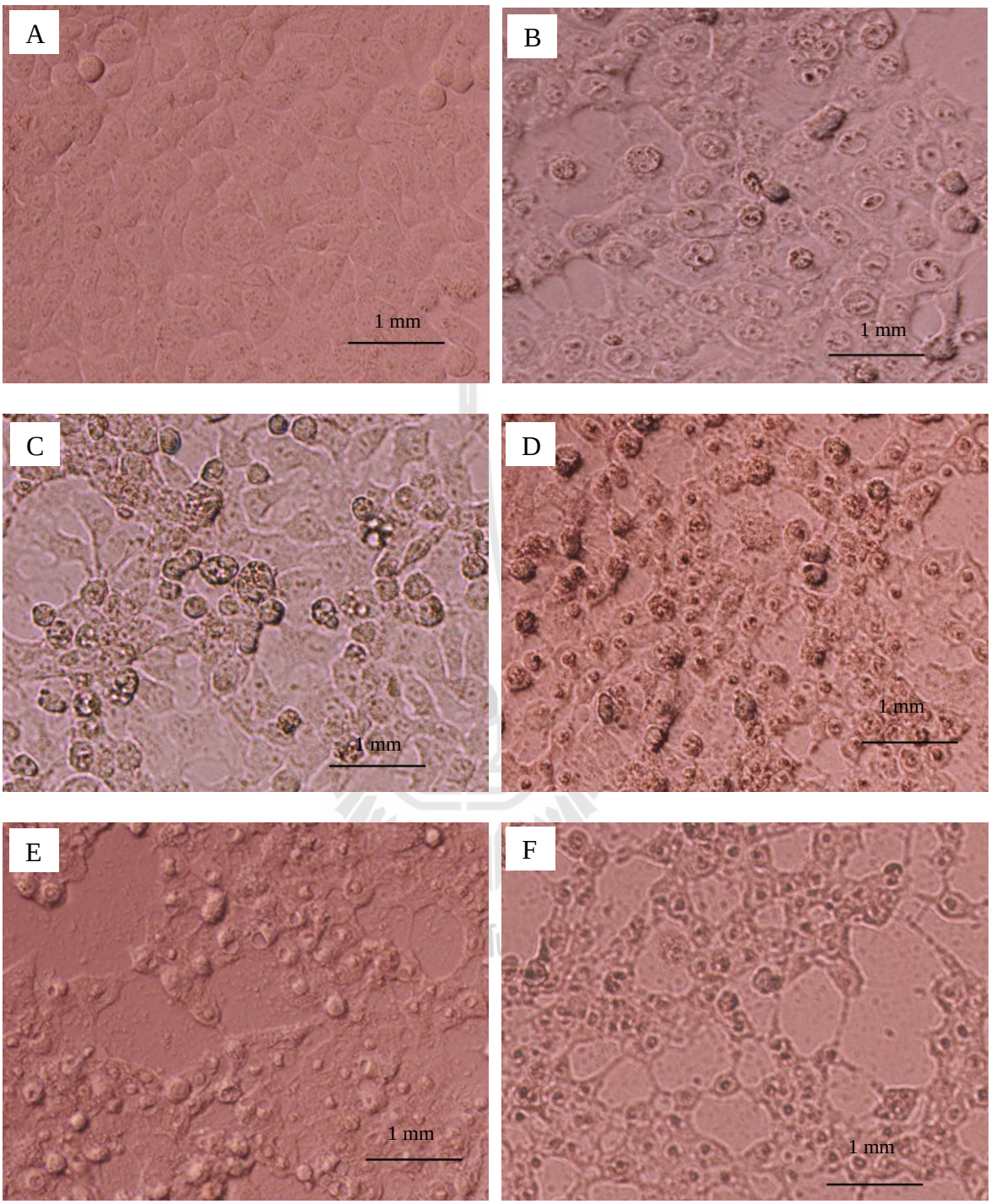

Figure 5.5 Morphology of MCF7 cells under bright field microscope when treated with SgLE (*S. gratum*) for 24 h. A, 0.2% DMSO (control); B, resveratrol 80 µg/mL; C-F, SgLE: 100, 150, 200, and 300 µg/mL, respectively (Magnified × 100).

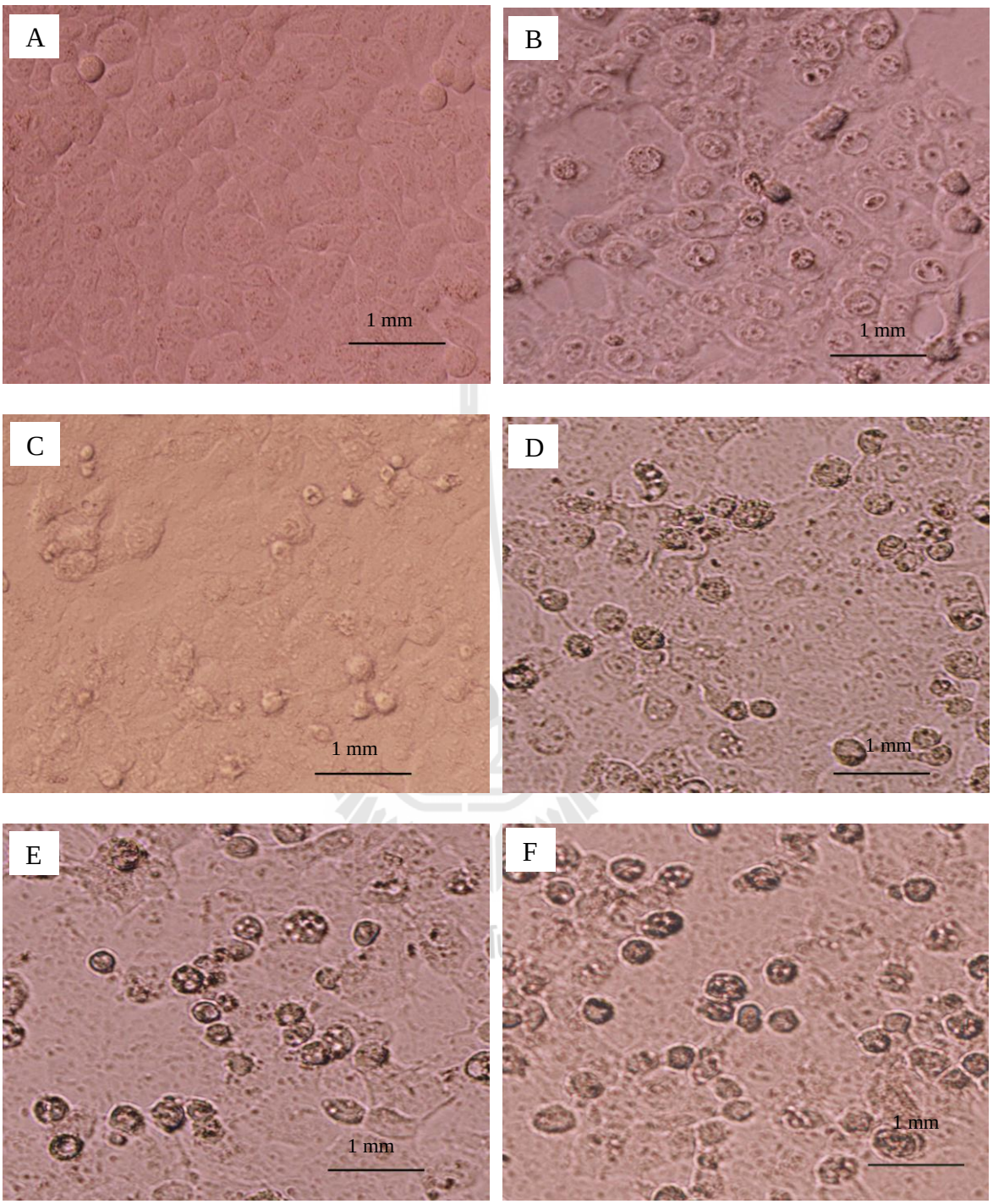

Figure 5.6 Morphology of MCF7 cells under bright field microscope when treated with StLE (*S. terebinthifolius*). A, 0.2% DMSO (control); B, resveratrol 80 µg/mL; C-F, StLE: 100, 200, 300, and 400 µg/mL for 24 h, respectively (Magnified × 100).

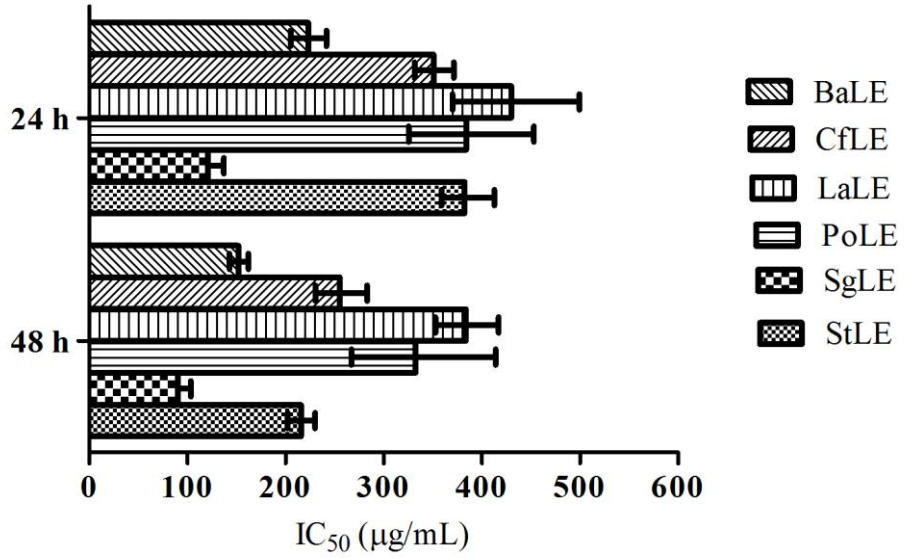

Figure 5.7 The IC_{50} values (Mean ± 95% CI, n = 6) of the 6 vegetable leaf extracts on MCF7 cells proliferation at 24 and 48 h.

5.4.2 Nuclear morphological changes of MCF7 cells

The blue fluorescent Hoechst dye, Hoechst 33258, was used to stain the nuclei acid for detecting the morphological features associated with late apoptosis. Nuclear morphological changes of MCF7 cells were observed under fluorescent light microscope, the percentage of condensed and fragmented nuclei were calculated and summarized in Tables 5.3, 5.4, and 5.5. Control cells showed round and homogenous nuclei, whereas apoptotic-treated cells showed condensed and fragmented nuclei (arrow) (Figures 5.8-5.13). Resveratrol was serves as the positive control in this study since its apoptotic effects on MCF7 cells has been reported (Li et al., 2006). All vegetable extracts induced the condensation and/or fragmentation of nuclei (apoptotic-like nuclei) with dose- and time-dependent and with difference in morphology. BaLE induced a dense and fragmented nuclei which seem like induced by resveratrol (Figure 5.8) with EC_{50} values of 330.8, 237.4, and 159.9 g/mL at 12 h, 24 h, and 48 h, respectively. CfLE, LaLE, and PoLE, exhibited similar in the ability to induce DNA fragmented and in nuclear morphological features (Figures 5.9,

5.10 and 5.11). They showed no significant difference ($p > 0.05$) from control when treated at 100 µg/mL for 12 h, 24 h, 48 h and produced significantly different ($p < 0.05$) when treated ≥ 300 µg/mL at 12 h and ≥ 200 µg/mL at 24 h and 48 h, respectively. The EC_{50} values of CfLE, LaLE, and PoLE were 454.50, 567.40, and 446.02 µg/mL at 12 h, 407.50, 472.22, and 295.80 µg/mL at 24 h and 310.70, 402.61, and 319.20 µg/mL at 48 h, respectively. Among the extracts, SgLE was the highest DNA fragmented inducing ability which seems to be more dose-dependent than time-dependent manner with 33.46%, 38.90% and 35.48% when treated 100 µg/mL and 69.92%, 81.87%, and 94.50% fragmented nuclei when treated 200 µg/mL at 12 h, 24 h, and 48 h, respectively. EC_{50} values of SgLE were 136.60, 118.21, and 112.80 µg/mL at 12 h, 24 h, and 48 h, respectively. The fragmented nuclei of SgLE were same as resveratrol but smaller in the nuclear boundary (Figure 5.12). StLE demonstrated dose-dependent and time-dependent until 24 h incubation, with EC_{50} values of 408.11, 290.20, and 279.63 µg/mL at 12 h, 24 h, and 48 h, respectively. The apoptotic-like nuclei were significantly observed ($p < 0.05$) at StLE 200 µg/mL as shown in Tables 5.2, 5.3, 5.4 and Figure 5.13. Among the extracts, SgLE was the highest ability to induce apoptotic-like nuclei at 12 h, 24 h, and 48 h. The second was BaLE which showed no different from the other four extracts at 12 h but different at 24 h and 48 h. The other four extracts showed no different in IC_{50} values but it was more likely to follow this orders StLE > PoLE > CfLE > LaLE at 12 h and 24 h, and StLE > CfLE > PoLE > LaLE at 48 h, respectively (Figure 5.14).

Table 5.2 Apoptotic nuclei (%) of MCF7 cells treated with the 6 vegetable leaf extracts for 12 h.

Conc.	% Condensed or fragmented nuclei*					
(µg/mL)	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
100	2.26 ± 0.68^{d}	1.17 ± 0.51^{e}	0.92 ± 0.59^{d}	1.66 ± 0.56^{f}	33.46 ± 6.32^{c}	5.75 ± 0.81^{f}
200	28.31 ± 2.41^{bc}	7.07 ± 2.77^{e}	2.56 ± 1.93^{d}	6.91 ± 2.00^{f}	69.92 ± 5.68^{b}	31.14 ± 6.66^{d}
300	31.40 ± 8.12^{b}	29.12 ± 7.07^{d}	14.77 ± 2.58^{c}	31.15 ± 4.50^{d}	91.02 ± 4.68^{a}	40.11 ± 4.71^{c}
400	93.00 ± 6.56^{a}	44.82 ± 4.15^{c}	42.12 ± 4.71^{b}	46.79 ± 4.06^{c}	100.00^{a}	46.28 ± 3.34^{c}
600	100.00^{a}	60.10 ± 5.96^{b}	52.18 ± 11.38^{ab}	62.38 ± 2.82^{b}	100.00^{a}	62.37 ± 2.82^{b}
800	100.00^{a}	79.70 ± 13.93^{a}	59.59 ± 13.27^{a}	73.85 ± 6.07^{a}	100.00^{a}	73.85 ± 6.07^{a}
Resv	23.46 ± 6.40^{c}	23.46 ± 6.40^{d}	23.46 ± 6.40^{c}	23.46 ± 6.40^{e}	23.46 ± 6.40^{d}	23.46 ± 6.40^{e}
Control	0.00^{d}	0.00^{e}	0.00^{d}	0.00^{f}	0.00^{e}	0.00^{f}
EC_{50}*	330.80	454.50	567.40	446.02	136.6	408.1
	(308.30-354.80)	(408.00-506.20)	(483.50-667.20)	(409.61-485.74)	(126.10-148.02)	(358.23-464.84)

*presented as mean ± SD, n = 6, statistical analysis was performed by SPSS program, ANOVA ($p < 0.05$), alphabets: a-g in column.
**presented as mean (95% confidence interval). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; Resv, Resveratrol 80 µg/mL; Control, 0.2% DMSO.

Table 5.3 Apoptotic nuclei (%) of MCF7 cells treated with the 6 vegetable leaf extracts for 24 h.

Conc.	% Condensed or fragmented nuclei*					
(µg/mL)	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
100	2.96 ± 3.95^{e}	1.33 ± 0.91^{f}	0.85 ± 0.55^{e}	0.09 ± 0.08^{e}	38.90 ± 19.24^{d}	8.58 ± 3.16^{e}
200	30.68 ± 9.91^{d}	25.22 ± 3.95^{e}	6.62 ± 4.68^{de}	25.12 ± 8.14^{d}	81.87 ± 4.68^{b}	34.84 ± 7.64^{d}
300	79.24 ± 3.63^{b}	31.05 ± 4.12^{e}	11.74 ± 4.08^{d}	54.47 ± 6.28^{c}	100.00^{a}	51.78 ± 8.51^{c}
400	99.59 ± 0.70^{a}	44.14 ± 2.62^{d}	42.31 ± 3.00^{c}	68.44 ± 5.73^{b}	100.00^{a}	64.02 ± 16.24^{bc}
600	100.00^{a}	91.07 ± 6.28^{b}	62.71 ± 8.58^{b}	72.07 ± 5.73^{b}	100.00^{a}	75.96 ± 3.00^{b}
800	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	96.39 ± 5.10^{a}
Resv	65.04 ± 8.05^{c}	65.04 ± 8.05^{c}	65.04 ± 8.05^{b}	65.04 ± 8.05^{b}	65.04 ± 8.05^{c}	65.04 ± 8.05^{bc}
Control	0.13 ± 0.22^{e}	0.13 ± 0.22^{f}	0.13 ± 0.22^{e}	0.13 ± 0.22^{e}	0.13 ± 0.22^{e}	0.13 ± 0.22^{e}
EC_{50}**	237.40	407.50	472.22	295.80	118.21	290.20
	(226.00-249.30)	(368.70-450.40)	(435.06-512.62)	(262.80-333.00)	(106.32-131.55)	(252.32-333.83)

*presented as mean ± SD, n = 6, statistical analysis was performed by SPSS program, ANOVA ($p < 0.05$), alphabets: a-g in column.

**presented as mean (95% confidence interval). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; Resv, Resveratrol 80 µg/mL; Control, 0.2% DMSO.

Table 5.4 Apoptotic nuclei (%) of MCF7 cells treated with the 6 vegetable leaf extracts for 48 h.

Conc.	% Condensed or fragmented nuclei*					
(µg/mL)	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
100	13.72 ± 6.62^{c}	4.28 ± 1.70^{e}	1.11 ± 0.77^{f}	2.79 ± 0.72^{f}	35.48 ± 6.39^{c}	9.97 ± 0.45^{f}
200	72.48 ± 3.65^{b}	29.36 ± 5.72^{d}	18.41 ± 9.04^{e}	12.05 ± 4.46^{e}	94.50 ± 0.73^{a}	31.94 ± 4.03^{e}
300	78.67 ± 6.68^{b}	45.61 ± 7.17^{c}	31.23 ± 7.02^{d}	46.00 ± 6.68^{d}	100.00^{a}	56.81 ± 4.08^{d}
400	100.00^{a}	69.14 ± 1.30^{b}	56.21 ± 11.01^{c}	70.22 ± 5.63^{c}	100.00^{a}	70.95 ± 5.98^{c}
600	100.00^{a}	100.00^{a}	61.89 ± 1.01^{c}	84.07 ± 4.76^{b}	100.00^{a}	90.35 ± 2.78^{b}
800	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
Resv	76.42 ± 2.51^{b}	76.42 ± 2.51^{b}	76.42 ± 2.51^{b}	76.42 ± 2.51^{c}	76.42 ± 2.51^{b}	76.42 ± 2.51^{c}
Control	0.00^{d}	0.00^{e}	0.00^{f}	0.00^{f}	0.00^{d}	0.00^{g}
EC_{50}**	159.94	310.70	402.61	319.20	112.80	279.63
	(147.82-173.00)	(293.40-328.90)	(356.31-454.93)	(305.30-333.72)	(109.60-116.10)	(266.41-293.31)

*presented as mean ± SD, n = 6, statistical analysis was performed by SPSS program, ANOVA ($p < 0.05$), alphabets: a-g in column.

**presented as mean (95% confidence interval). BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract; Resv, Resveratrol 80 µg/mL; Control, 0.2% DMSO.

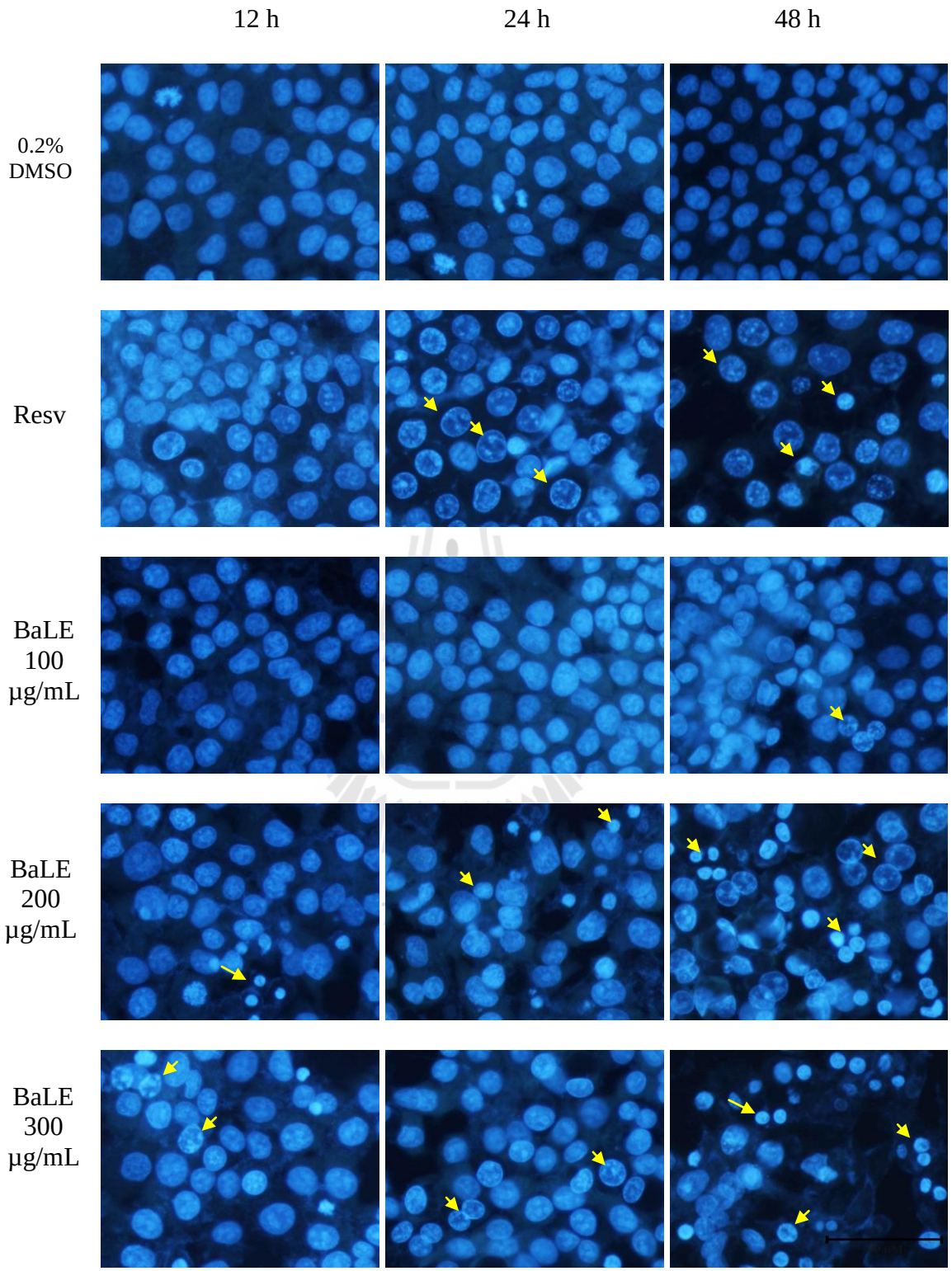

Figure 5.8 Nuclear morphological changes of MCF7 cells treated with 0.2% DMSO, resveratrol (Resv) 80 µg/mL, and BaLE (*B. acutangula*) 100, 200, 300 µg/mL for 12, 24, and 48 h. The condensed or fragmented nuclei are indicated as yellow arrow (Magnified × 200).

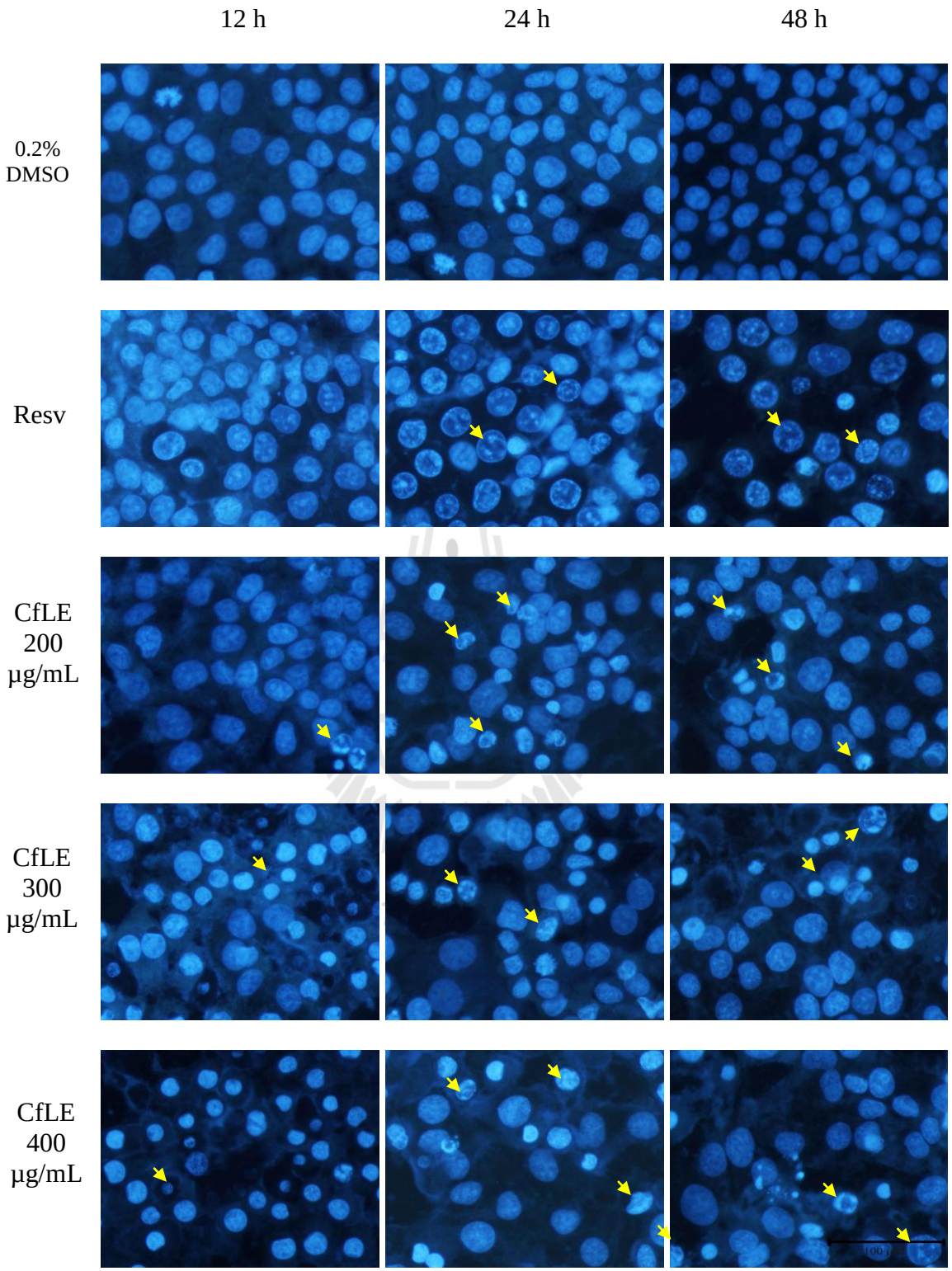

Figure 5.9 Nuclear morphological changes of MCF7 cells treated with 0.2% DMSO, resveratrol (Resv) 80 µg/mL, and CfLE (*C. formosum*) 200, 300, 400 µg/mL for 12, 24, and 48 h. The condensed or fragmented nuclei are indicated as yellow arrow (Magnified × 200).

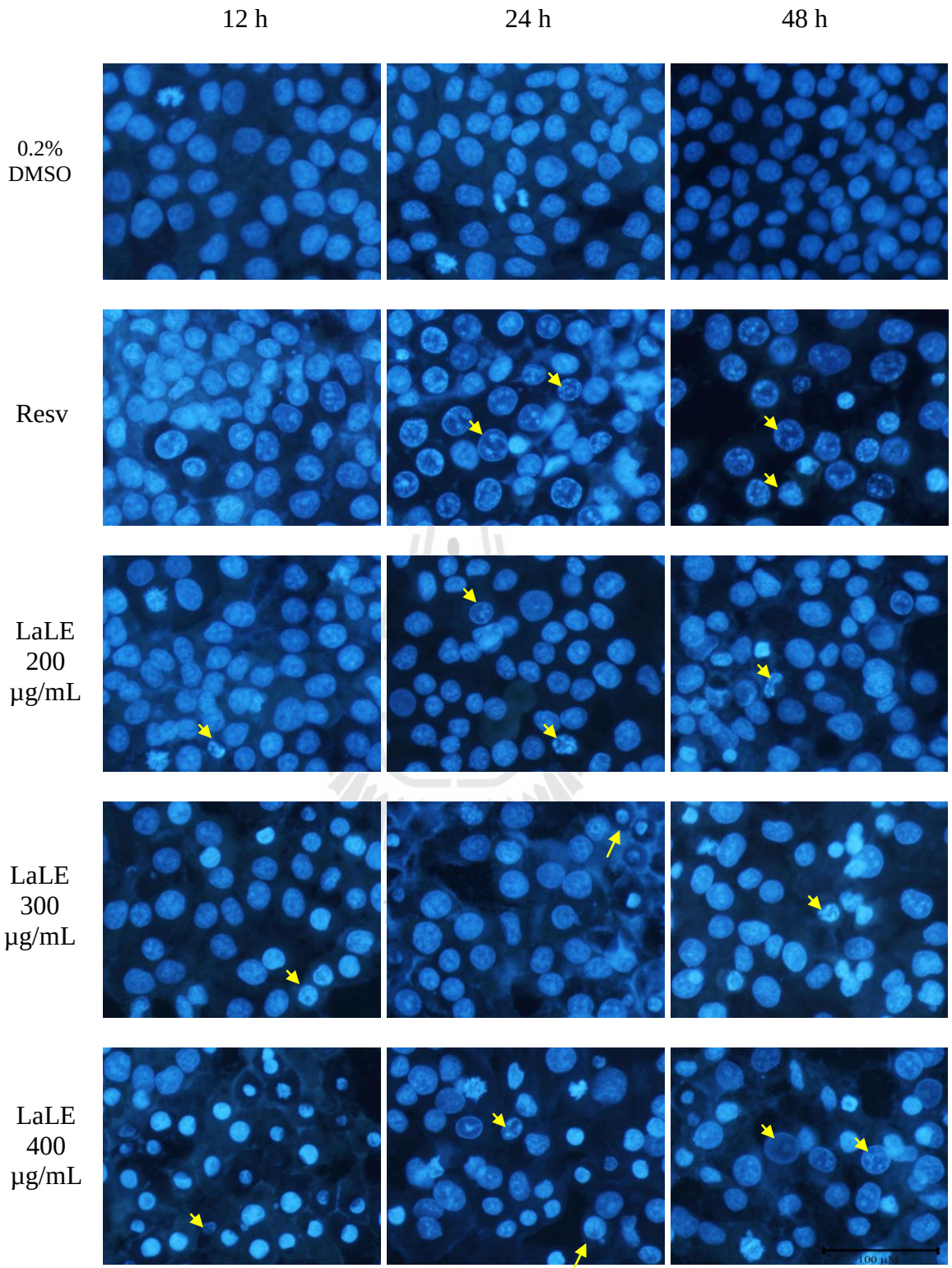

Figure 5.10 Nuclear morphological changes of MCF7 cells treated with 0.2% DMSO, resveratrol (Resv) 80 µg/mL, and LaLE (*L. aromatica*) 200, 300, 400 µg/mL for 12, 24, and 48 h. The condensed or fragmented nuclei are indicated as yellow arrow (Magnified × 200).

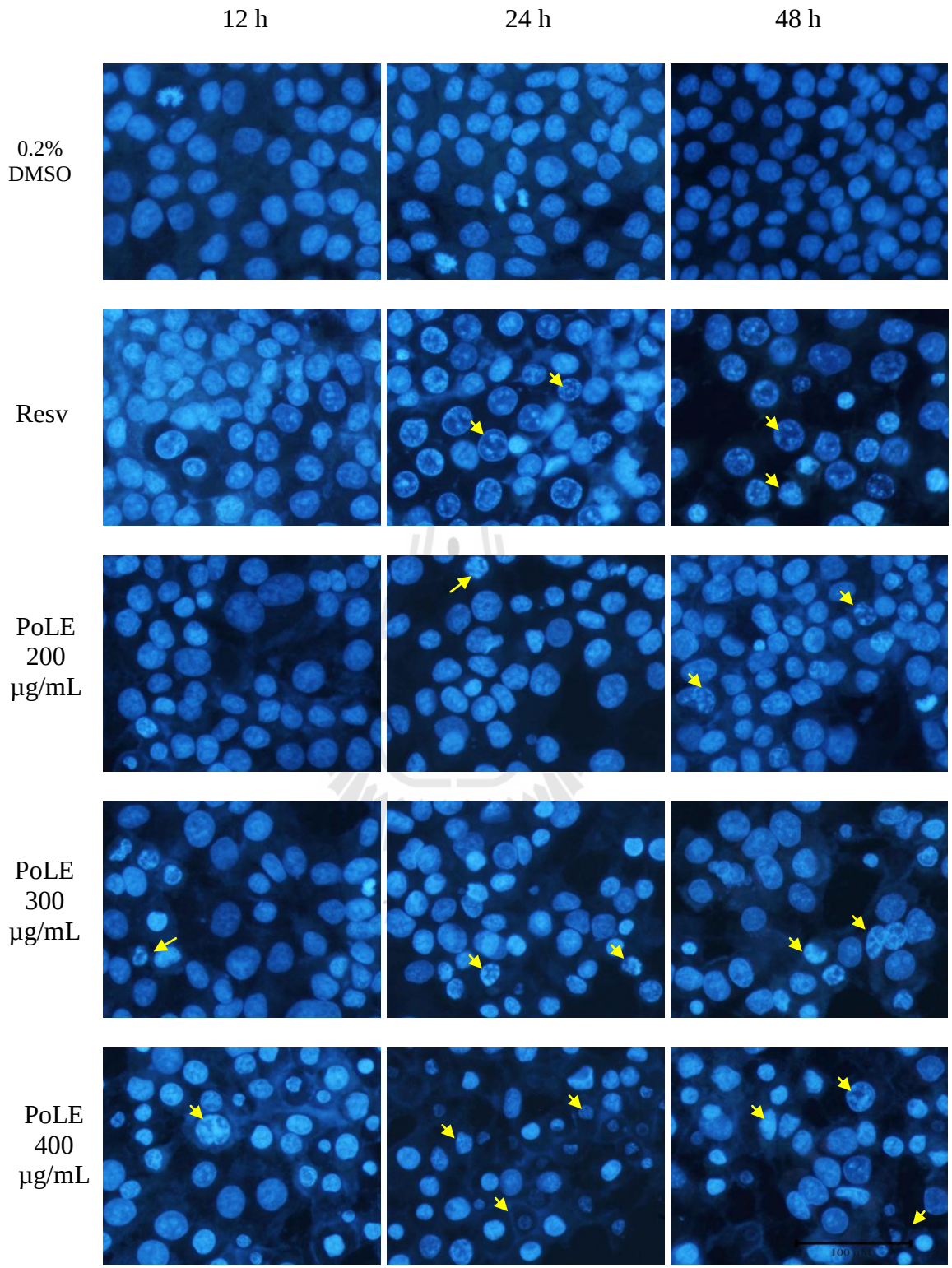

Figure 5.11 Nuclear morphological changes of MCF7 cells treated with 0.2% DMSO, resveratrol (Resv) 80 µg/mL, and PoLE (*P. odoratum*) 200, 300, 400 µg/mL for 12, 24, and 48 h. The condensed or fragmented nuclei are indicated as yellow arrow (Magnified × 200).

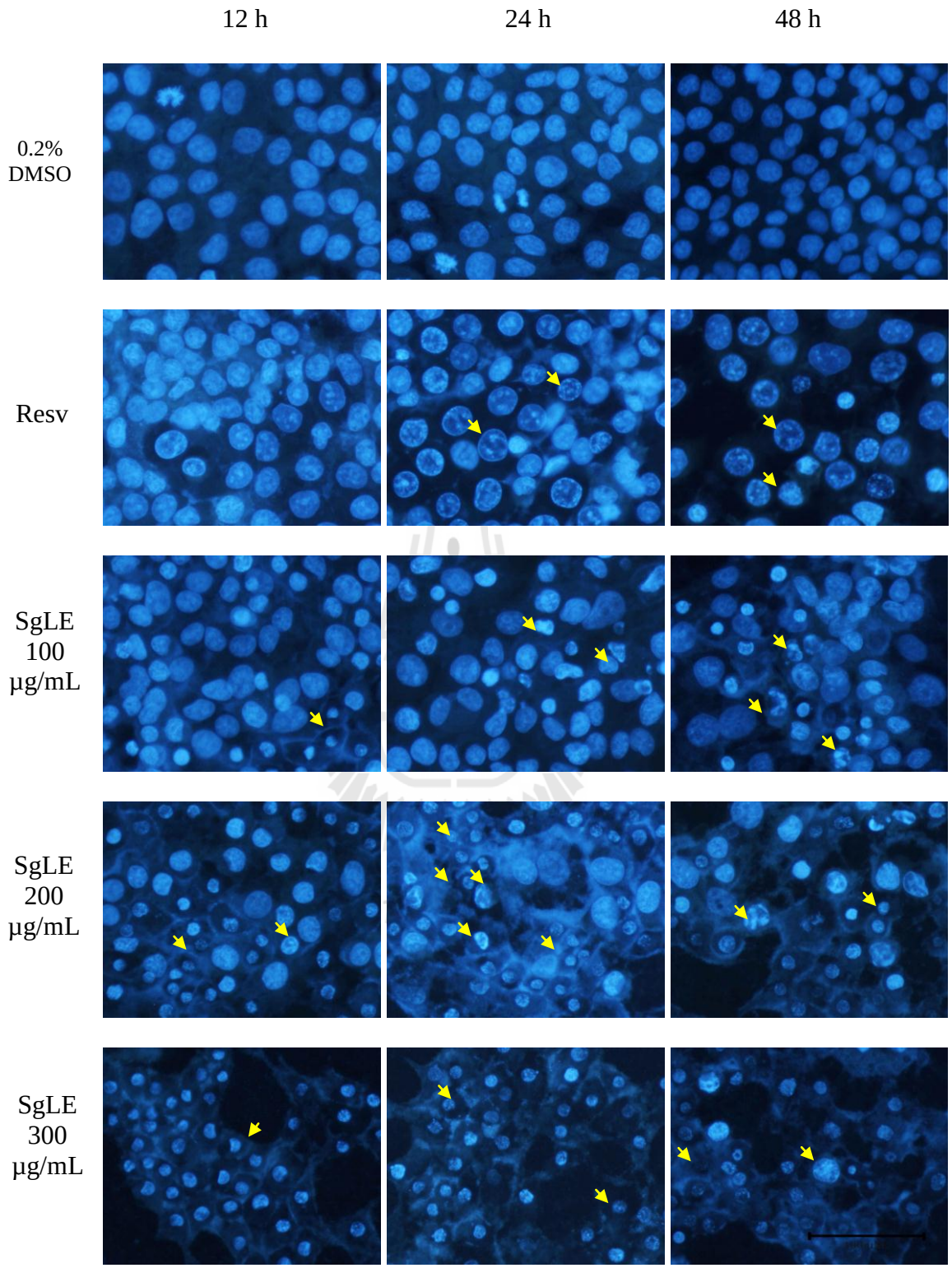

Figure 5.12 Nuclear morphological changes of MCF7 cells treated with 0.2% DMSO, resveratrol (Resv) 80 µg/mL, and SgLE (*S. gratum*) 100, 200, 300 µg/mL for 12, 24, and 48 h. The condensed or fragmented nuclei are indicated as yellow arrow (Magnified × 200).

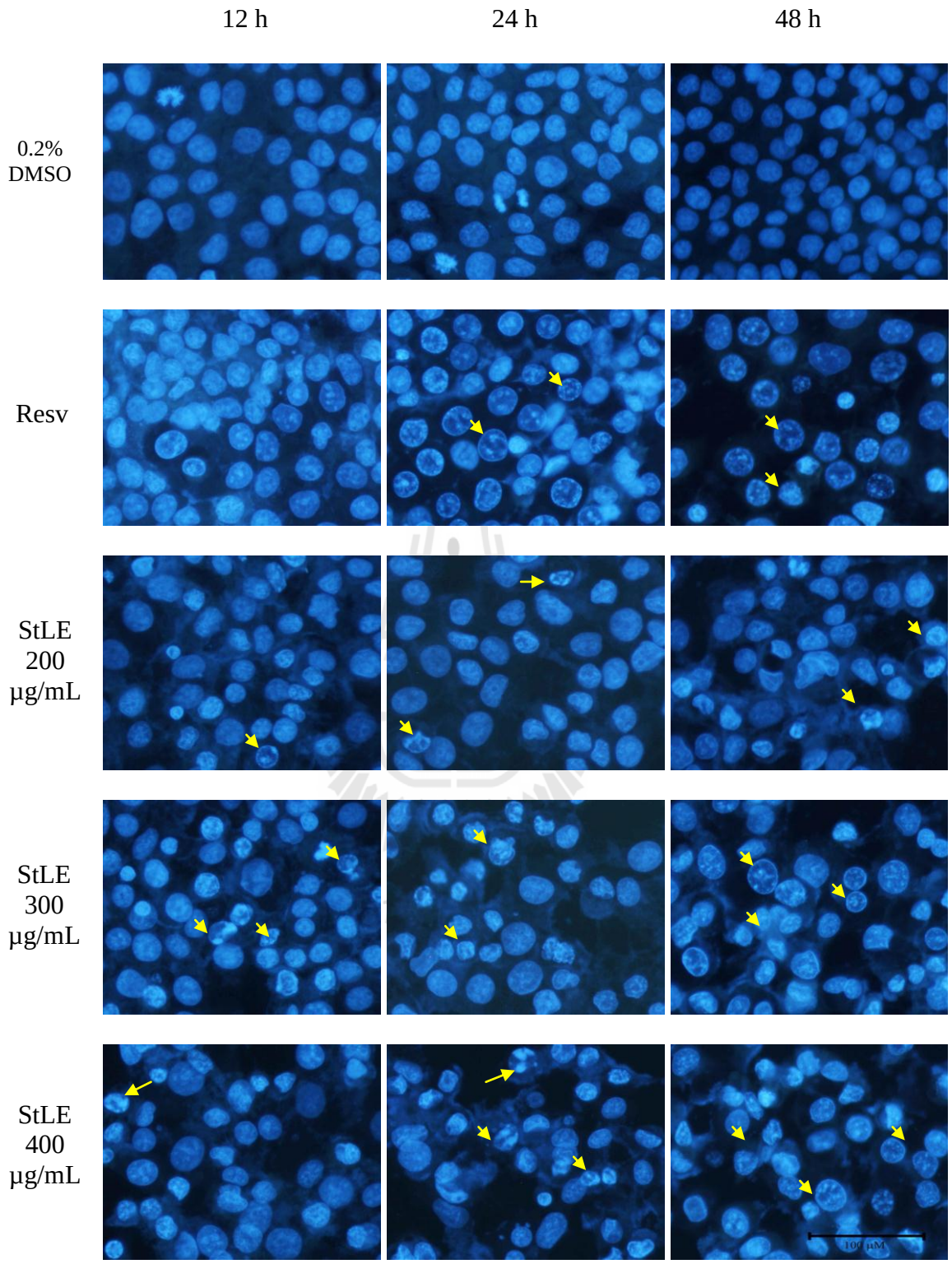

Figure 5.13 Nuclear morphological changes of MCF7 cells treated with 0.2% DMSO, resveratrol (Resv) 80 µg/mL, and StLE (*S. terebinthifolius*) 200, 300, 400 µg/mL for 12, 24, and 48 h. The condensed or fragmented nuclei are indicated as yellow arrow (Magnified × 200).

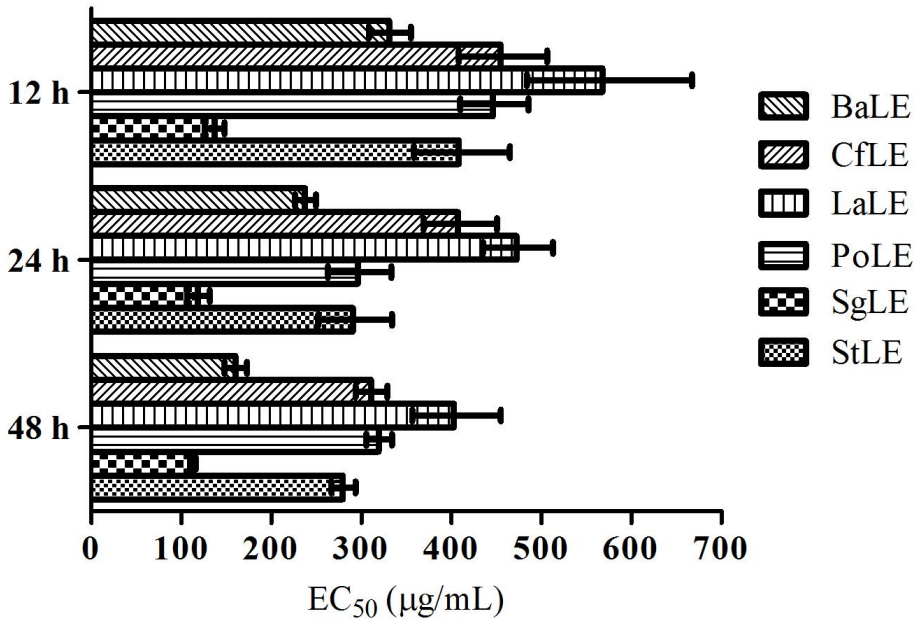

Figure 5.14 The EC_{50} values (Mean ± 95% CI, n = 6) of the 6 vegetable leaf extracts on induction of fragmented nuclei.

According to the characteristic of MCF7 cell line which lost of caspase-3 owing to a 47-base pair deletion within exon 3 of the CASP-3 gene. This deletion results in the skipping of exon 3 during pre-mRNA splicing, thereby aborating translation of the CASP-3 mRNA (Janicke, Sprengart, Wati, and Porter, 1998). Effector caspase (caspase-3, -6, -7) are required for the characteristic morphological feature of apoptosis such as chromatic condensation and nuclear fragmentation, but caspase-3 and caspase-6 are believed to cleave structural proteins such as nuclear lamins (the interior of nuclear envelope) (Slee, Adrain, and Martin, 2001). Thus, MCF7 cancer cells exposed to apoptotic treatment, could undergo apoptosis by the caspase-3 deficiency pathway (nuclear condensation and fragmentation), but did not display some of the distinct morphological features typical of apoptotic cells such as nuclear and membrane blebing and form the apoptotic bodies as observed in this study.

5.4.3 The effects on DNA fragmentation of MCF7 cells

DNA fragmentation or DNA ladder formation as another feature of the cell undergoing apoptosis was detected when treated with the 6 vegetable extracts and results are demonstrated in Figures 5.15-5.20. Resveratrol was used as the positive control for DNA ladder formation. Six-extracts showed different profiles in DNA fragmentation, BaLE, SgLE, and StLE seem to exhibit DNA ladder formation, whereas CfLE, LaLE, and PoLE seem to induce the high molecular weight (HMW) DNA fragmentation (~ 23,130 bp) and mononlucleosome (180 bp). BaLE produced the intense DNA ladder formation and some diffused smear at 400 µg/mL, 12 h, while the diffused smear and oligonucleosomes above 564 pb marker were detected at 100, 200, 300, 400 µg/mL at 24 h, implying the concomitant of necrotic and apoptotic DNA fragments might occurred at high concentration and prolong treatment (Figure 5.15). CfLE, LaLE, and PoLE with 400-800 µg/mL at 24 h induce HMW DNA fragmentation (Figures 5.16, 5.17, and 5.18), but no DNA ladder formation were observed. SgLE actively induced a ladder-like pattern of DNA fragmentation consisting of multiple of cleaved mono- (~ 180 bp) and oligo-nucleosomes (~ 360, ~ 540, ~ 720 bp and so on) as same as the resveratrol, with 300 µg/mL at 12 h, and 200, 300 µg/mL at 24 h, respectively (Figure 5.19), indicating the apoptotic-inducing activity. StLE induced HMW DNA fragmentation (above 2,027 bp marker) at 300 and 400 µg/mL, and high number of oligo-nucleosome fragments (ladder formation) (under 2,027 bp marker) at 600 and 800 µg/mL, 24h, respectively (Figure 5.20). Therefore, it was concluded that SgLE highly induced DNA ladder formation of MCF7 cells. BaLE could induce apoptosis and concomitant with necrosis in high concentration (400 µg/mL) and prolong treatment. CfLE, LaLE, and PoLE were less active on inducing the DNA ladder formation, but induced HMW fragments of MCF7 cells.

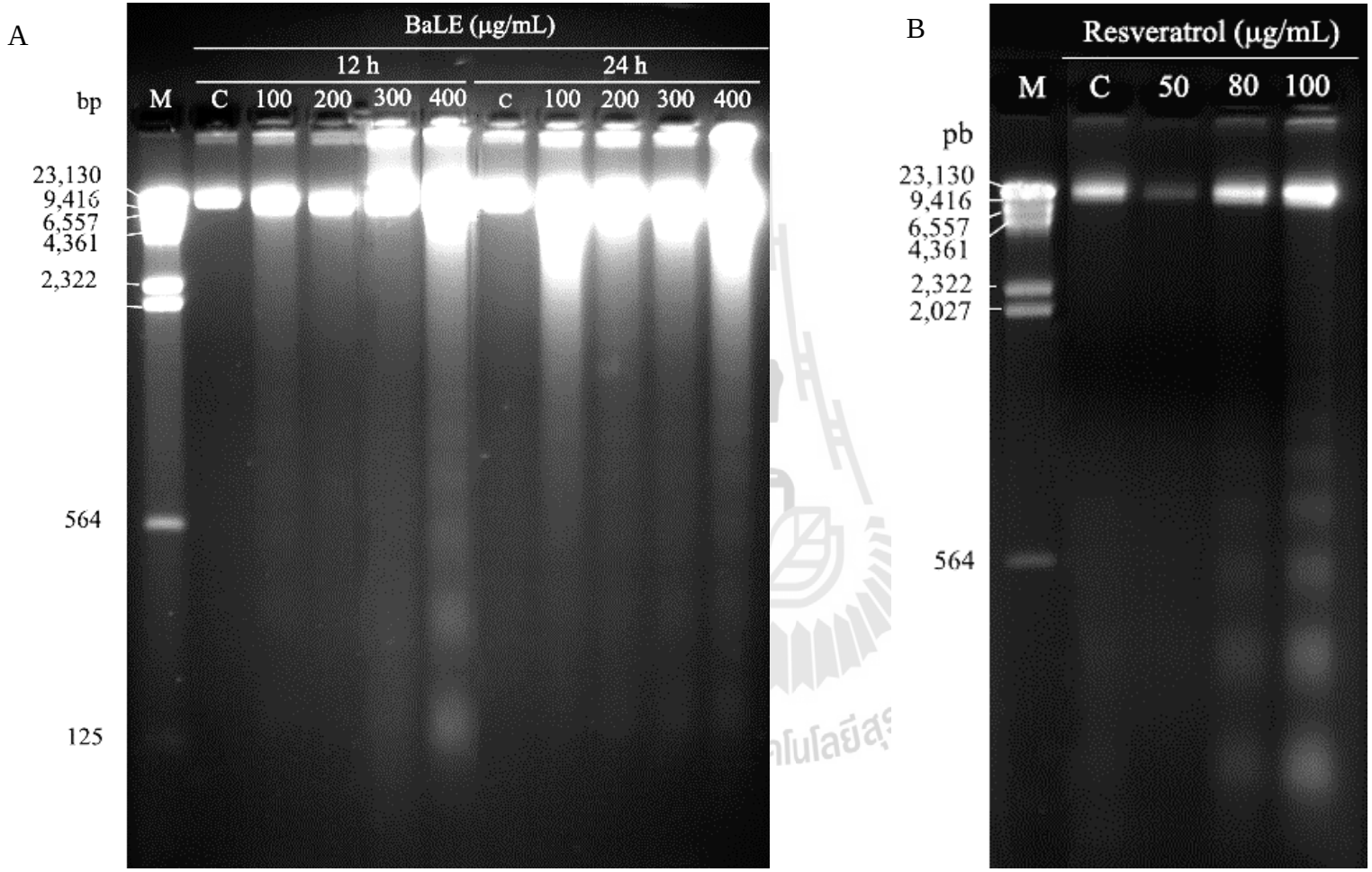

Figure 5.15 DNA fragmentation of MCF7 cells treated with various concentrations of *B. acutangula* (BaLE) at 12 h and 24 h (A) and resveratrol at 24 h (B). DNA (10 µg) was electrophoresed on 1.5% agarose gel for 90 min.

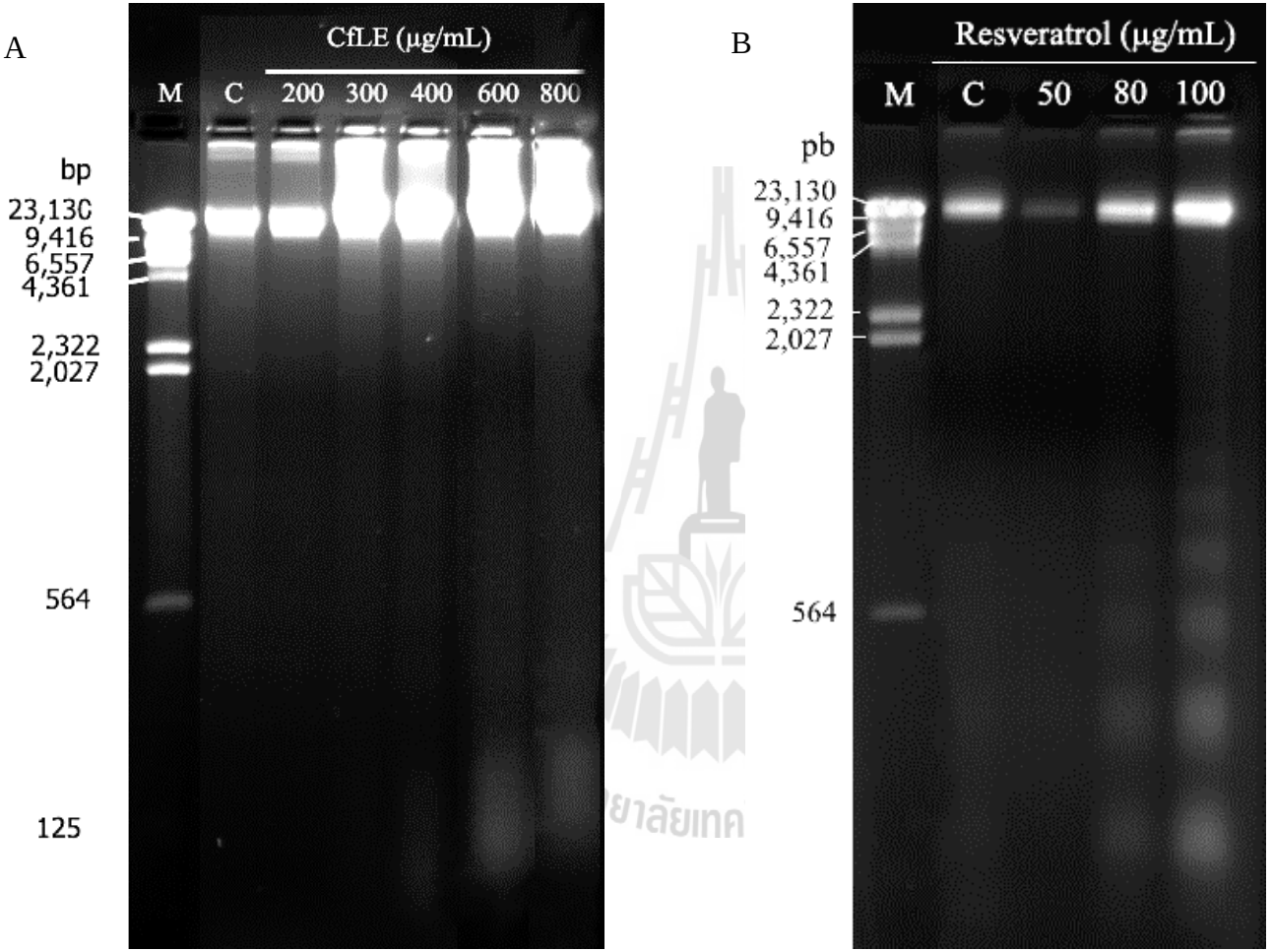

Figure 5.16 DNA fragmentation of MCF7 cells treated with various concentrations of *C. formosum* (CfLE) at 24 h (A) and resveratrol at 24 h (B). DNA (10 µg) was electrophoresed on 1.5% agarose gel for 90 min.

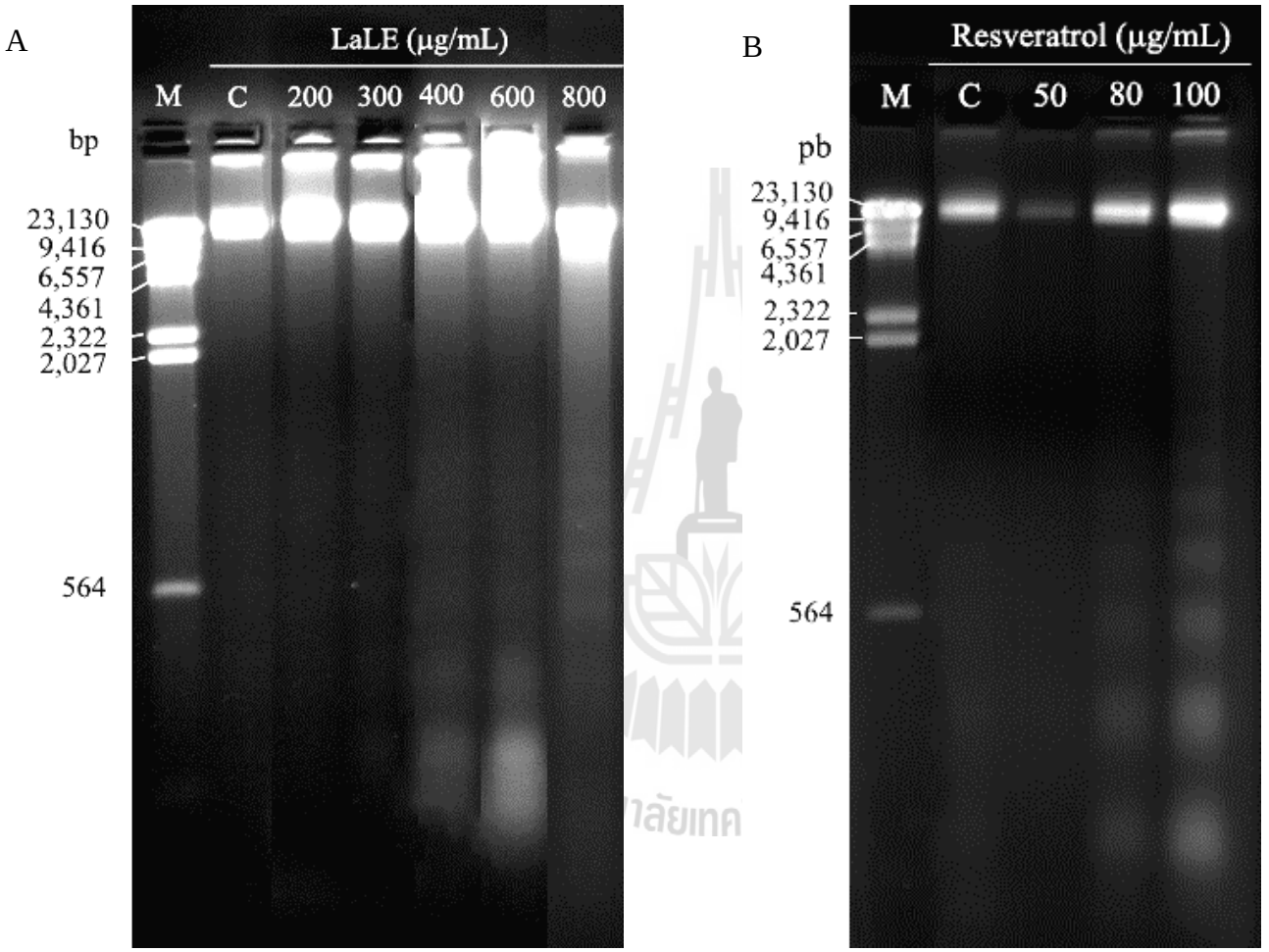

Figure 5.17 DNA fragmentation of MCF7 cells treated with various concentrations of *L. aromatica* (LaLE) at 24 h (A) and resveratrol at 24 h (B). DNA (10 µg) was electrophoresed on 1.5% agarose gel for 90 min.

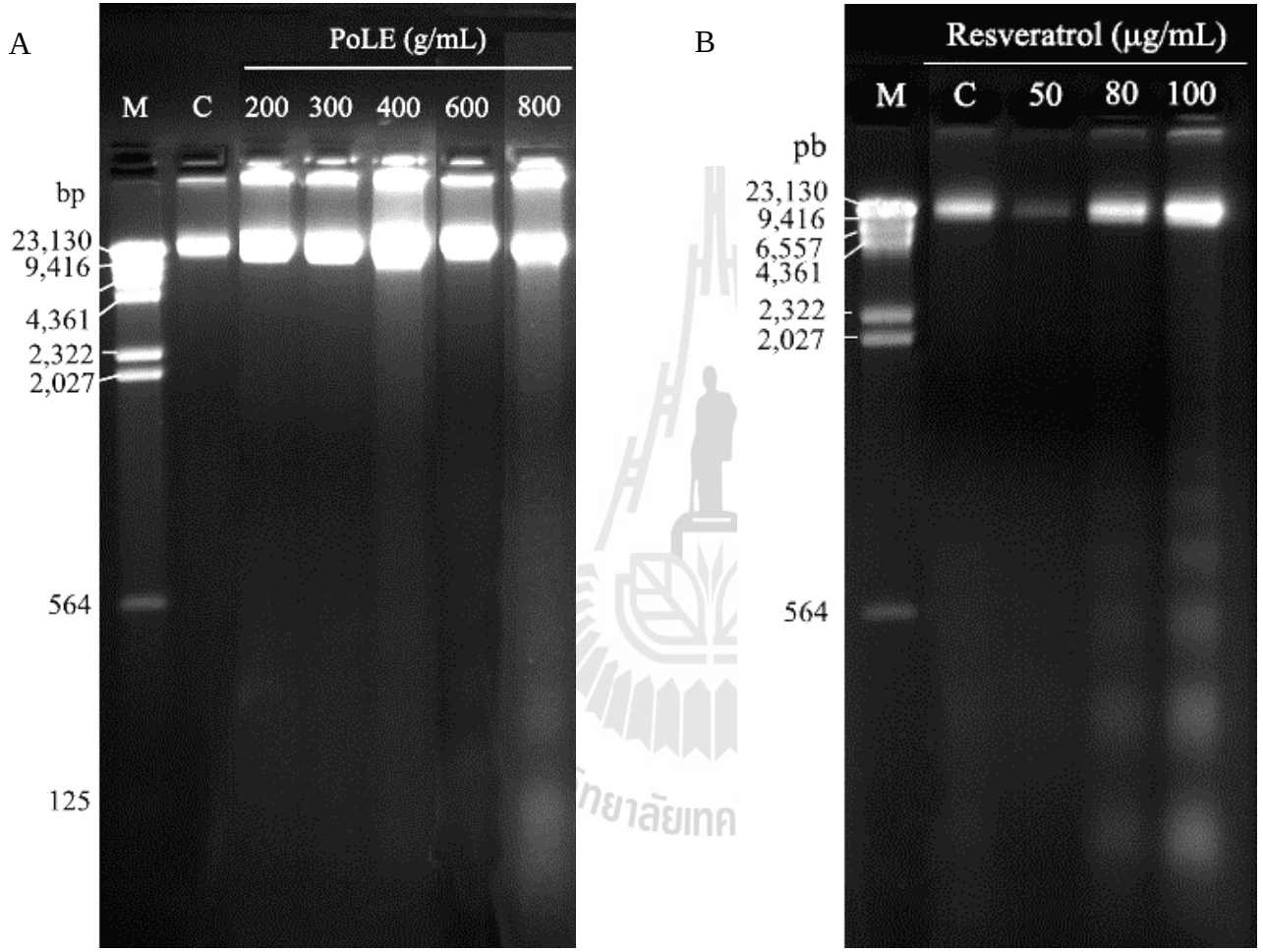

Figure 5.18 DNA fragmentation of MCF7 cells treated with various concentrations of *P. odoratum* (PoLE) at 24 h (A) and resveratrol at 24 h (B). DNA (10 µg) was electrophoresed on 1.5% agarose gel for 90 min.

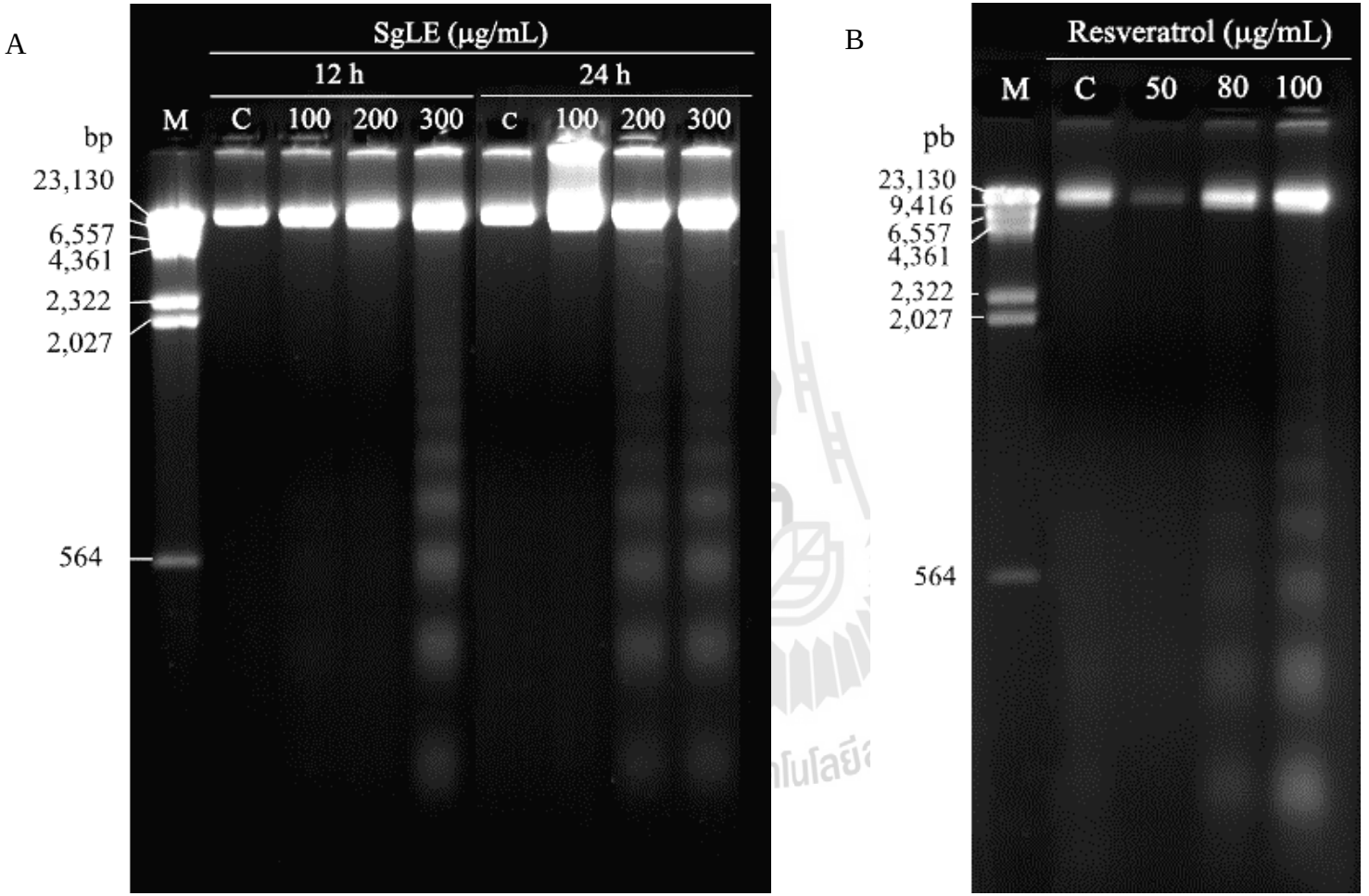

Figure 5.19 DNA fragmentation of MCF7 cells treated with various concentrations of *S. gratum* (SgLE) at 12 h and 24 h (A) and resveratrol at 24 h (B). DNA (10 µg) was electrophoresed on 1.5% agarose gel for 90 min.

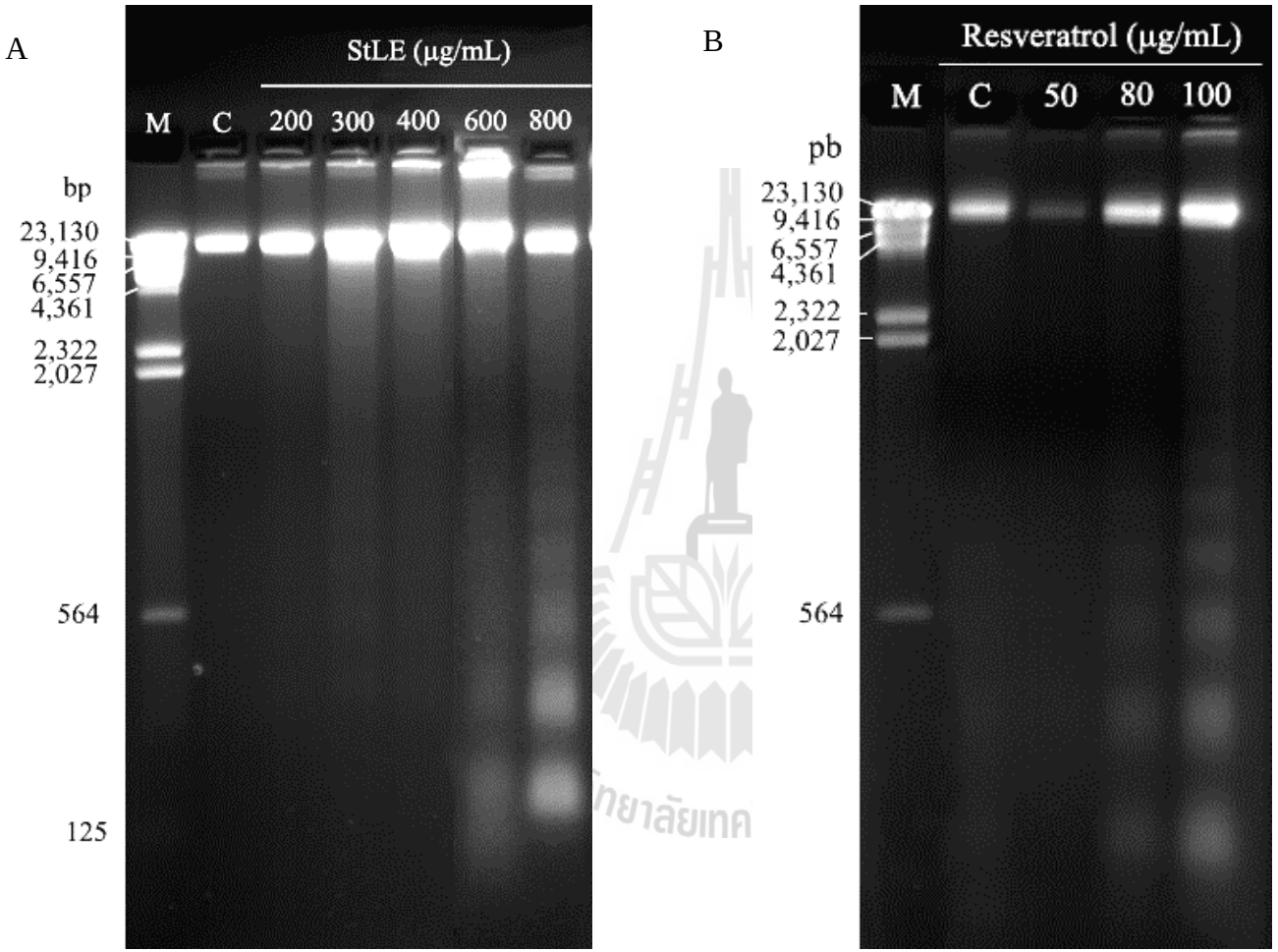

Figure 5.20 DNA fragmentation of MCF7 when treated with various concentrations of *S. terebinthifolius* (StLE) at 24 h (A) and resveratrol at 24 h (B). DNA (10 µg) was electrophoresed on 1.5% agarose gel for 90 min.

During late apoptotic induction, the endonuclease attacks the linker regions, internucleosomal cleavage and results in the formation of DNA fragments of 180-200 base pairs and multiples (DNA ladder formation) in contrast to necrosis which DNA cleavage events are random and show histone degradation (Duval and Wyllie, 1986), producing a diffuse smear on DNA electrophoresis. CFF45 (DNA fragmentation factor-45)/ICAD (inhibitor of caspase-activated DNase) is cleaved by proteolytic enzyme, caspase-3, which release the active endonuclease CAD (DFF40/CAD) before proceeding of apoptotic internucleosomal DNA fragmentation (DNA ladder formation) (Liu, Zou, Slaughter, and Wang, 1997). In a cell-free assay, caspase-3, -6, -7, -8, and granzyme B initiated the DEVD (benzoyloxycarbonyl-Asp-Glu-Val-Asp) cleaving caspase activity, DFF45/ICAD inactivation, and DNA fragmentation, but the calcium-dependent proteinase calpain and cathepsin (the lysosomal proteinase) failed to initiate these events. Moreover, only the caspase-3 and caspase-7 are directly inactivated DFF45/ICAD and promoted DNA fragmentation in an *in vitro* DFF40/CAD assay, but the endogenous levels of caspase-7 failed to inactivate CFF45/ICAD in caspase-3 null MCF7 cells and extracts. Although, caspase-3 may be the primary regulator of apoptotic DNA fragmentation, caspase-7 might be responsible for this apoptotic hallmark in caspase-3 deficient MCF-7 cells during apoptosis inducing by death stimuli such as pyrrolo-1,5-benzoxazepine (PBOX-6) (Mc Gee et al., 2002). Apoptosis inducing factor (AIF), which release from the mitochondria and then translocates to nucleus, could induce HMW DNA fragmentation (~ 50 kb) in a caspase-independent manner (Susin et al., 1999). Therefore, these could be say that caspase-7 might contributed to the DNA ladder formation in caspase-3 deficient MCF7 cells exposed to SgLE and StLE, and the caspase-7 and necrotic factor might concomitant in BaLE. Whereas, AIF or calpain (calcium-dependent proteinase) or cathepsin or might responsible for the DNA fragmentation in MCF7 cells treated with CfLE, LaLE, and PoLE.

5.4.4 The induction of hypodiploid, subG$_0$, in cell cycle profile

To investigate whether the 6 vegetable extracts can induce apoptosis, a subG$_0$ DNA peak which has been suggested to be the apoptotic DNA was tested by flow cytometer. The typical flow cytometric profile of DNA content and cell cycle behavior of MCF7 cells with or without treatment are demonstrated in Figures 5.21-5.26. The percentage of cells with hypodiploid DNA content in subG$_0$ peak when treated with the extracts was increased with dose- and time-dependent (Tables 5.5 and 5.6). Cell cycle arrest activity was detected in MCF7 cells treated with StLE, but not detected in the other extracts. The subG$_0$ population in BaLE was found to increase but no significant different from control ($p > 0.05$) which were 4.26%, 4.96%, and 9.58% at 12 h, and 2.52%, 10.52%, and 9.28% at 24 h for 200, 300, and 400 µg/mL, respectively (Figure 5.21, and Tables 5.5 and 5.6). The clumping cells found after detached and fixed cells in BaLE treatment, render to the lower number detecting in subG$_0$ phase. The effective concentration, 400 µg/mL of CfLE, LaLE, and PoLE exhibited a significant different from control ($p < 0.01$) in the percentages of subG$_0$ cells which were 46.87%, 21.44%, and 25.50% at 24 h (Table 5.6 and Figures 5.22, 5.23, and 5.24). The percentage of apoptotic (subG$_0$) MCF7 cells in SgLE, the highest activity, significantly increased ($p < 0.01$ and $p < 0.001$) which were 8.61%, 22.66%, and 80.15% for 12 h and 20.54%, 52.51%, and 80.80% for 24 h when treated at 100, 200, and 300 µg/mL, respectively (Figure 5.25). After incubating for 24 h, StLE demonstrated the significant increasing in number of apoptotic cells (%) when treated at effective concentration of 400 µg/mL ($p < 0.001$) which was 25.60% (Figure 5.26). Interestingly, StLE at lower dose, 200 and 300 µg/mL, significantly increased the percentage of cells in G_0/G_1 phase ($p < 0.01$) which were 77.83% and 72.03% at 12 h and 73.18% and 72.21% at 24 h, respectively, indicating the cell cycle arrest at G_0/G_1 phase. These findings were agreement with Queires et al. (2006) study which reported that

polyphenol fraction from the leaf of *S. terebinthifolius* induce G_0/G_1 cell growth arrest and cell apoptosis in the androgen-insensitive DU145 prostate carcinoma cells.

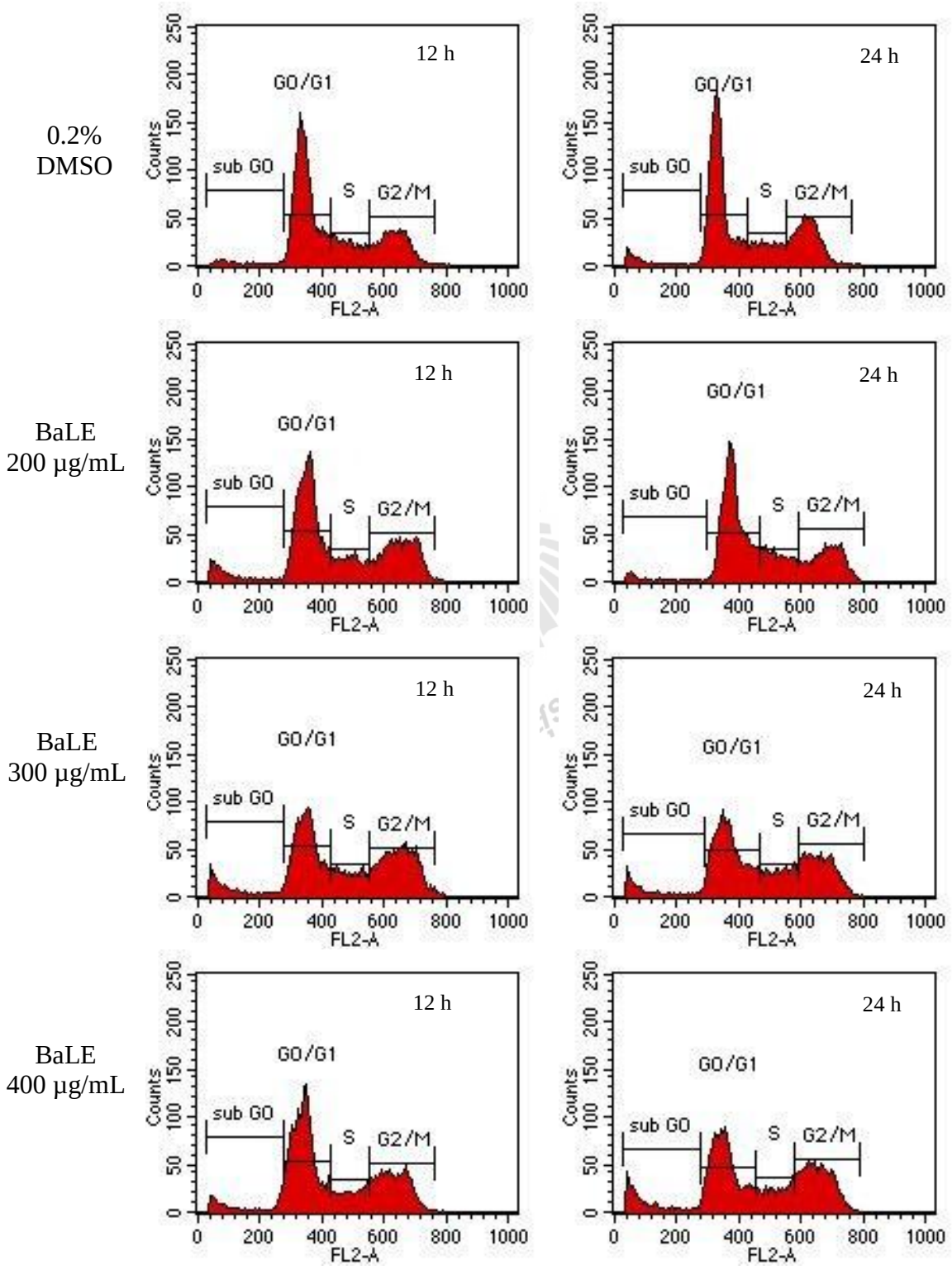

Figure 5.21 Cell cycle histograms of MCF7 cells treated with BaLE (*B. acutangula*) for 12 h and 24 h.

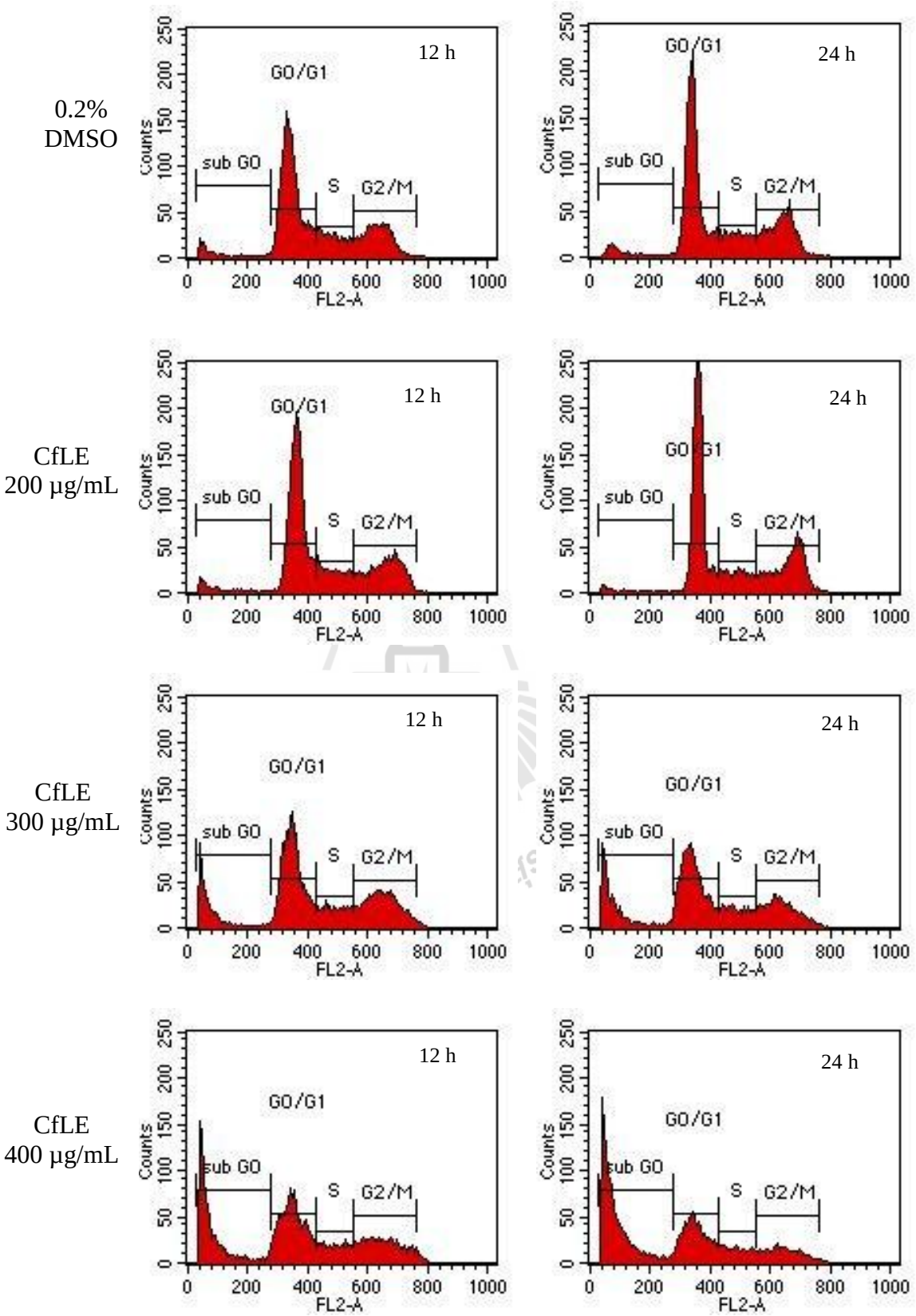

Figure 5.22 Cell cycle histograms of MCF7 cells treated with CfLE (*C. formosum*) for 12 h and 24 h.

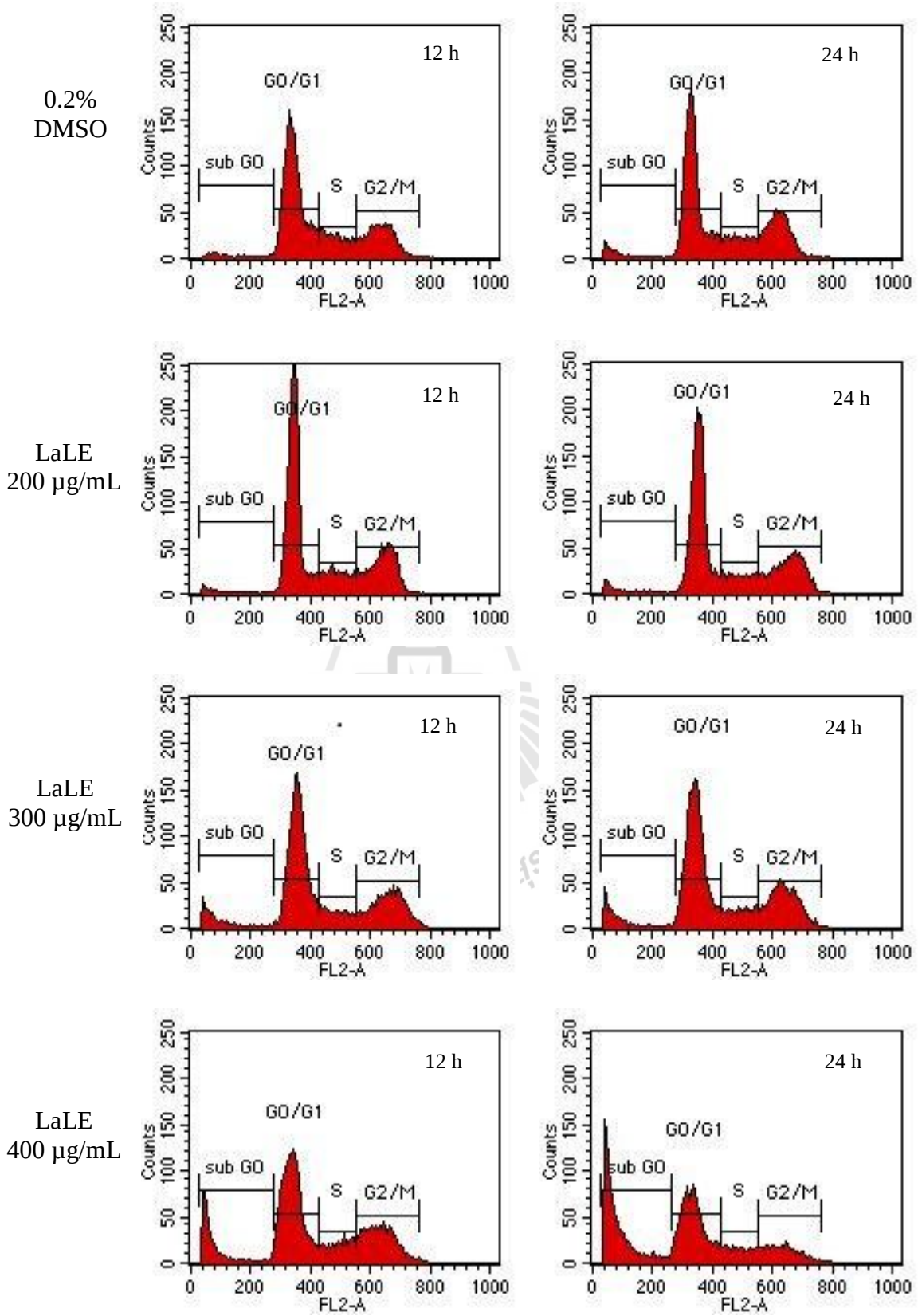

Figure 5.23 Cell cycle histograms of MCF7 cells treated with LaLE (*L. aromatica*) for 12 h and 24 h.

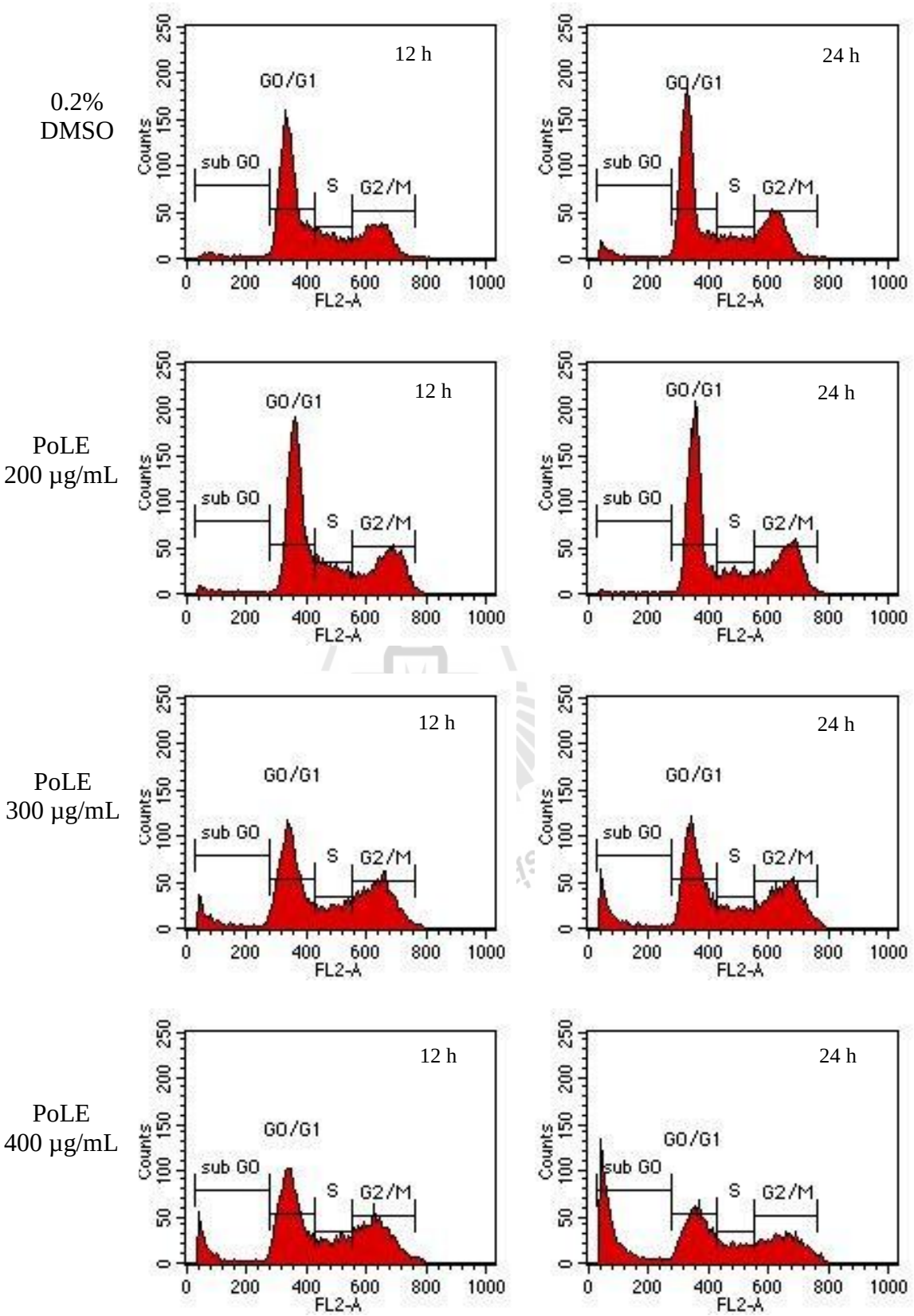

Figure 5.24 Cell cycle histograms of MCF7 cells treated with PoLE (*P. odoratum*) for 12 h and 24 h.

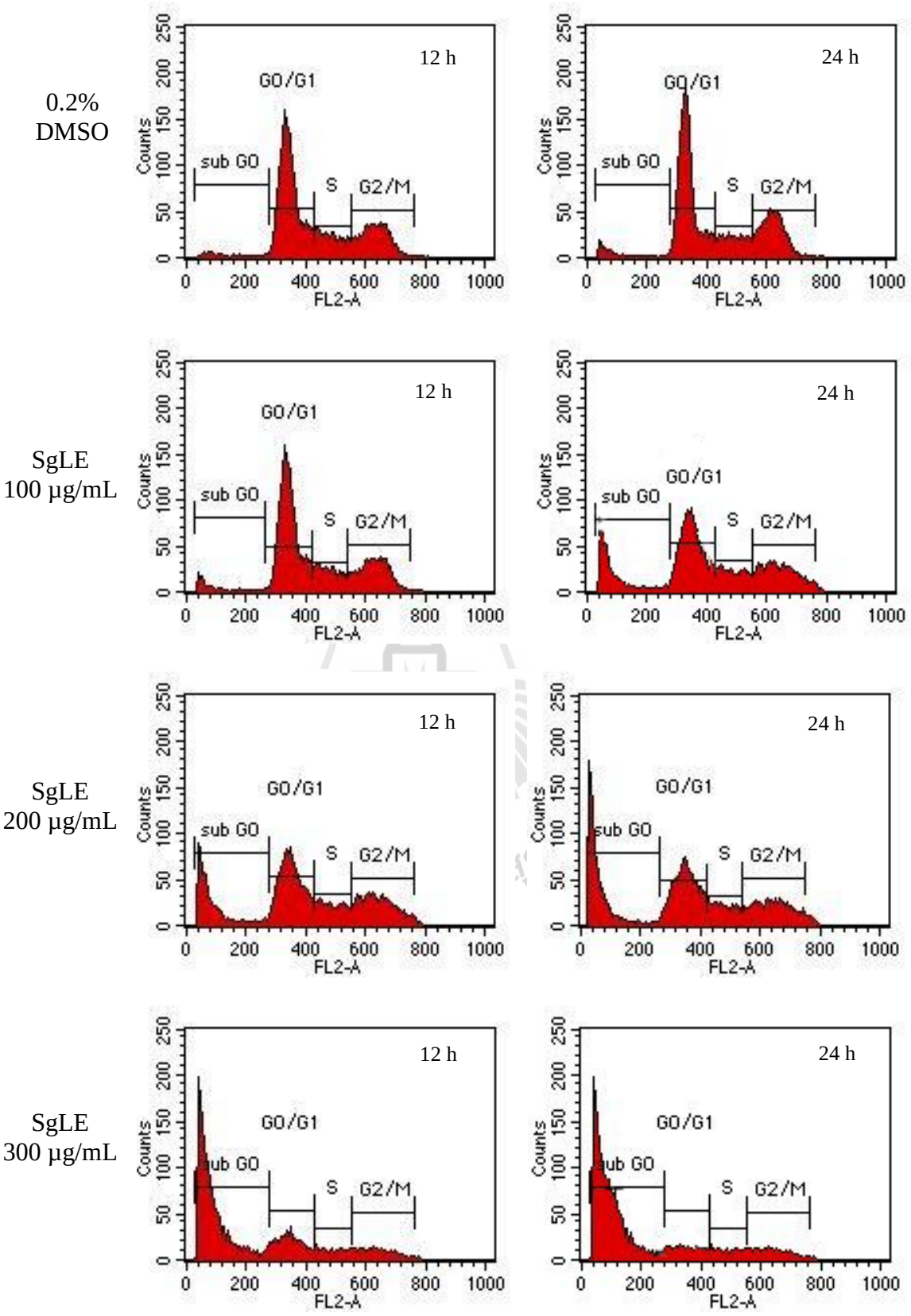

Figure 5.25 Cell cycle histograms of MCF7 cells treated with SgLE (*S. gratum*) for 12 h and 24 h.

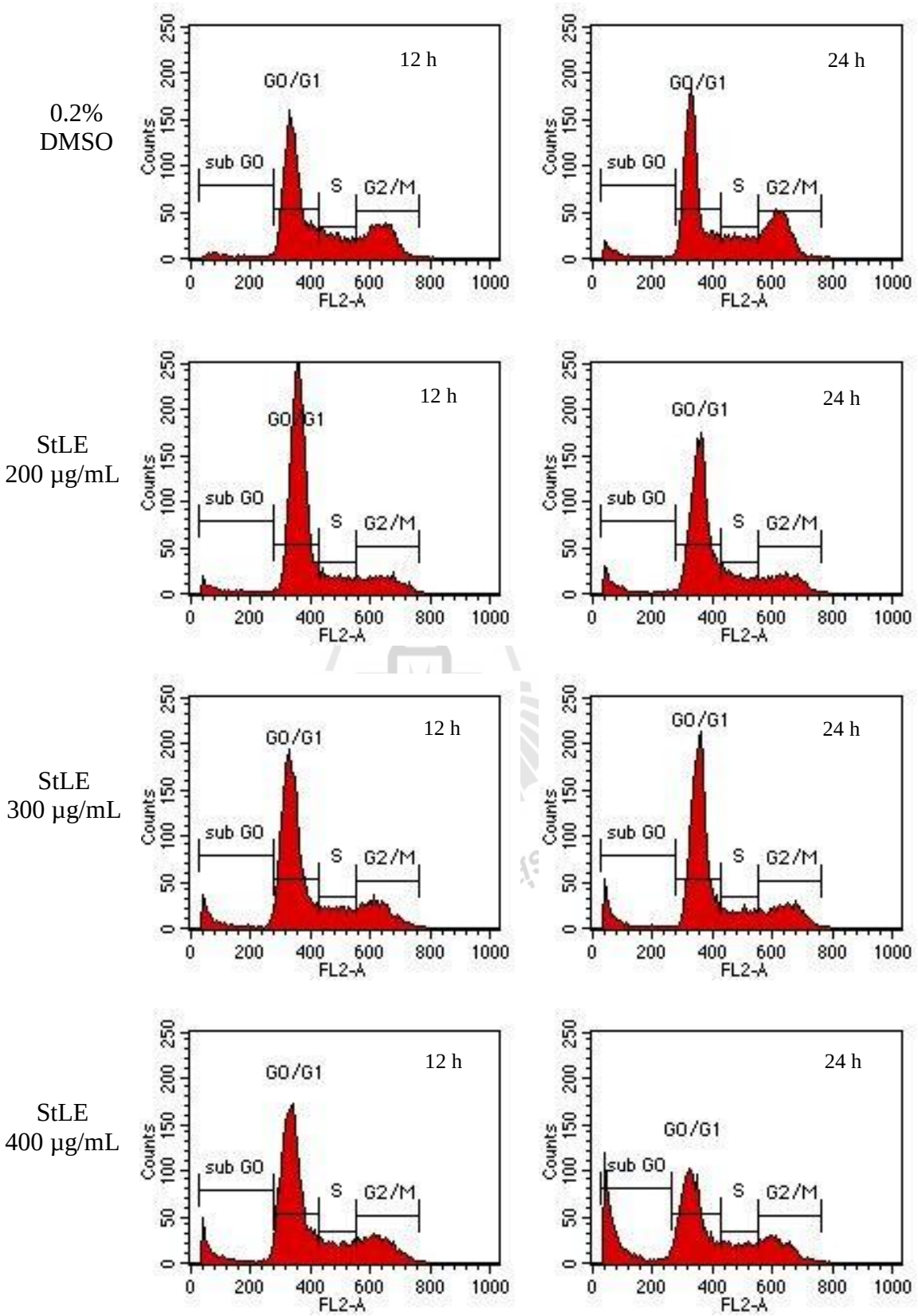

Figure 5.26 Cell cycle histograms of MCF7 cells treated with StLE (*S. terebinthifolius*) for 12 h and 24 h.

Table 5.5 Percentage of cells in each phase of MCF7 cells treated with the 6 vegetable leaf extracts at 12 h.

	Conc. (μg/mL)	Percentage of cells 12 h SubG$_0$	G_0/G_1	S	G_2/M
0.2% DMSO		2.40 ± 0.04	57.16 ± 0.68	15.51 ± 1.49	25.16 ± 1.03
BaLE	200	4.26 ± 1.02^{ns}	52.92 ± 2.98^{ns}	13.56 ± 2.56^{ns}	29.43 ± 1.40^{ns}
	300	4.96 ± 1.45^{ns}	51.77 ± 4.00^{ns}	12.67 ± 2.54^{ns}	30.85 ± 7.82^{ns}
	400	9.58 ± 2.86*	48.16 ± 5.16**	11.50 ± 0.37**	30.48 ± 3.22*
CfLE	200	1.84 ± 1.35^{ns}	59.58 ± 2.04^{ns}	14.98 ± 1.60^{ns}	23.80 ± 1.75^{ns}
	300	8.55 ± 3.27^{ns}	51.59 ± 3.99^{ns}	13.14 ± 2.80^{ns}	26.81 ± 1.58^{ns}
	400	30.63 ± 1.95***	37.01 ± 1.14***	11.30 ± 0.76**	20.94 ± 1.64^{ns}
LaLE	200	2.68 ± 1.76^{ns}	57.06 ± 3.29^{ns}	14.59 ± 1.03^{ns}	25.85 ± 0.41^{ns}
	300	4.84 ± 1.28^{ns}	57.65 ± 2.75^{ns}	12.30 ± 2.68^{ns}	25.68 ± 0.40^{ns}
	400	9.76 ± 3.55*	52.55 ± 3.92^{ns}	11.44 ± 1.78**	26.56 ± 1.12^{ns}
PoLE	200	1.94 ± 0.71^{ns}	57.74 ± 2.33^{ns}	13.56 ± 0.42^{ns}	27.02 ± 2.07^{ns}
	300	4.41 ± 1.42^{ns}	49.05 ± 2.27*	12.28 ± 1.71^{ns}	34.41 ± 0.70**
	400	7.79 ± 0.78^{ns}	45.72 ± 1.21**	13.99 ± 1.70^{ns}	32.54 ± 3.20**
SgLE	100	8.61 ± 3.68*	54.61 ± 4.51^{ns}	12.28 ± 1.64^{ns}	24.50 ± 3.20^{ns}
	200	22.66 ± 4.46***	43.87 ± 2.46**	13.59 ± 1.52^{ns}	19.83 ± 5.28^{ns}
	300	80.15 ± 1.81***	12.66 ± 2.78***	5.16 ± 0.53***	3.44 ± 0.47***
StLE	200	2.84 ± 0.08^{ns}	77.83 ± 2.43***	10.21 ± 1.98*	9.29 ± 0.58***
	300	4.42 ± 1.71^{ns}	72.03 ± 4.23**	10.47 ± 1.16*	13.42 ± 3.49*
	400	7.36 ± 3.08^{ns}	66.24 ± 1.04*	10.89 ± 0.96**	15.93 ± 3.79*

Data presented as mean ± SD, n = 3. Statistical analysis compared to control (0.2% DMSO): ns, no significant different; *, 0.05; **, 0.01; ***, 0.001, in column. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

Table 5.6 Percentage of cells in each phase of MCF7 cells treated with the 6 vegetable leaf extracts at 24 h.

	Conc. (µg/mL)	Percentage of cell in each phase (24 h)			
		$SubG_0$	G_0/G_1	S	G_2/M
0.2% DMSO		2.13 ± 0.50	57.17 ± 0.66	14.21 ± 1.84	26.66 ± 2.68
BaLE	200	2.52 ± 1.43^{ns}	51.69 ± 1.23*	16.56 ± 1.68^{ns}	28.82 ± 2.26^{ns}
	300	10.52 ± 7.73^{ns}	42.44 ± 2.62***	13.93 ± 4.85^{ns}	33.19 ± 0.80*
	400	9.28 ± 1.76^{ns}	40.76 ± 2.22***	12.75 ± 1.48^{ns}	37.15 ± 1.40*
CfLE	200	1.98 ± 1.28^{ns}	57.90 ± 0.33^{ns}	13.87 ± 0.34^{ns}	26.38 ± 1.89^{ns}
	300	11.19 ± 8.14^{ns}	52.26 ± 1.17^{ns}	12.97 ± 0.25^{ns}	23.92 ± 6.40^{ns}
	400	46.87 ± 6.80***	31.33 ± 5.33***	8.92 ± 1.37*	13.18 ± 1.69**
LaLE	200	2.61 ± 1.85^{ns}	55.64 ± 1.28^{ns}	14.04 ± 1.82^{ns}	27.78 ± 2.31^{ns}
	300	3.99 ± 1.63^{ns}	56.59 ± 1.88^{ns}	13.61 ± 1.51^{ns}	25.96 ± 0.82^{ns}
	400	21.44 ± 6.38**	47.63 ± 1.69*	11.01 ± 1.58^{ns}	19.92 ± 3.67^{ns}
PoLE	200	1.85 ± 0.83^{ns}	55.14 ± 1.34^{ns}	12.63 ± 0.89^{ns}	28.40 ± 0.12^{ns}
	300	10.21 ± 1.02^{ns}	44.02 ± 1.26**	12.14 ± 0.76^{ns}	32.45 ± 0.37*
	400	25.50 ± 6.20**	34.92 ± 3.47***	11.93 ± 1.17^{ns}	26.78 ± 3.79^{ns}
SgLE	100	20.54 ± 6.51***	43.21 ± 2.03***	13.80 ± 1.23^{ns}	21.86 ± 7.21^{ns}
	200	52.51 ± 8.30***	24.29 ± 4.85***	8.73 ± 2.40**	14.30 ± 1.14***
	300	80.80 ± 1.71***	11.90 ± 2.36***	4.85 ± 0.65**	3.32 ± 0.39***
StLE	200	3.74 ± 1.36^{ns}	73.18 ± 4.15***	12.04 ± 0.61^{ns}	11.22 ± 2.15**
	300	5.17 ± 3.77^{ns}	72.21 ± 5.60***	10.78 ± 0.78^{ns}	11.98 ± 2.55***
	400	25.60 ±10.69***	51.62 ± 3.58^{ns}	8.73 ± 3.08*	14.14 ± 6.27**

Data presented as mean ± SD, n = 3. Statistical analysis compared to control (0.2% DMSO): ns, no significant different; *, 0.05; **, 0.01; ***, 0.001, in column. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

5.4.5 The apoptotic induction of MCF7 cells *via* cytochrome c release

The disruption of the mitochondrial membrane results in the release of cytochrome c into the cytosol which can be detected by directly probed with anti-cytochrome c-FITC and analyzed with flow cytometer. The anti-IgG FITC isotype control was used as the unfluorescence stained cells (unfilled), while normal cells, intact cytochrome c, probed with anti-cytochrome c-FITC antibody was almost showed the fluorescence intensity in 10^2-10^3 (Figure 5.27). The reducing in the FITC intensity, implicate for the releasing of cytochrome c. The results showed that treatment of MCF7 cells with the 6 extracts induced the release of cytochrome c from the mitochondria with time-dependent manner (Figures 5.27, 5.28, and 5.29). BaLE dramatically induced the cytochrome c release which was 27.94% and 41.23% when treated with 200 µg/mL and 39.75% and 64.89% when treated with 300 µg/mL at 12 h and 24 h, respectively (Table 5.7). CfLE, LaLE, PoLE, and StLE exhibited the similar activity; the cytochrome c release (%) when treated with 300 µg/mL was increased with times which were 5.85%, 8.68%, 3.92%, and 7.24% at 12 h and 53.93%, 49.03%, 40.35%, and 32.10% at 24 h, respectively. SgLE was the highest activity, 39.29% and 71.94% of cytochrome c release was observed when treated at 200 µg/mL for 12 h and 24 h.

The interaction of pro-apoptotic (Bax, Bad, and Bid) and anti-apoptotic proteins (Bcl-2 and Bcl-xL), at the surface of mitochondria, disrupts the normal function of the anti-apoptotic proteins and lead to the formation of pores in the mitochondria and then releasing cytochrome c and other pro-apoptotic molecules. Moreover, the overexpression of pro-apoptotic protein (Bax, Bak) appears to regulate inner mitochondria membrane disruption by increasing the amount of Ca^{2+} releasable from ER, resulting in the mitochondria permeability transition and release of mitochondrial intermembrane space apoptotic protein such as cytochrome c (Kuwana and Newmeyer, 2003). In addition, calpain enzyme cleaved Bcl-2 and Bid (anti-apoptotic protein) and then induced cytochrome c

release from isolated mitochondria (Gil-Parrado et al., 2002). When cytochrome c release in the cytoplasm, it is able to form the so-called “apoptosome” and induce the classical caspase-dependent apoptotic pathway.

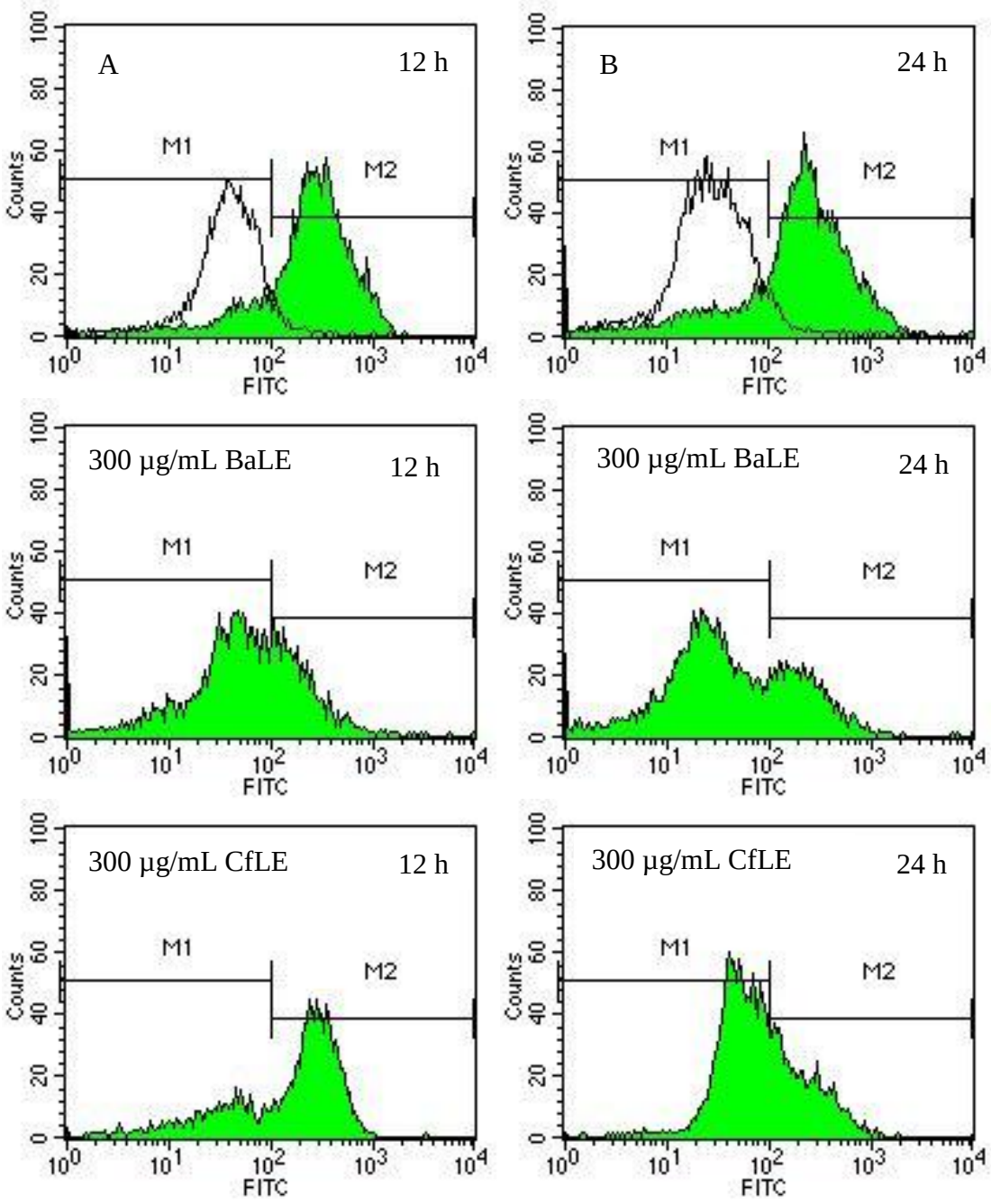

Figure 5.27 Cytochrome c release histrogram of MCF7 cells with untreated isotype control (no filled) and untreated FITC control (filled) (A, B). BaLE, *B. acutangula*; CfLE, *C. formosum.*

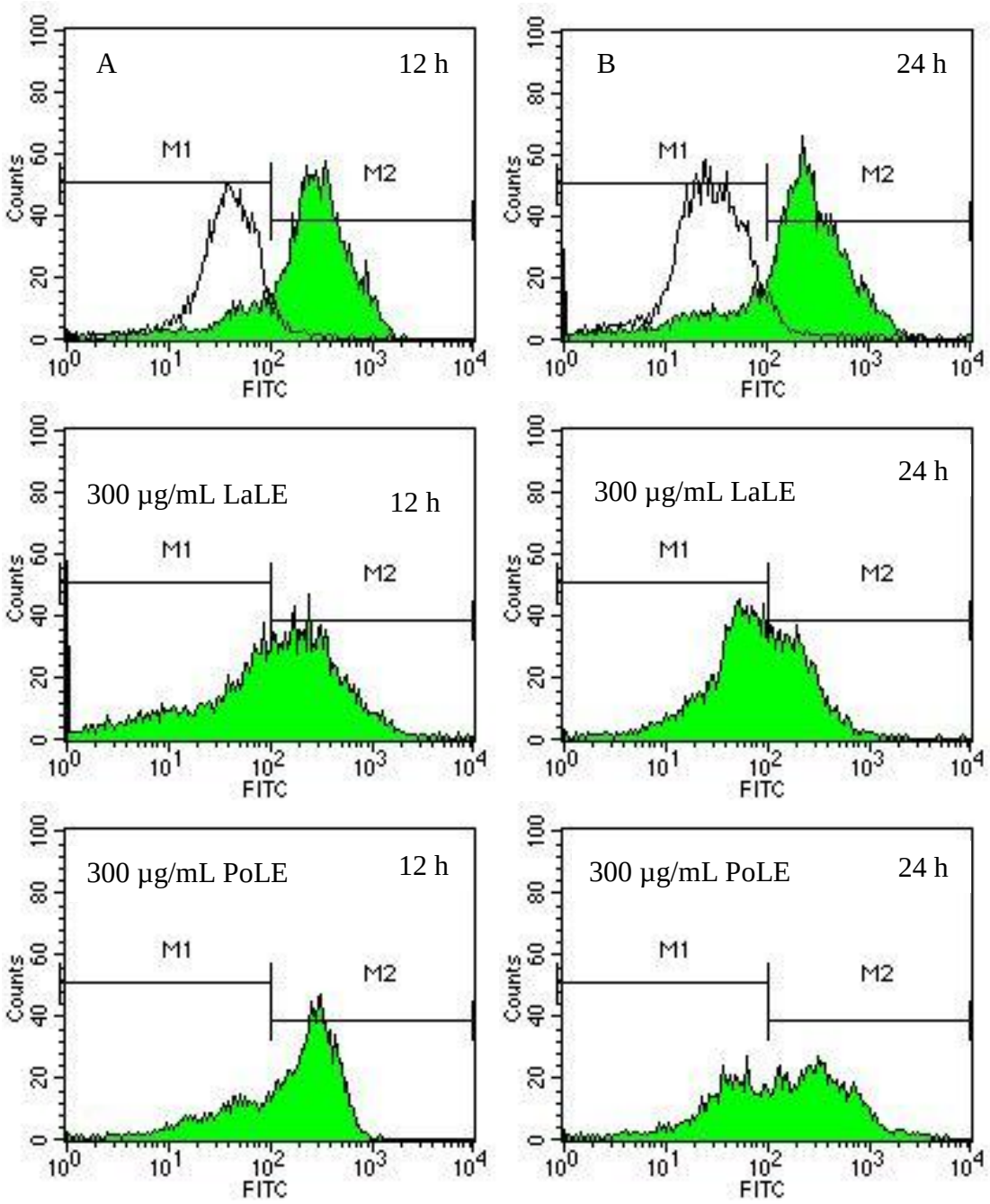

Figure 5.28 Cytochrome c release histrogram of MCF7 cells with untreated isotype control (no filled) and untreted FITC control (filled) (A, B). LaLE, *L. aromatica*; PoLE, *P. odoratum*.

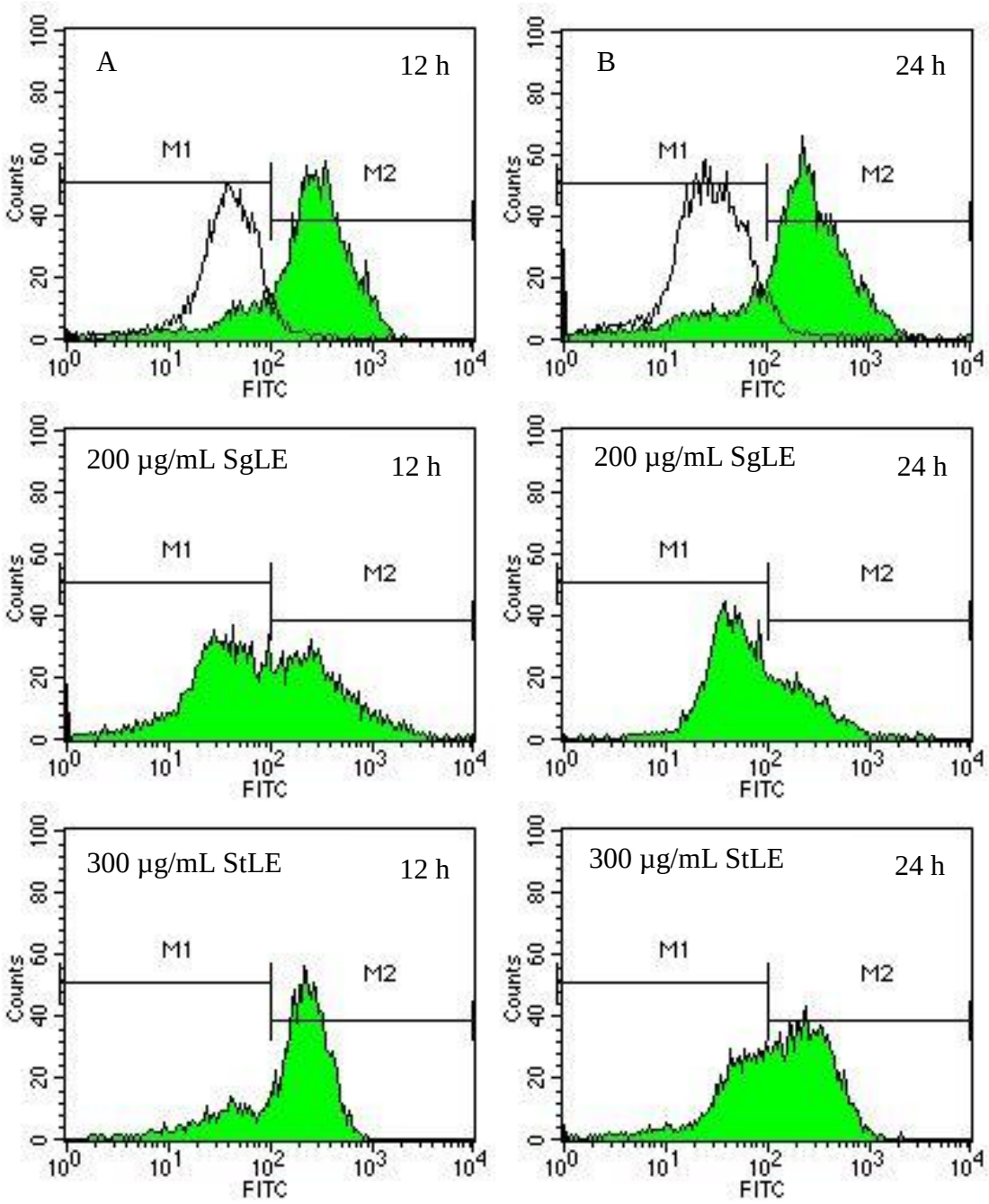

Figure 5.29 Cytochrome c release histrogram of MCF7 cells with untreated isotype control (no filled) and untreted FITC control (filled) (A, B). SgLE, *S. gratum*; StLE, *S. terebinthifolius.*

Table 5.7 Cytochrome c release (%) of MCF7 cells treated with the 6 vegetable leaf extracts for 12 h and 24 h.

Conc. (µg/mL)	Cytochrome c release (%)						
	0.2% DMSO	BaLE	CfLE	LaLE	PoLE	SgLE	StLE
12 h							
	0.34						
200		27.94 ± 2.43***	-	-	-	39.29 ± 5.29***	-
300		39.75 ± 5.46***	5.85 ± 1.98ns	8.68 ± 2.27ns	3.92 ± 0.76ns	75.67 ± 1.43***	7.24 ± 1.89ns
24 h							
	1.32						
200		41.23 ± 2.74***	-	-	-	71.94 ± 0.30***	-
300		64.89 ± 3.09***	53.93 ± 2.69***	49.03 ± 6.44***	40.35 ± 8.51***	-	32.10 ± 6.98***

Data presented as mean ± SD, n = 3, Statistical analysis compared to control (0.2% DMSO): ns, no significant different; *, 0.05; **, 0.01; ***, 0.001, in each 12 h and 24 h. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf ethanolic extract.

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5.4.6 The effect on apoptotic proteins of MCF7 cells

To investigate whether the 6 vegetable extracts were involved in apoptotic induction of MCF7, the apoptotic mediated proteins: poly (ADP-ribose) polymerase (PARP), cleaved-PARP, procaspase-7, cleaved caspase-7 (an active form, p20 and p10), Bcl-2, and Bax levels were determined by western blot. The results showed that treatment of 6-extracts on MCF7 cells induced an atypical proteolysis of PARP resulting in a ~ 60 kDa fragment which was a unique cleavage on caspase-independent cell death pathway (Tagliarino, Pink, Dubyak, Nieminen, and Boothman, 2001) (Figures 5.30-5.33). BaLE induced a decreasing in PARP and increasing in atypical cleaved-PARP (~ 60 kDa), but not detected in a classical cleaved-PARP (~ 89 kDa), with no changed in procaspase-7 and active caspase-7, involving the caspase-independent cell death pathway. Furthermore, the treatment with BaLE 200 and 300 µg/mL for 12 h and 24 h, reduced the expression of Bcl-2 and increased the Bax protein but reduced in Bax level at 400 µg/mL. CfLE, LaLE, and PoLE exhibited the similar results in protein expression. They showed a decrease in PARP, and an increase in cleavage PARP (~ 60 kDa) expression, with a small detection of a classical cleaved-PARP (~ 89 kDa) at 300 and 400 µg/mL. They also cleaved the procaspase-7 into the active caspase-7 form which explained by the decreasing in procaspase-7 and increasing in caspase-7 p20 and p10 subunit. The decrease in Bcl-2 and no change in Bax levels at 300 µg/mL, but a decrease in Bax at 400 µg/mL were observed in CfLE, LaLE, and PoLE. SgLE could induced MCF7 cell death by both caspase-independent and dependent pathway. MCF7 cells treated with SgLE showed an increase in PARP and cleavage PARP (~ 60 kDa) with dose-dependent, implying the oxidative stress occurred which then might induced cell death by non caspase involving mechanisms. The changing in caspase-7 level (decreased in pro-caspase-7, increased in active caspase-7 subunit), increasing in Bax, decreasing in Bcl-2 levels and increasing in cleaved-PARP

(~ 89 kDa) were detected in SgLE, implying the caspase-dependent apoptotic pathway. The intense of PARP, small amount of cleaved-PARP (~ 89 kDa) and cleaved-PARP (~ 60 kDa), with no change in caspase-7 (pro- and active form), Bax, Bcl-2 levels were observed in StLE 300 and 400 µg/mL at 24 h (Figures 5.31 and 5.33), indicating the caspase-independent cell death.

One of the nuclear proteins cleaved during apoptosis is the DNA repair associated enzyme, poly (ADP-ribose) polymerase (PARP). PARP1 is a nuclear enzyme that functions in the routine repair of DNA damage. The proteolytic actions of suicidal proteases such as caspases, calpains, cathepsins on PARP-1 generated several specific cleavage fragments with different molecular weight (Chaitanya, Alexander, and Babu, 2010). The characteristic and site of PARP cleavage could help in understanding the effect of antitumor agent on the process of cell death. During a classical apoptosis or caspase-dependent cell death, PARP-1 is cleaved by caspase-3 or -7 resulting in the formation of an 89 kDa catalytic fragment and a 24 kDa DNA binding domain fragment (Nicholson and Thornberry, 1997). The losing of DNA binding capacity of 89 kDa and irreversible binding of the 24 kDa fragment (non-function) to DNA strand breaks lead to the attenuation of DNA repair with conserving cellular ATP pool. Moreover, PARP-1 can also be cleaved by calpains and cathepsins (lysosomal enzyme) which participate in caspase-independent cell death (Chaitanya et al., 2010). Calpains, Ca^{2+} activated non lysosomal cyateinyl protease, play crucial roles in caspase-independent cell death. In contrast to caspase, calpain induced the cleavage of PARP at distinct site: the ~ 60 kDa fragment was recognized. In caspase-3 deficient MCF7 cells, an unique atypical PARP cleavage (~ 60 kDa fragment) can occurred in response of antitumor agent such as β-lapachone (Tagliarino et al., 2003). The neutral Ca^{2+}-dependent cysteine protease, µ-calpain has been reported for the certain aspects of cytotoxicity through caspase-independent cell death pathway. The induction of excessive cytosolic Ca^{2+} with the loss of ATP in cells might leads to protease activation (presumably

activation of calpain or a calpain-like protease) and, thus endonuclease (DFF40) activation, resulting in apoptosis (Tagliarino et al., 2001). In addition, lysosomal protease, cysteine protease cathepsins also targets on the PARP-1. The prominent 50 kDa fragment of PARP-1 (necrotic fragment) was found during necrotic cell death. The cleavage of PARP-1 at 35, 40, and 50 kDa fragments is also observed after necrotic induction of human leukemia HL-60 cells (Duriez and Shah, 1997). Cathepsin-B and D produce active PARP-1 fragments with molecular weights of 55 kDa and 42 kDa and also inactive PARP-1 fragment with molecular weights of 74 and 62 kDa (Duriez and Shah, 1997).

PARP-1 also plays a central role in a caspase-independent apoptosis pathway mediated by apoptosis-inducing factor (AIF). PARP-1 activity raises intra-mitochondrial Ca^{2+} levels, activating μ-calpain to release AIF from mitochondria (Virág and Szabó, 2002; Yu et al., 2002). Nevertheless, the overactivation of PARP-1 results in high NAD^{+} consumption (depleting ATP pools) leads to passive necrotic cell death (Lemaire, Andréau, Souvannavong, Adam, 1998). Apart from activation of calpain, Ca^{2+} also promotes PARP-1 hyper-activation through production of ROS and peroxynitrite leading to cell death (Bentle, Reinicke, Bey, Spitz, and Boothman, 2006).

One possible explanation for the MCF7 cell which missing caspase-3 (primary role) is that the abundant compensatory mechanism for example: the deletion of caspase-3 and -9 results in the compensatory activation of caspase-7 and -6 (Zheng et al., 2000). Furthermore, the effector caspases may have distinct roles in specific pathways, such as ER stress for caspase-7 (Rao et al., 2001) but have redundant functions in the majority of the apoptotic events and thus, abolition of all effector may be required to observe complete inhibition of all apoptotic feature (Degterev et al., 2003).

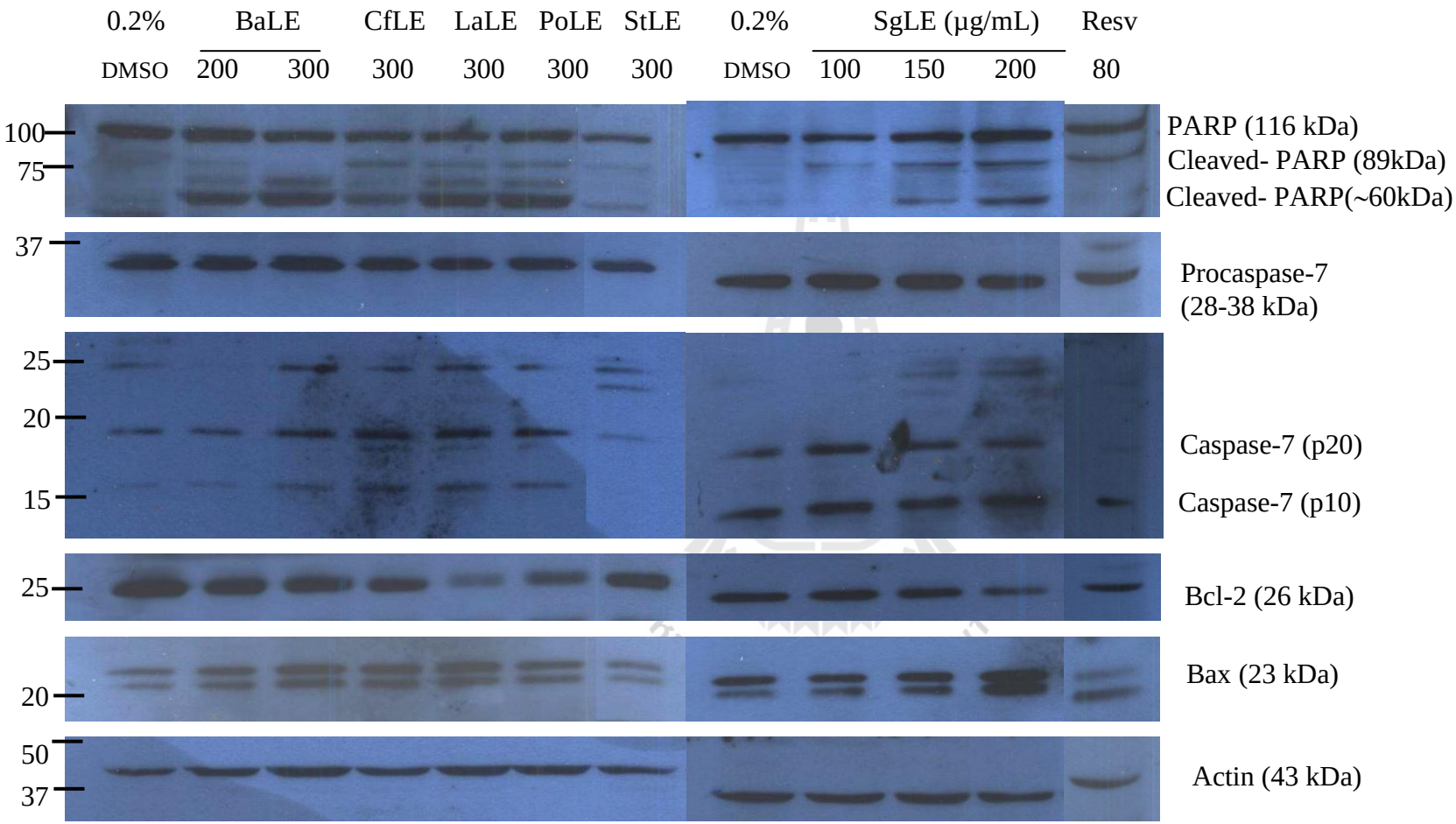

Figure 5.30 Apoptotic proteins of MCF7 cells treated with the 6 vegetable leaf extracts for 12 h. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf extract; Resv, resveratrol.

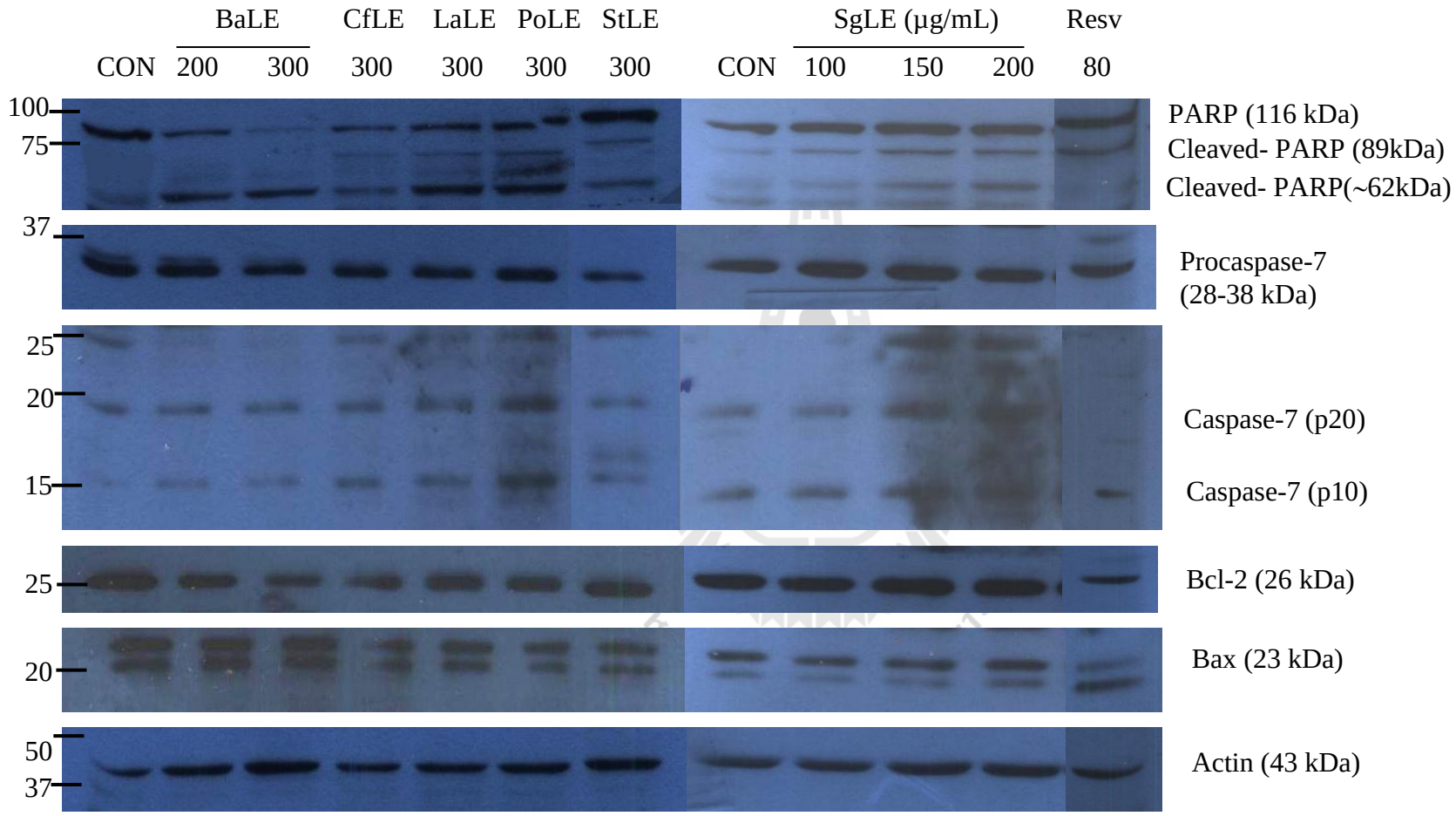

Figure 5.31 Apoptotic proteins of MCF7 cells treated with the 6 vegetable leaf extracts for 24 h. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf extract; Resv, resveratrol.

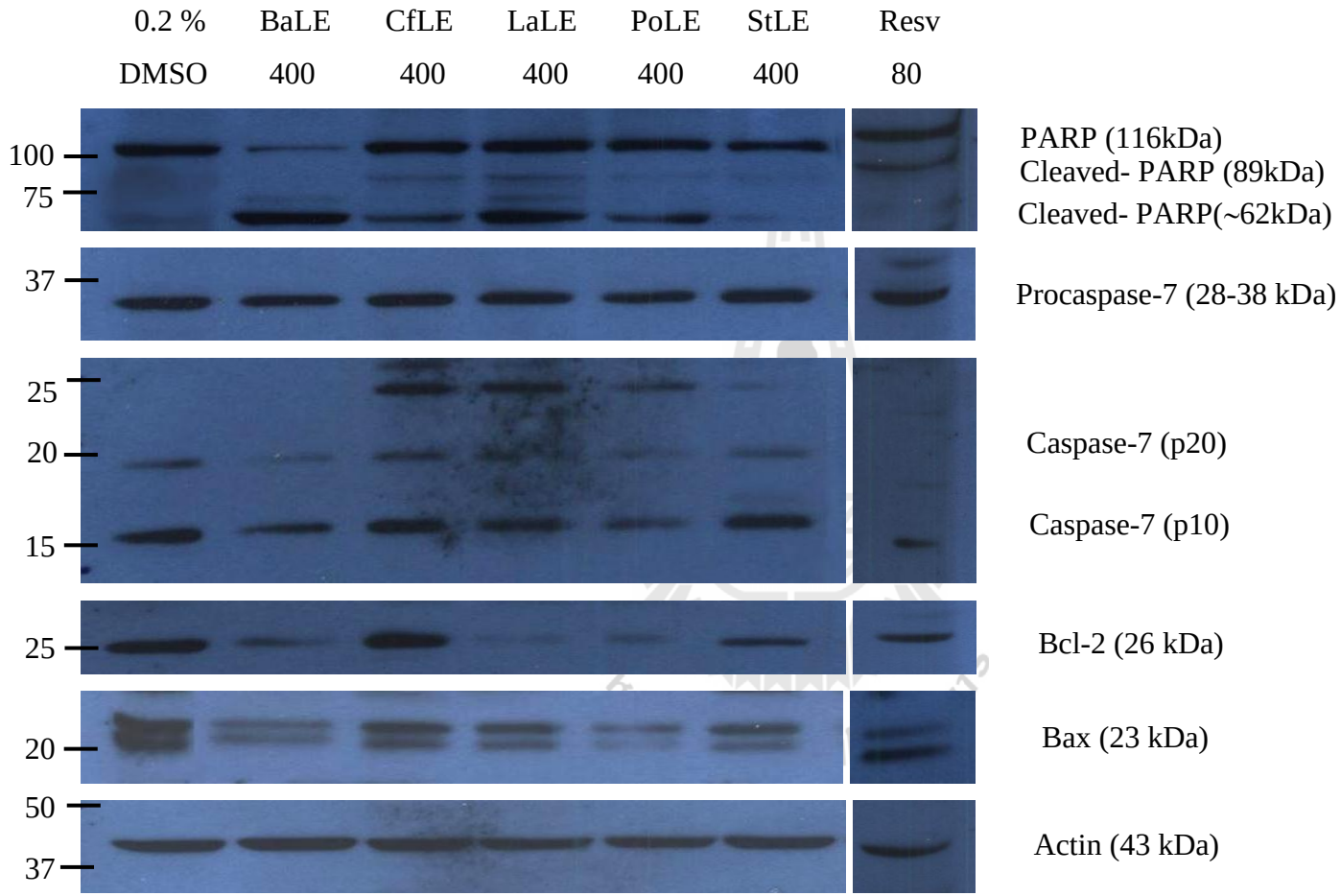

Figure 5.32 Apoptotic proteins of MCF7 cells treated with the 5 vegetable leaf extracts at 400 µg/mL for 12 h. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf extract; Resv, resveratrol.

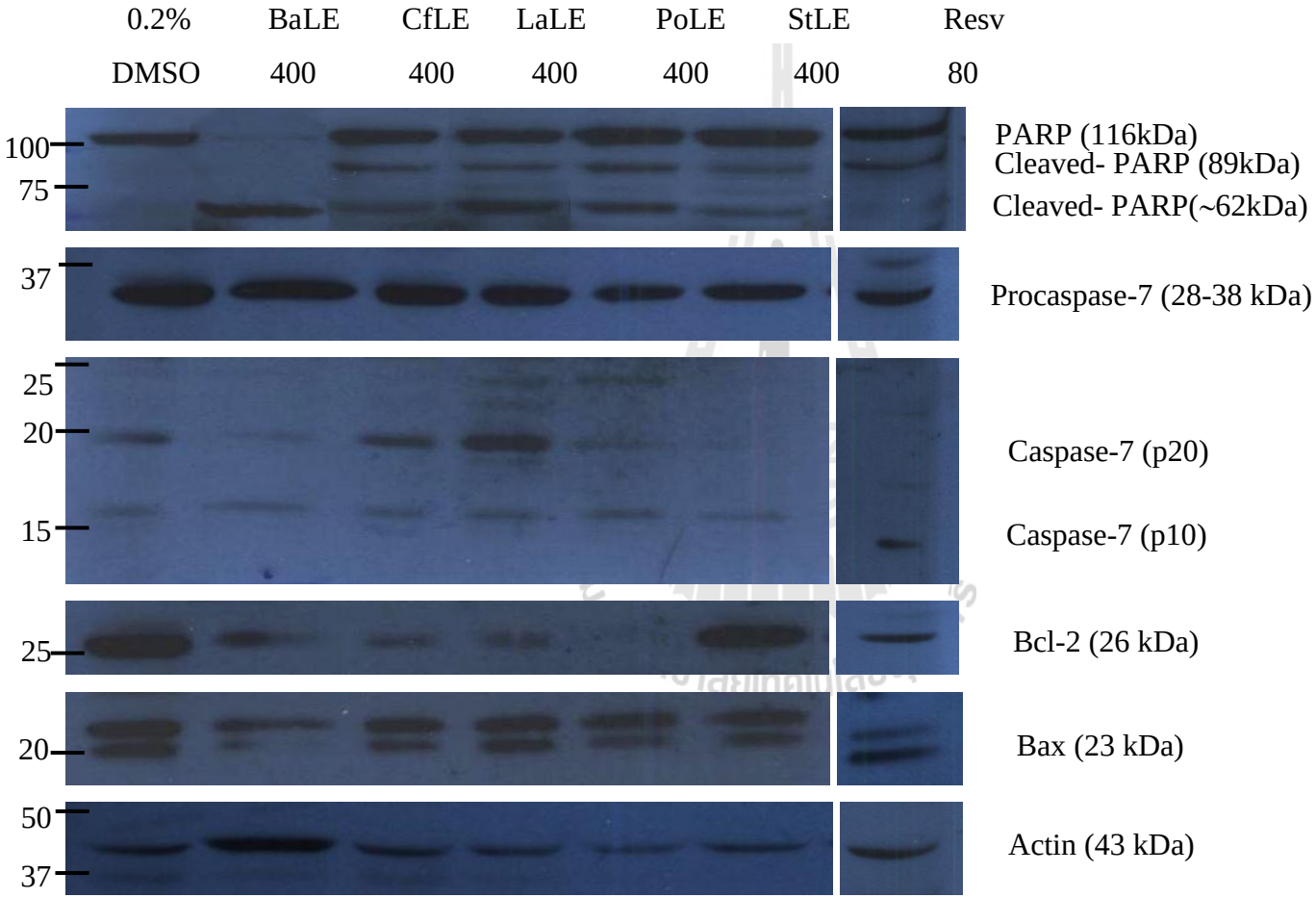

Figure 5.33 Apoptotic proteins of MCF7 cells treated with the 5 vegetable leaf extracts at 400 µg/mL for 24 h. BaLE, *B. acutangula*; CfLE, *C. formosum*; LaLE, *L. aromatica*; PoLE, *P. odoratum*; SgLE, *S. gratum*; and StLE, *S. terebinthifolius* leaf extract; Resv, resveratrol.

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The leaf of *B. acutangula*, *C. formosum*, *L. aromatica*, *P. odoratum*, *S. gratum*, and *S. terebinthifolius* extracts could induce MCF7 cells undergoing caspase-independent or necrosis cell death. The cytotoxic effects of BaLE on MCF7 were caspase-indenpedent cell death or necrotic pathway at high concentration and prolong treatment. The formation of diffused smear and oligonucleosome DNA, the completely cleavage of PARP into ~ 60 kDa which observed in BaLE could be the products from those mentioned mechanisms of cell death. CfLE, LaLE, and PoLE exhibited the similar activities on inducing MCF7 cell death which were observed by the nuclear morphological changed, DNA fragmentation and induction of subG_0 phase, cytochrome c release and cleaved-PARP (both ~ 89 kDa and ~ 60 kDa fragments). The possible mechanisms of action could be induced *via* caspase-independent cell death through releasing of AIF which explained by large fragmented nuclei (Hoechst stain) along with the formation of HMW DNA fragment (agarose-gel electrophoresis). However, more studies on the proteins involving in caspase-independent cell death through AIF activation are need. Another mechanism might induced by calpain activity which explained by the cleavage of PARP into ~ 60 kDa (atypical) fragments and the reduction in Bcl-2 levels. SgLE, high cytotoxic activity, was likely rendered through caspase-independent pathway and caspase-dependent pathway (*via* caspase-7 activity). The possible mechanism of SgLE might elevate the Ca^{2+} levels leading to the activation of PARP-1 and calpain enzyme. PARP-1 could then cleaved by calpain into ~ 60 kDa fragment which observed by Western blotting. Caspase-dependent cell death could be induced concomitant along with the non caspase mechanism which explained by the increasing in Bax, reducing in Bcl-2, and modulation of caspase-7 (decreased in procaspase-7 and increased in active caspase-7). The effect of StLE on MCF7 cells was different from the others. StLE induced the cell cycle arrest at G_0/G_1 phase when treated with low concentration (200-300 µg/mL) and induced cell death at high concentration (≥ 400 µg/mL) in caspase-independent pathway.

5.5 Conclusions

The 6 vegetable leaf extracts, *B. acutangula*, *C. formosum*, *L. aromatica*, *P. odoratum*, *S. gratum*, and *S. terebinthifolius*, exhibited the anti-proliferative effects or cytotoxic effects on MCF7 cells with dose- and time-dependent manner. SgLE was the highest effects which showed IC_{50} values of 120.70 and 90.12 µg/mL at 24 h and 48 h, followed by BaLE. No significant different in IC_{50} values of the other four extracts but CfLE and StLE seem to be more effective than PoLE and LaLE at 12 h and 48 h.

The 6 vegetable leaf extracts induced the condensation and fragmentation of nuclei (apoptotic-like nuclei) with some different in morphology. BaLE, CfLE, LaLE, and PoLE produced the dense and fragmented nuclei. SgLE induced the fragmented nuclei with smaller in the nuclear boundary. The fragmented nuclei in StLE seem like the nuclei induced with resveratrol. SgLE was the highest activity for inducing the fragmented nuclei of MCF7 cells with IC_{50} values of 136.60, 118.21, and 112.80 µg/mL for 12 h, 24 h, and 48 h and inducing DNA ladder formation. Secondly, BaLE effectively induces fragmented nuclei and showed significant different ($p < 0.05$) from the other four extracts at 24 h and 48 h. BaLE could induce apoptosis and concomitant with necrosis in high concentration and prolong treatment as observed by the diffused smear pattern of DNA fragmentation. CfLE, LaLE, PoLE, and StLE could also induce fragmented nuclei with IC_{50} values ranged from 408.11-567.40 µg/mL, 290.20-472.22 µg/mL and 279.63-402.61 µg/mL at 12 h, 24 h, and 48 h, respectively, however, StLE and CfLE seem to be effective than PoLE and LaLE. CfLE, LaLE, and PoLE produced high molecular weight DNA fragments, whereas StLE showed DNA ladder at 600-800 µg/mL. Flow cytometric analysis revealed that all treatments, except BaLE, induced the population in subG_0 phase, apoptotic cells, with dose- and time-dependent manner. Interestingly, low concentration of StLE, 200-300 µg/mL, induced cells growth arrest in G_0/G_1 phase, and induced apoptotic cell death at 400 µg/mL.

The effects of the 6 vegetable leaf extracts on caspase-3 deficient MCF7 cells were induced through caspase-independent pathway. The cytotoxic effects of BaLE were caspase-indenpedent cell death or necrotic pathway. CfLE, LaLE, and PoLE might possible induce cell death by triggering AIF release or calpain activity. SgLE, high cytotoxic activity, could induce by both caspase-independent and caspase-dependent pathway. StLE affected on the cell cycle arrest and caspase-independent cell death pathway.

Caspase-independent cell death involving mitochondria, lysosmoes, and ER which might be a possible mechanism for MCF7 cells death induced by the extracts. The signals from the different organelle are linked and may act both upstream and downstream of each other. It has been postulated that the dominant cell death phenotype is determined by the relative speed of the available death programs, and only the fastest and most effective pathway is usually evident (Bröker et al., 2005)

The studies on molecular levels involving cell death provide the valuable information on the molecular cytotoxic effect of the 6-vegetable leaf extract on MCF7 breast cancer cells. Among them, SgLE might be applicable use for anti-tumor agent but the cytotoxic effect on another cells and more detail on mechanism of action should be further study.

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CHAPTER VI

CONCLUSIONS

Dietary and herbal plants have highly received attention all over the world. According to local cuisine and pharmacology, they are important as dietary supplements, providing bioactive compounds. Therefore the main objective of this study was to elucidate the capacity of dietary plants which are commonly consumed as vegetables in traditional daily diet. In the present study, the leaf of the six local Thai vegetables, *Barringtonia acutangula* (phak jig); *Cratoxylum formosum* (phak tiew); *Limnophila aromatica* (phak khayaeng); *Polygonum odoratum* (phak phai); *Syzygium gratum* (phak mek); and *Schinus terebinthifolius* (phak matumkhaeg) were evaluated for the phytochemicals, antioxidant activities, cytotoxicity on normal and cancer cells, and the cell death pathway. The six local Thai vegetable extracts contained a variety of phytochemicals which mostly were phenolic compounds and terpenes. StLE, BaLE, and SgLE contained high total phenolics of 438.73, 377.28, and 303.70 µg GAE/mg, rich in tannins but low flavonoid contents which were 39.80, 15.47, and 37.24 µg CAE/mg. Whereas, CfLE, PoLE, and LaLE had total phenolics of 313.81, 216.74, and 152.41 µg GAE/mg and rich in total flavonoids 251.36, 117.30, and 112.35 µg CAE/mg, respectively. Essential oils and tannins were found in all extracts with different levels. Saponins were high in StLE and BaLE, and some detected in LaLE and SgLE. Terpenoids were found in SgLE and small amount in LaLE.

The activities of the 6-local Thai vegetable leaf extract were divided into 2 groups as regarding to their antioxidant capacity. Group I with high antioxidant activity were BaLE, SgLE, and StLE, and group II with low antioxidant activity were CfLE, LaLE, and PoLE. The reducing power, DPPH radical scavenging and inhibition of microsome lipid

peroxidation of group I was similar to trolox, catechin and ascorbic acid and significant higher than ($p < 0.05$) that of group II. Group I (BaLE, SgLE, and StLE) exhibited high FRAP activity with 8.76, 7.74, and 8.09 μmolFe^{2+}/mg, high DPPH scavenging ability with EC_{50} values of 6.67, 8.85, and 5.54 μg/mL, and high lipid peroxidation (LPO) inhibition activity with EC_{50} values of 3.92, 3.54, and 6.50 μg/mL, respectively. In cell-base antioxidant assay using *t*-BuOOH to induced ROS generation in HepG2 cells, the 6 vegetable extracts exhibited some antioxidative effects at low concentrations but produced somewhat cytotoxicity at high concentrations which might be due to the complex of cytoprotective and cytotoxic compounds in each plant extract. However, the 6 vegetable extracts could inhibit ROS generation when compare to the *t*-BuOOH induced control. BaLE and StLE at 20 μg/mL, and SgLE 25 μg/mL presented significant reduction of ROS production ($p < 0.05$) with 26.40%, 12.34%, and 17.99% inhibition. LaLE, PoLE, and CfLE 100 μg/mL was significantly reduce ROS production ($p < 0.001$) with 35.60%, 29.00%, and 28.30%, respectively.

The local Thai vegetable leaf extracts possessed cytotoxicity differently to normal cells, small organisms, and cell lines which could depend upon the different phytochemical components. CfLE possessed the high toxicity prominently on small organisms, *Artemia salina*, with LC_{50} values of 37.00 μg/mL. BaLE induced cytotoxic against *A. salina* nauplii, human normal lymphocytes, MCF7 breast cancer, and HepG2 liver cancer cells with similar IC_{50} values ranging from 112.70-196.40 μg/mL. However, BaLE as well as StLE had more selective effects on Jurkat cells with IC_{50} values of 66.91 and 75.36 μg/mL, respectively. The high cytolytic properties of BaLE and StLE to erythrocytes might relate to their cytotoxic effects and was likely to be the effects of saponins. SgLE was selective toxic on MCF7 cells with IC_{50} values of 66.71 μg/mL, and had a slight effect on erythrocyte membrane. Of noted, moderate to high amount of terpenoids were detected only in SgLE, it could be the reason for the specificity on MCF7 breast cancer cells.

The study on molecular mechanism of cell death was elucidated to obtain informative data on the 6 vegetable leaf extracts. The 6 extracts exhibited anti-proliferative effects or cytotoxic effects on MCF7 cells depending on doses and times. SgLE was the most active effects on MCF7 cells which showed IC_{50} values of 120.70 µg/mL and 90.12 µg/mL at 24 h and 48 h, followed by BaLE (IC_{50} values of 223.00 µg/mL and 152.20 µg/mL at 24 h and 48 h), respectively. The 6 vegetable leaf extracts could induce condensed and fragmented nuclei (apoptotic-like nuclei) with some different in morphology and DNA fragmentation pattern. SgLE actively induced the fragmented nuclei with smaller in the nuclear boundary which showed the IC_{50} values of 136.60, 118.21, and 112.80 µg/mL for 12 h, 24 h, and 48 h, and showed the intense DNA ladder formation at 200 µg/mL, 24 h. BaLE produced dense and fragmented nuclei at 100-300 µg/mL, but might induce apoptosis and concomitant with necrosis at 400 µg/mL as observed by the release of nucleic acid from nucleus (data not shown) and the diffused smear DNA pattern. CfLE, LaLE, PoLE and StLE also induced the condensed and fragmented nuclei with IC_{50} values ranged from 408.11-567.40 µg/mL, 290.20-472.22 µg/mL and 279.63-402.61 µg/mL at 12 h, 24 h, and 48 h, respectively. However, StLE and CfLE seem to be more effective than PoLE and LaLE. Furthermore, they showed some difference in DNA pattern, CfLE, LaLE, and PoLE produced high molecular weight DNA fragments, whereas StLE showed DNA ladder at 600-800 µg/mL. Interestingly, StLE could trigger cell cycle dependent cells death of MCF7 cells (G_0/G_1 arrest), whereas the other five extracts were cell cycle independent cell death.

The effects of the 6 vegetable leaf extracts on caspase-3 deficient MCF7 cells was induced through caspase-independent or necrosis pathways which might be the actions of cytosolic protein, calpain, or lysosomal protein, cathepsin. Because of the complexity in the molecular mechanism of actions and the link between caspase-dependent, caspase-independent and also necrosis, the quite hard to justified the mode of cell death induced by these extracts. Further investigations are needed for SgLE, the most active extract.

However, these studies on antioxidant activities, cytotoxicity and the molecular levels involving cell death provide the informative data on the beneficial effect of local Thai vegetables which may possibly be consumed as sources of potential antioxidant and possible be chemopreventive agent.

APPENDICES

APPENDIX A

SPRAY REAGENT FOR THIN LAYER CHROMATOGRAPHY

A.1 Dragendorff reagent with hydrochloric acid (modified)

- Solution A : 0.3 g bismuth subnitrate, 1 mL of 25% HCl, 5 mL water
- Solution B : 3 g potassium iodide in 5 mL water

Spray reagent : 5 mL of A + 5 mL of B + 5 mL of 12% HCl + 100 mL water

A.2 Potassium hydroxide (KOH)

- 5% ethanolic potassium hydroxide, spray and evaluate in visible or in UV 366 nm

A.3 Iron (III) chloride reagent ($FeCl_3$)

- 10% iron (III) chloride aqueous solution, spray and evaluate in visible

A.4 Kedde reagent

- 5 mL of 3% ethanolic 3,5-dinitrobenzoic acid are mixed with 5 mL of 2 M NaOH. The plate is sprayed with 10 mL of freshly prepared mixture, evaluated in visible.

A.5 ρ-Anisaldehyde/sulfuric acid (H_2SO_4) reagent

- 0.5 mL anisaldehyde mixed with 10 mL of glacial acetic acid, followed by 85 mL of methanol and 5 mL of concentrated H_2SO_4, in that order. The TLC plate is sprayed with 10 mL, heated at 100ºC for 5-10 min then evaluated in visible or UV 366 nm.

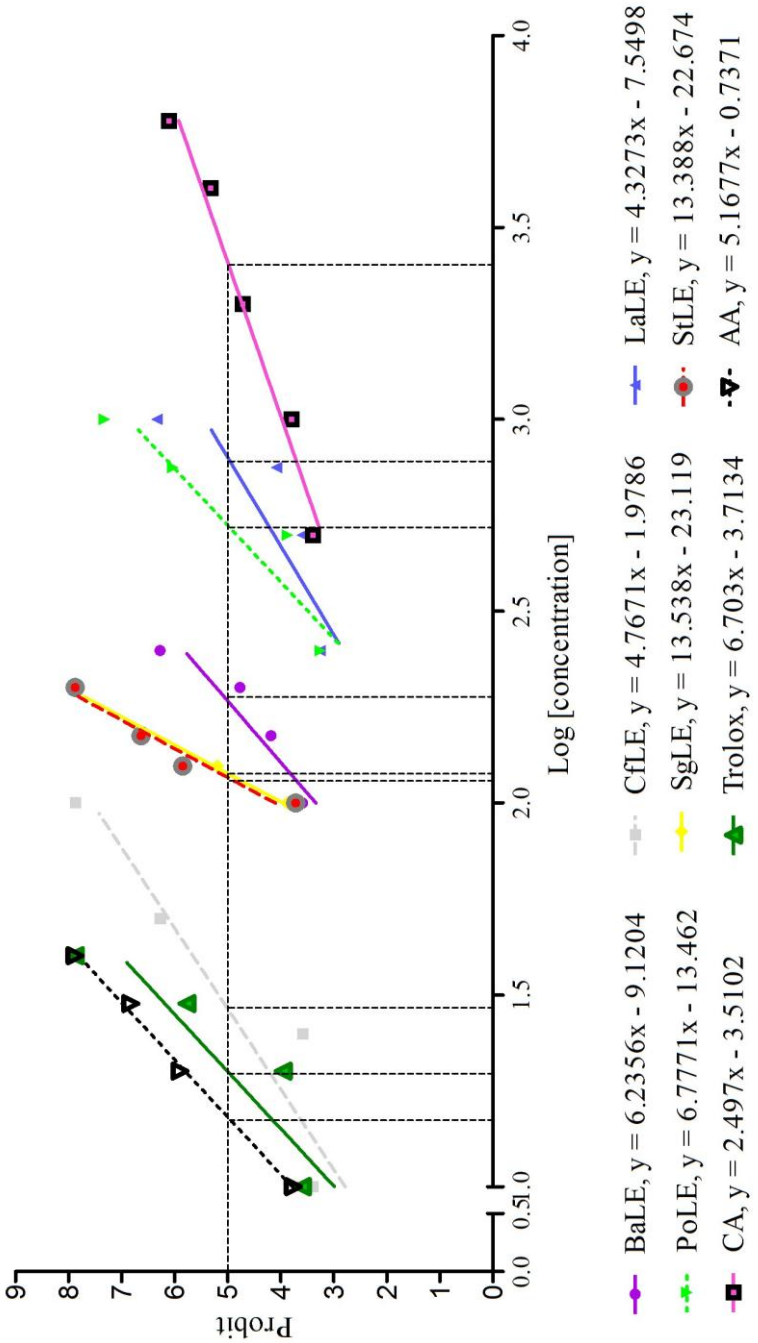
A.6 Vanillin/sulfuric acid (H_2SO_4) reagent (modified)

- 5% ethanolic H_2SO_4 mixed with 1% ethanolic vanillin (1:1). The plate is sprayed with 10 ml, heated at 110ºC for 5-10 min then evaluated in visible.

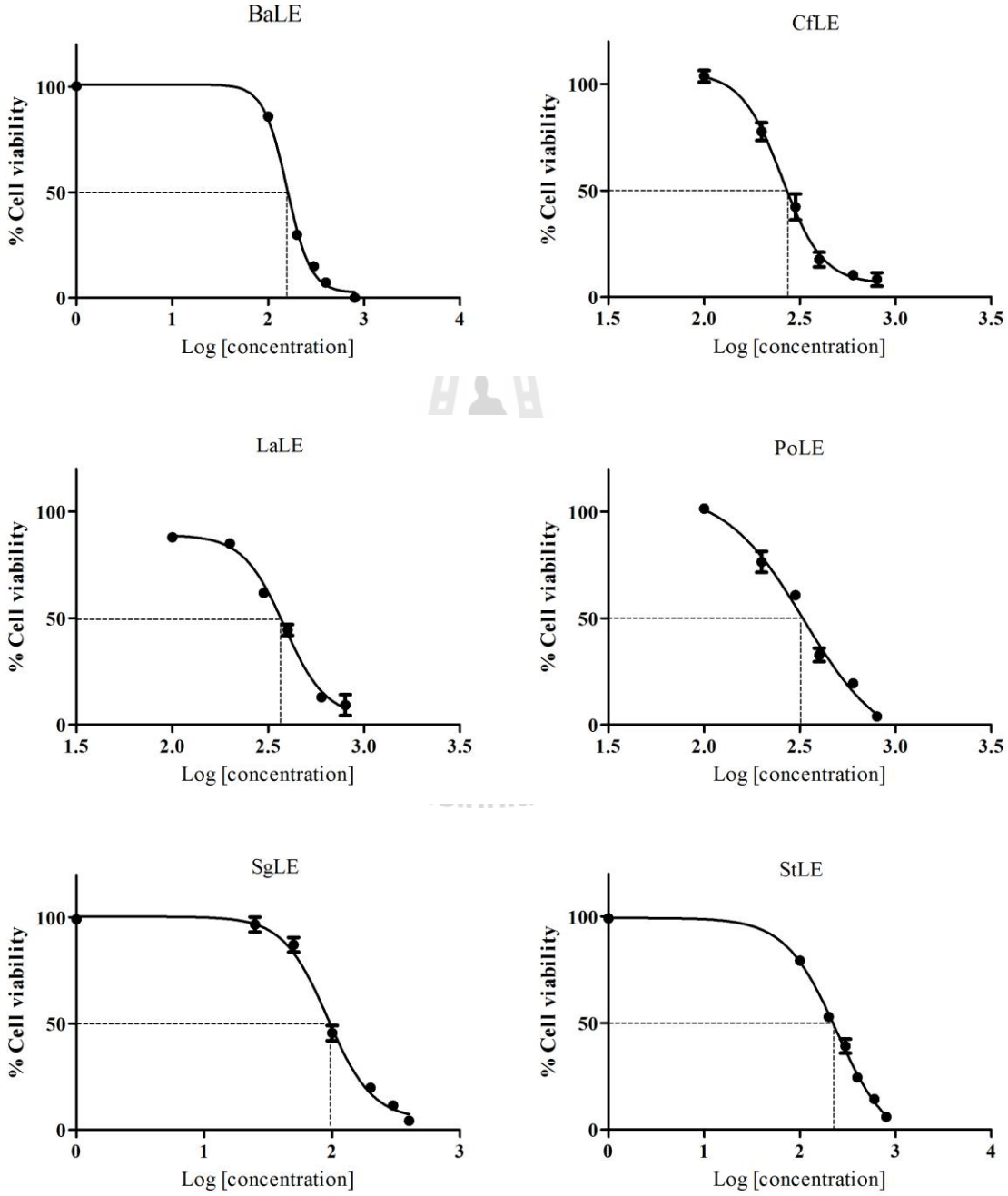
APPENDIX B

DOSE RESPONSE CURVE OF THE EXTRACTS

B.1 The effects of the 6 vegetable leaf extracts and standard antioxidant on brine shrimp lethality.



B2. The anti-proliferative effects of the 6 vegetable leaf extracts on MCF7 breast cancer cells at 48 h incubation.



APPENDIX C

PREPARATION OF REAGENTS

C1 Hoechst 33258 staining

- Phosphate buffer saline (PBS), pH 7.4
  - Potassium dihydrogen phosphate (KH_2PO_4) 0.144 g
  - Sodium phospahe dibasic heptahydrate ($Na_2HPO_4.7H_2O$) 0.795 g
  - NaCl 9.0 g

  Adjust volume with DI water to 1000 mL, filter sterile (store at 4ºC).
- Fixing solution (4% *p*-formaldehyde)
  - 37% *p*-formaldehyde 1.6 mL
  - PBS 13.2 mL
- Hoechst staining solution
  - Hoechst 33258 (1 mg/mL) 120 µL
  - 10% TritonX-100 240 µL
  - PBS 11.64 mL

  (Store at -20ºC)

C2 DNA fragmentation

- Lysis buffer (10 mL)
  - 10 mM Tris-HCl, pH 8.0 5.0 mL
  - 0.5 M EDTA 0.4 mL
  - 5 M NaCl 0.2 mL
  - 10% TritonX-100 0.5 mL

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Adjust volume to 10 mL with DI water (store at 4ºC).

- Reconstituted proteinase K (100 mL)
  - Tris base 0.6055 g
  - $CaCl_2.2H_2O$ 0.1470 g

  Adjust pH to 8.0 and bring the volume to 100 mL with DI water (store at 4ºC).
- 3 M Sodium acetate, pH 5.8 (20 mL)
  - $CH_3COONa.3H_2O$ 8.1648 g

  Adjust pH to 5.8 and bring the volume to 20 mL with DI water (store at 4ºC).
- TE buffer (100 mL)
  - Tris base 0.1576 g
  - 0.5 M EDTA 0.2 mL

  Adjust pH to 8.0 and make volume to 100 mL (store at 4ºC).
- TBE buffer solution (10X)
  - Boric acid 55 g
  - Tris base 108 g
  - 0.5 M EDTA, pH 8.0 40 mL
  - DI water 800 mL

  Adjust volume to 1 L (store at 4ºC).

C3 Cell cycle assay

- 3.8 mM Sodium citrate in PBS buffer, pH 8.0 (100 mL)
  - Sodium citrate 0.1274 g

  Add PBS, adjust pH to 8.0 and bring the volume to 100 mL with PBS.
- Propidium iodide (PI) solution (1 mg/mL) (1 mL)
  - PI 1 mg
  - DI water 1 mL

  Store at 4ºC, working solution is diluted with sodium citrate buffer.

C4 Western blot

- RIPA buffer
  - 100 mM Tris, pH 7.5 2,500 µL
  - 5 M NaCl 150 µL
  - 100 mM EDTA 50 µL
  - 10% SDS 50 µL
  - 10% Sodium deoxycholate 250 µL
  - 10% TritonX-100 500 µL
  - DI water 1,250 µL
- 200 mM Sodium orthovanadate (Na_3VO_4) (20 mL)
  - Na_3VO_4 0.7356 g

  Dissolve in DI water ~ 10 mL, set pH to 9.0 with NaOH. Boil until colorless, and cool to room temperature. Set pH to 9.0 again and boil until colorless. Repeat this cycle until the solution remains at pH 9.0 (Aliquot and store at -20ºC).
- Complete lysis buffer
  - RIPA buffer 950 µL
  - 200 mM PMSF 5 µL
  - 200 mM Na_3VO_4 5 µL
  - Protease inhibitor cocktail (25X) 40 µL
- Sample loading buffer (4X)
  - 1 M Tris-Hcl, pH 6.8 2,500 µL
  - SDS 1 g
  - Glycerol 4,000 µL
  - β-Merceptoethanol 1,000 µL
  - 5% Bromophenol blue 100 µL
  - DI water 2,400 µL

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Store at -20ºC. Make to 1X in sample mixture and boil for 5 min.

- 30% Acrylamide (29.1:0.9)
  - Acrylamide 29.1 g
  - Bis-acrylamide 0.9 g

  Dissolve in 100 mL of DI water and filter (store at 4ºC).

- 1.5 M Tris-Hcl, pH 8.8 (500 mL)
  - Tris base 90.86 g
  - DI water 400 mL

  Adjust pH to 8.8 with 6 M HCl and bring to 500 mL with DI water (store at 4ºC).

- 0.5 M Tris-Hcl, pH 6.8
  - Tris base 15.14 g
  - DI water 200 mL

  Adjust pH to 6.8 with 6 M HCl and bring to 250 mL with DI water (store at 4ºC).

- Running buffer (10X)
  - Tris base 30.28 g
  - Glycine 144 g
  - SDS 10 g

  Adjust volume to 1000 mL with DI water, and filter (store at 4ºC). Do not adjust the pH, it should be around 8.1-8.3.

- Transfer buffer (1X)
  - Tris base 3.03 g
  - Glycine 14.4 g
  - MeOH 200 mL

  Adjust volume to 1000 mL with DI water, and filter (store at 4ºC).

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- 0.1% Tween20 in TBS (TBST)
  - Tris base 1.2114 g
  - NaCl 8.776 g
  - Tween-20 1 mL

  Add 800 mL DI water, adjust pH to 8 and bring to 1000 mL (store at 4ºC.
- Blocking solution (5%)
  - Bovine serum albumin (BSA) 1 g
  - TBST 0.1% Tween20 20 mL
- Resolving gel (12%) (for 2 gels, 15 mL)
  - 30% Acrylamide 6 mL
  - 1.5 M Tris-HCl, pH 8.8 3.75 mL
  - DI water 4.98 mL
  - 10% SDS 150 µL
  - 10% Ammonium persulfate (AP) 112.5 µL
  - TEMED 7.5 µL
- Stacking gel (4%) (for 2 gels, 10 mL)
  - 30% Acrylamide 1.34 mL
  - 0.5 M Tris-HCl, pH 6.8 2.5 mL
  - DI water 6 mL
  - 10% SDS 100 µL
  - 10% Ammonium persulfate (AP) 50 µL
  - TEMED 10 µL

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