THE EFFECTS OF MINTWEED (*HYPTIS SAUVEOLENS* (L.) POIT) EXTRACTS ON PROLIFERATION AND APOPTOSIS OF A HUMAN JURKAT LEUKEMIA CELL LINE

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อิทธิพลของสารสกัดแมงลักคา (*HYPTIS SAUVEOLENS* (L.) POIT) ต่อการเพิ่มจำนวนและการตายแบบอะพ็อบโทซิสของเซลล์มะเร็งของมนุษย์สายพันธุ์เม็ดเลือดขาวเจอร์แคท

นางสาวสุมาลี มุสิกา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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มหาวิทยาลัยเทคโนโลยีสุรนารี
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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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สุมาลี มุสิกา : อิทธิพลของสารสกัดแมงลักคา (*HYPTIS SAUVEOLENS* (L.) POIT) ต่อการเพิ่มจำนวนและการตายแบบอะพ็อบโทซิสของเซลล์มะเร็งของมนุษย์สายพันธุ์เม็ดเลือดขาวเจอร์แคท (THE EFFECTS OF MINTWEED (*HYPTIS SAUVEOLENS* (L.) POIT) EXTRACTS ON PROLIFERATION AND APOPTOSIS OF A HUMAN JURKAT LEUKEMIA CELL LINE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กรกช อินทราพิเชฐ, 177 หน้า.

สารสกัดใบและเมล็ดแมงลักคาด้วยเอทานอลและน้ำ เมื่อนำมาตรวจสอบชนิดของสารพฤกษเคมี คุณสมบัติการต้านอนุมูลอิสระ ความเป็นพิษต่อเซลล์ คุณสมบัติการเพิ่มจำนวนของเซลล์มะเร็งเม็ดเลือดขาวสายพันธุ์เจอร์แคทและเซลล์เม็ดเลือดขาวปกติของคน และวิถีการตายแบบอะพ็อบโทซิสของเซลล์ ผลการทดลองพบว่า สารสกัดใบแมงลักคาด้วยเอทานอล มีปริมาณสารฟีนอลิกและฟลาโวนอยด์สูงที่สุด ปริมาณสารฟีนอลิกและฟลาโวนอยด์ในสารสกัดใบแมงลักคาด้วยเอทานอล สารสกัดใบแมงลักคาด้วยน้ำ สารสกัดเมล็ดแมงลักคาด้วยเอทานอล และสารสกัดเมล็ดแมงลักคาด้วยน้ำ คือ 370.02 ± 7.10 319.45 ± 8.67 135.92 ± 2.17 และ 77.02 ± 2.05 mg GAE/g ของสารสกัดแห้ง ตามลำดับ ปริมาณสารฟลาโวนอยด์ คือ 278.81 ± 3.40 240.81 ± 5.01 86.28 ± 0.67 และ 15.38 ± 0.21 mg CAE/g ของสารสกัดแห้ง ตามลำดับ การตรวจสารพฤกษเคมีของสารสกัดแมงลักคา พบว่ามีสารกลุ่ม เทอร์พีนอยด์ น้ำมันหอมระเหย แทนนิน ซาโปนิน แอลคาลอยด์ แต่ไม่พบสารกลุ่ม คูมาริน คาร์ดิแอ็กกลัยโคไซด์ แอนทราควิโนน คุณสมบัติการต้านอนุมูลอิสระของสารสกัดใบแมงลักคาด้วยเอทานอลมีความสามารถต้านอนุมูลอิสระสูงที่สุด ความสามารถในการรีดิวซ์เฟอริก คือ 8.52 ± 0.44 μM $FeSO_4$/mg ของสารสกัดแห้ง ความสามารถในการจับอนุมูลอิสระ 2, 2-diphenyl-1-picrylhydrazyl (DPPH) มีค่า IC_{50} เท่ากับ 9.26 ± 0.08 μg/mL สารสกัดใบแมงลักคาด้วยเอทานอลมีความสามารถต้านอนุมูลอิสระต่างจากสารมาตรฐานคาเทชินและกรดแอสคอร์บิคอย่างมีนัยสำคัญที่ $p < 0.05$ การวิเคราะห์กิจกรรมสลายไขมันด้วยวิธี TBARS แสดงการต้านอนุมูลอิสระของสารสกัดใบแมงลักคาด้วยเอทานอลและน้ำเหมือนกับคาเทชิน และสูงกว่าสารสกัดเมล็ดแมงลักคาด้วยเอทานอลและน้ำอย่างมีนัยสำคัญที่ $p < 0.05$

สารสกัดใบแมงลักคาด้วยเอทานอล มีความเป็นพิษต่อไรทะเล (*Artemia salina*) มีค่า LC_{50} เท่ากับ 360.48 (344.02-381.05) μg/mL และยับยั้งการเพิ่มจำนวนเซลล์มะเร็งเม็ดเลือดขาวของคนสายพันธุ์เจอร์แคท โดยมีค่า IC_{50} เท่ากับ 553.52 ± 14.07 μg/mL ในขณะที่สารสกัดใบแมงลักคาด้วยเอทานอล ชักนำการเพิ่มจำนวนเซลล์ในเซลล์เม็ดเลือดขาวปกติของคน การชักนำการตายแบบอะพ็อบโทซิสของเซลล์มะเร็งเม็ดเลือดขาวสายพันธุ์เจอร์แคทวิเคราะห์โดยการย้อมการแตกหัก

ของนิวเคลียสด้วยสี Hoechst 33258 และวิเคราะห์การแตกหักของดีเอ็นเอโดยอะกาโรสเจลอิเล็กโทรโฟไรซิส พบว่า สารสกัดใบแมงลักคาด้วยเอทานอล ชักนำให้เกิดการตายแบบอะพ็อบโทซิส โดยบ่งชี้ได้จากเส้นใยโครมาตินที่หดแน่น การแตกหักของนิวเคลียส และชิ้นดีเอ็นเอแตกหักแบบขั้นบันได สารสกัดใบแมงลักคาด้วยเอทานอลทำให้เซลล์มะเร็งเม็ดเลือดขาวสายพันธุ์เจอร์แคทตายที่ 24 ชั่วโมง โดยการเปลี่ยนแปลงรูปร่างของนิวเคลียสซึ่งขึ้นอยู่กับความเข้มข้นของสารสกัด และการแตกหักของดีเอ็นเอแบบขั้นบันไดเริ่มพบที่ 6 ชั่วโมง หลังบ่มเซลล์กับสารสกัดที่ความเข้มข้น 600 μg/mL

นอกจากนั้น การวิเคราะห์โปรตีนโดยวิธี Western blot แสดงว่า สารสกัดแมงลักคาสามารถชักนำให้เกิดการตายแบบอะพ็อบโทซิสในเซลล์มะเร็งเม็ดเลือดขาวสายพันธุ์เจอร์แคทโดยการเพิ่มระดับของโปรตีนชักนำให้เกิดการตาย caspase-9 และ Bax และมีการลดระดับของโปรตีนต้านการตาย Bcl-2 จึงสรุปได้ว่า สารสกัดใบแมงลักคาด้วยเอทานอล มีฤทธิ์มากที่สุดจากสารสกัดทั้งหมดในการต้านอนุมูลอิสระ และชักนำให้เกิดการตายแบบอะพ็อบโทซิสในเซลล์มะเร็งเม็ดเลือดขาวสายพันธุ์เจอร์แคท ดังนั้น มีความเป็นไปได้ที่สารสกัดใบแมงลักคาด้วยเอทานอล จะเป็นแหล่งของสารต้านอนุมูลอิสระ และสารป้องกันการเกิดมะเร็ง

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SUMALEE MUSIKA : THE EFFECTS OF MINTWEED (*HYPTIS SAUVEOLENS* (L.) POIT) EXTRACTS ON PROLIFERATION AND APOPTOSIS OF A HUMAN JURKAT LEUKEMIA CELL LINE. THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D. 177 PP.

HYPTIS SAUVEOLENS/ PHYTOCHEMICALS/ ANTIOXIDANT/ CYTOTOXICITY/ CELL PROLIFERATION/ APOPTOSIS

Mintweed leaf ethanolic and water extracts (MLE/e, MLE/w), mintweed seed ethanolic and water extracts (MSE/e, MSE/w) were evaluated for the phytochemical properties, antioxidant activities, cytotoxicity, proliferative effects on Jurkat leukemia cells and normal human lymphocyte cells and the pathway of apoptotic cell death. The results showed that MLE/e had the highest total phenolic compounds (TPC) and flavonoid contents (TFC). TPC of MLE/e, MLE/w, MSE/e and MSE/w were 370.02 ± 7.10, 319.45 ± 8.67, 135.92 ± 2.17 and 77.02 ± 2.05 mg gallic acid equivalent (GAE)/g dried extract and TFC of MLE/e, MLE/w, MSE/e and MSE/w were 278.81 ± 3.40, 240.81 ± 5.01, 86.28 ± 0.67 and 15.38 ± 0.21 mg catechin equivalent (CAE)/g dried extract, respectively. Phytochemical screening of the extracts revealed the existence of terpeniods, essential oils, tannins, saponins and alkaloids whereas coumarins, cardiac glycosides and antraquinones were not detected. Antioxidant activity of the extracts, which was determined by a FRAP assay showed that MLE/e possessed the highest ferric reducing activity of 8.52 ± 0.44 μM $FeSO_4$/mg dried extract. DPPH method, MLE/e also showed the highest antioxidant activity with an IC_{50} of 9.26 ± 0.08 μg/mL. The antioxidant activity of MLE/e was significantly different

from those of the standards, catechin and ascorbic acid at $p < 0.05$. TBARS assay for lipid peroxidation activity demonstrated that the antioxidant activity of MLE/e and MLE/w exhibited similar property of catechin and their activities were significantly higher than MSE/e and MSE/w ($p < 0.05$).

MLE/e showed the highest cytotoxicity on *Artemia salina* with LC_{50} value of 360.48 (344.02-381.05) µg/mL and also inhibited the growth of Jurkat human cancer cells with IC_{50} values of 553.52 ± 14.07 µg/mL whereas it induced the proliferative property on normal human lymphocytes. Induction of apoptotic cell death in Jurkat cells was analyzed by using Hoechst 33258 staining assay and DNA fragmentation assay. MLE/e induced apoptotic cell death as indicated by chromatin condensation and nuclear fragmentation and nucleosomal DNA ladder pattern analyzed by agarose gel electrophoresis. MLE/e caused death to Jurkat cells at a significant level by changing nuclear morphology at 24 h with dose dependent manner and DNA ladders began to appear at 6 h after the cells were treated at 600 µg/mL.

In addition, Western blot analysis showed that induction of apoptosis of Jurkat cells by mintweed extracts were accompanied by increasing caspase-9, pro-apoptotic Bax and decreasing Bcl-2 levels. It is concluded that MLE/e is the most active extract of all the mintweed extracts in antioxidant and apoptotic activities on Jurkat cells. It may possibly be a potential source of antioxidants and chemopreventive agents against carcinogenesis.

School of Biology
Academic Year 2012

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LIST OF ABBREVIATIONS

µg/mL	=	Microgram per milliliter
µL	=	Microliter
µM	=	Micromolar
ANOVA	=	Analysis of variance
Bp	=	Basepair
BSA	=	Bovine serum albumin
CAE	=	Catechin equivalent
CLS	=	Cell line services
DMSO	=	Dimethylsulfoxide
DNA	=	Deoxyribonucleic acid
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	=	Median effective concentration
ECL	=	Enhanced chemiluminescence
EDTA	=	Ethylenediaminetetraacetic acid
EB	=	Ethidium bromide
FBS	=	Fetal bovine serum
FRAP	=	Ferric reducing antioxidant power
GAE	=	Gallic acid equivalent
h	=	Hour
HEPES	=	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid

LIST OF ABBREVIATIONS (Continued)

IC_{50}	=	Median inhibition concentration
kDa	=	Kilodalton
LD_{50}	=	Median lethal dose
min	=	Minute
mL	=	Milliliter
MLE/e	=	Mintweed leaf ethanolic extract
MLE/w	=	Mintweed leaf water extract
MSE/e	=	Mintweed seed ethanolic extract
MSE/w	=	Mintweed seed water extract
PBS	=	Phosphate buffered saline
R_f	=	Retention factor
RNase A	=	Ribonuclease A
ROS	=	Reactive oxygen species
RPMI 1640	=	Roswell Park Memorial Institute number 1640
SD	=	Standard deviation
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFC	=	Total flavonoid contents
TLC	=	Thin layer chromatography
TPC	=	Total phenolic contents
v/v	=	volume by volume
w/v	=	weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Reactive oxygen species (ROS) are normal oxidant by-products of aerobic metabolism. ROS includes the major radical species such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), highly reactive hydroxyl radical ($^{\bullet}OH$), and peroxyl radicals ($ROO^{\bullet-}$) (Cross et al., 1987). These free radicals are extremely harmful to living organisms in the way that they attack different constituents of cells which lead to ageing, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts, inflammation and cancer. Cancer is one of the causes of disease related deaths despite the significant advancement in treatment of cancers made over the past decades (Chabner and Roberts, 2005). Treatment and management of cancers involve surgical intervention, radiotherapy and chemotherapy. The traditional medical system based on natural products continues to play an important role in the treatment of many diseases including cancer. Such natural products are alkaloids, essential oils, diterpenes, etc. Natural antioxidants from plants are reported to prevent the radical chain reactions of oxidation by inhibiting the initiation and propagation steps, which leads to the termination of the reactions and delay in the degradation reaction (Matthaus, 2002). Many tropical plants have interesting biological properties with potential therapeutic applications. Mintweed, *Hyptis sauveolens* (L.) Poit belongs to the family Lamiaceae, commonly known as

"maeng lak kha" in Thailand. This plant is a fast-growing perennial herb, found in dense clumps along roadsides. Its branches and semi-woody stems can reach a height of 2 meters. The plant has a characteristic minty smell when it is crushed. The plant naturally grows as weed in open areas, found throughout Thailand. *H. sauveolens* is used for traditional medicine for the treatment of various illness such as the respiratory tract, infection, colds, pain, fever, cramps, and skin diseases. In addition, the leaves of *H. sauveolens* are used as a stimulant, carminative, sudorific, galactogogue, and as a cure for parasitic cutaneous disease. The plant has been traditionally used as a pesticide, antibacterial, antimicrobial, antifungal, antiplasmodial, and anti-inflammatory activities. Ethanolic extract of *H. suaveolens* also possesses potent anticancer activity against Ehrlich ascites carcinoma by activating an apoptotic pathway (Gurunagarajan and Pemaiah, 2011). The major chemical composition in *H. sauveolens* oil is a terpenoid group. It contains 1,8-cineole and β-caryophyllene from distillation (Luz et al., 1984; Peerzada, 1997). Azevedo et al. (2001) reported that the essential oils of *H. sauveolens* are sabinene, limonene, biclyclogermacrene, β-phellandrene and 1,8-cineole. Oliveira et al. (2005) also reported that the composition of essential oil of *H. sauveolens* is 1,8-cineole, (*E*)-caryophyllene, spathulenol. Monoterpenes inhibit the proliferation of human colon cancer cells by modulating cell cycle-related protein expression (Bardon et al., 2002). Herbal diterpenoids induce growth arrest and apoptosis in colon cancer cells with an increase in the expression of the nonsteroidal anti-inflammatory drug-activated gene (Ko et al., 2007). In addition, oridonin, a di-terpenoid compound, induced apoptosis of Jurkat cells (Liu et al., 2004). Lupane triterpenes showed inhibitory effects on leukemia, melanoma and neuroblastoma cell growth (Hata et al.,

2003). Lanostanoid triterpenes from *Laetiporus sulphureus* induced apoptosis of HL-60 human myeloid leukemia cells (Len, Quintana, Rivera, Estvez and Bermejo, 2004). Fernandes et al. (2003) reported that pentacyclic triterpenes from Chrysobalanaceae species inhibited the growth and induced apoptosis of K562, an erythroleukemia cell line. Furthermore, betulinic acid, pentacyclic lupine-type triterpene, could significantly inhibit the proliferation of Jurkat cells by arresting cells in G0/G1 phase and inducing apoptosis (Chen et al., 2008). These bioactive compounds of *H. sauveolens* may inhibit proliferation of cancer cell by inducing apoptosis. Therefore, the aim of this study is to determine the phytochemical properties, antioxidant activities, cytotoxicity, proliferative effect and apoptotic induction by the extract of *H. sauveolens* on Jurkat human cancer cells.

1.2 Research objectives

The objectives of this study were as follows:

1. To determine the total phenolic compounds (TPC), the total flavonoid contents (TFC), the phytochemical screening and the antioxidant activities of *H. sauveolens* extract
2. To investigate the cytotoxic effect of extract of *H. sauveolens* on *Artemia salina* and proliferative effect on human Jurkat, lymphocytes cancer cell and normal human lymphocytes
3. To evaluate the effect of the extracts of *H. sauveolens* on apoptotic induction of human Jurkat, leukemia cells

1.3 Research hypotheses

1. *H. sauveolens* extracts contain a high total phenolic compounds, total flavonoid content, and antioxidant activities.
2. *H. sauveolens* extracts are toxic to Jurkat, lymphocyte cancer cells, but exhibit non- toxicity or less cytotoxic effects on normal lymphocytes.
3. *H. sauveolens* extracts can induce apoptosis in human Jurkat lymphocyte cancer cells.

1.4 Scope and limitations of the study

The leaves of *H. sauveolens* were collected from the plants grown on the grounds of Suranaree University of Technology (SUT) in Nakhon Ratchasima, Thailand in October 2008. The seeds were collected in January 2009 from the same place. The leaves and seeds of *H. sauveolens* were extracted in water and 70% ethanol by using the Soxhlet extraction apparatus. Some phytochemical properties and antioxidant activities of *H. sauveolens* were determined. Cytotoxicity was investigated on Jurkat cells and human normal lymphocytes. The apoptotic induction of Jurkat cells was observed by Hoechst staining and DNA fragmentation. Apoptotic proteins were observed by the Western blot assay.

1.5 Expected results

The extract of *H. sauveolens* contains high total of phenolic compounds, total flavonoid content, antioxidant activities, is non-toxic to normal cells, and inhibits the proliferation of Jurkat cells by inducing apoptosis on Jurkat cancer cells. These plants

may help in the design of more effective therapeutic strategies for the treatment of a variety of cancer and other diseases.

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CHAPTER II

LITERATURE REVIEW

2.1 Free radicals

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from oxygen represent the most important class of such species generated in living systems (Valko et al., 2006). Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) (Table 1). ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (HOCl, O_3, $ONOO^-$, $^1O_2$, H_2O_2). RNS is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as such nonradicals as HNO_2 and N_2O_3. $ONOO^-$ is often included in both categories. Reactive term is not always an appropriate term: H_2O_2, $NO^{\bullet}$ and $O_2^{\bullet-}$ react quickly with few molecules, whereas $OH^{\bullet}$ reacts quickly with almost everything. HOCl, $NO_2^{\bullet-}$, $ONOO^-$ and O_3 have intermediate reactivities. HOCl and NO_2Cl could also be regarded as reactive chlorinating species; HOBr as a reactive brominating species (Halliwell, 2005). ROS and RNS are: (i) generated during irradiation by UV light, by X-rays and by gamma rays; (ii) products of metal-catalyzed reactions; (iii) present as pollutants in the atmosphere; (iv) produced by

neutrophils and macrophages during inflammation; (v) by-products of mitochondria-catalyzed electron transport reactions and other mechanisms (Cadenas, 1989).

Table 2.1 Reactive species (Halliwell, 2005).

Reactive Oxygen Species (ROS)	
Radicals	*Nonradicals*
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO^{\bullet}$ (e.g. lipid peroxyl)	Hypobromous acid, HOBr
Alkoxyl, $RO^{\bullet}$	Ozone, O_3
Hydroperoxyl, $HO_2^{\bullet}$	Singlet oxygen $^1\Delta_g$
Reactive Nitrogen Species (RNS)	
Radicals	*Nonradicals*
Nitric oxide (nitrogen monoxide), $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO^{2+}
	(e.g. as nitryl chloride, NO_2Cl)
	Alkyl peroxynitrites, ROONO

ROS and RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (Valko et al., 2004). Beneficial effects of ROS involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of cell damage, including lipids, proteins and nucleic acids (Poli et al., 2004). The harmful effects of ROS are balanced

by the antioxidant action of non-enzymatic antioxidants, as well as antioxidant enzymes (Halliwell, 1996).

2.2 Oxidative damages to DNA, lipids and proteins

Overproduction of ROS (arising either from mitochondrial electrontransport chain or excessive stimulation of NADPH) results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA.

ROS, at high concentrations, can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko et al., 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell and Gutteridge, 1999). Permanent modification of genetic material resulting from these oxidative damage incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, as is well established, in various cancer tissues free radical-mediated DNA damage has occurred. ROS-induced DNA damage involves single or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Cooke et al., 2003). The most extensively studied DNA lesion is the formation of 8-hydroxyguanine (8-OH-G). The presence of oxidized DNA product is importance because it is both relatively easily formed and is mutagenic and carcinogenic. It is a

good biomarker of oxidative stress of an organism and a potential biomarker of carcinogenesis.

It is known that metal-induced generation of ROS results in the attack of not only DNA in the cell nucleus, but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems et al., 1995). The process of lipid peroxidation consists of three stages: initiation, propagation and termination. Once formed, peroxyl radicals ($ROO^{\bullet}$) can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Marnett, 1999). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE). MDA can react with DNA bases G, A and C to form adducts M1G, M1A and M1C, respectively. MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation (Marnett, 1999).

Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed. The side chains of all amino acid residues of proteins, in particularly, cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman, 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (-SH) and low molecular weight thiols, in particular GSH (S-glutathiolation). The concentration of carbonyl groups, generated by many different

mechanisms is a good measure of ROS-mediated protein oxidation. Advanced glycation end products (AGEs) is a class of complex products. They are the results of a reaction between carbohydrates and free amino group of proteins. Most of the AGEs are very unstable, reactive compounds and the end products are difficult to be completely analysed. The intermediate products are known as Amadori, Schiff Base and Maillard products. (Dalle-Donne et al., 2005).

2.3 ROS and human diseases

Oxidative stress has been implicated in various pathological conditions involving of cardiovascular diseases, atherosclerosis, hypertension, ischemia/ reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis, ageing, and cancer (Valko et al., 2006).

2.3.1 Cancer

Cancer is a group of more than 100 different diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread of cancer is not controlled, it can result in death. Such abnormal cell activity can be results of both external (chemical, radiation, and viruses) and internal (hormones, immune conditions, and inherited mutations) factors. Causal factors may act together, or in sequence, to initiate or promote carcinogenesis.

2.3.2 ROS, signal transduction and cancer

Cell signalling refers to the process that extracellular substances produce an intracellular response. Aberrant signalling mechanisms are related to various

disease states. Since one of the most fundamental processes regulated through signal transduction mechanisms is cell growth, alterations in the normal regulatory processes of cells may lead to cancer. The abnormal behavior of neoplastic cells can often be traced to an alteration in cell signalling mechanisms, such as receptor or cytoplasmic tyrosine kinases, altered levels of specific growth factors, intracellular processes for conveying membrane signals to the nucleus, portions of the transcription apparatus, and genes involved in the cell cycle and the regulation of DNA replication. It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways and are thus instrumental in the process of carcinogenesis (Poli et al., 2004). The mechanism of cell growth regulation is very complex and therefore the role of ROS in this process depends on the type and concentration of the particular radical involved. The activation of transcription factors including MAP-kinase/AP-1 and NF-κB pathways has a direct effect on cell proliferation and apoptosis (Valko et al., 2006). Abnormalities in growth factor receptor functioning are associated with the development of many cancers (Drevs et al., 2003). Several growth factor receptors (epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF)) are affected by ROS and carcinogenic metals such as nickel, arsenic, cobalt and beryllium. Activation of both EGF and VEGF results in increases in cellular Ca^{2+}. Increased expression of the EGF receptors and overexpression of the EGF receptor has been observed in lung and urinary cancers (Drevs et al., 2003).

The role of cellular oxidants and AP-1 activation is to increase cell proliferation. It has been demonstrated that c-fos and c-Jun are positive regulators of cell proliferation. Expression of c-fos and c-jun can be induced by a variety of

compounds, involving reactive radicals and non-genotoxic and tumor promoting compounds (various metals, carbon tetrachloride, phenobarbital, TPA, TCDD, alcohol, ionising radiation, asbestos) (Valko et al., 2006).

NF-κB regulates several genes involved in cell transformation, proliferation, and angiogenesis (Thannickal and Fanburg, 2000). NF-κB activation has been linked to the carcinogenesis process because of its role in differentiation, inflammation, and cell growth. Carcinogens and tumor promoters involving toxic metals, UV radiation, phorbol esters, asbestos, alcohol, and benzopyrene are among the external stimuli that activate NF-κB (Leonard et al., 2004). On the one hand, expression of NF-κB has been shown to promote cell proliferation, whereas on the other inhibition of NF-κB activation blocks cell proliferation.

The tumor suppressor protein p53 plays an important role in protecting a cell from tumorigenesis. p53 prevented DNA-damaged cells from dividing until either the chromosomal repair is effected or the cell undergoes apoptosis. ROS are enhanced through the action of p53-mediated transcription of apoptosis promoting genes; however, p53 also can promote the expression of many antioxidant genes that prevent apoptosis. Mutation in p53 leading to its inactivation found in more than half of human cancers p53 is activated by UV radiation, hypoxia, gamma-radiation, nucleotide deprivation and others. Many studies have been devoted to mutation in p53 caused by direct action of ROS or by carcinogenic metals (Hollstein et al., 1991).

2.3.3 The three stages model and mechanisms of carcinogenesis

Carcinogenesis is a complex multi-sequence process leading a cell from a healthy to a precancerous state and finally to an early stage of cancer. Two key

mechanisms have been proposed for the induction of cancer. One is an increased DNA synthesis and mitosis by non-genotoxic carcinogens which may induce mutations in dividing cells without repairment. Mutations may then clonally expand from an initiated preneoplastic cell state to a neoplastic cell state (Guyton and Kensler, 1993). Another mechanism attributes to an equilibrium between cell proliferation and cell death. If the damage to DNA is too great, it exists as an important process that eliminates altered cells selectively. This process is called apoptosis. During cell proliferation, protein p53 plays a primordial role, checking the integrity of the DNA. p53 triggers mechanisms that eliminate, the oxidized DNA bases that cause mutations. When cell damage is too great, p53 triggers cell death by apoptosis. Uncontrolled apoptosis can be harmful to an organism by leading to destruction of healthy cells (Hussain et al., 2003). More than half of cancers have defects in upstream or downstream genes of p53 function. The carcinogenic process can be described as an imbalance between cell proliferation and cell death shifted towards cell proliferation.

Epidemiological clues and animal experiments have shown that the process of carcinogenesis consists of multiple and distinct stages, each characterized by different underlying mechanisms namely, the initiation, promotion, and progression (Trueba et al., 2004). Cancer development is a multistage process. Chemical carcinogens interfere with various stages of this process and function through modifications of cellular and molecular events. Genotoxic agents are usually chemicals that directly damage to DNA, which in turn leads to mutation. A second category of carcinogenic compounds (non-genotoxic) functions through non-DNA or indirect-DNA reaction mechanisms. A multi-stage process such as cancer development

is characterised by cumulative action of multiple events occurring in a single cell and can be described by three stages namely, initiation, promotion and progression. ROS can act in all these stages of carcinogenesis (Klaunig and Kamendulis, 2004). The three stages model of carcinogenesis is shown in Figure 2.1. Initiation involves a non-lethal mutation in DNA that produces an altered cell followed by at least one round of DNA synthesis to fix the damage (e.g. 8-OH-G) produced during the initiation (Figure 2.1). If dividing cells are damaged for whatever reason, they are able to interrupt temporarily their cell cycle at stage G1, S, or G2, repair the damage, and resume division (Loft and Poulsen, 1996)

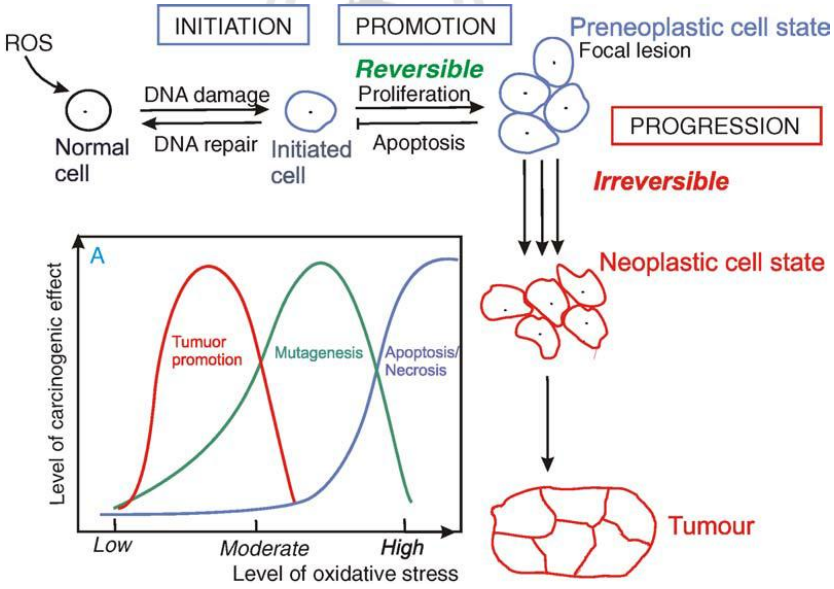

Figure 2.1 Three stages model of carcinogenesis and the level of carcinogenic effect vs. level of free radicals at various stages of carcinogenic process (Valko et al., 2006).

Oxidative DNA damage can occur via action of ROS, e.g. hydroxyl radicals, formed through the Fenton-type mechanism, along with other species. Several studies on benign tumors revealed an interesting correlation between the size of tumor and the amount of 8-OH-G adduct formation; the level of 8-OH-G may thus determine the transformation from benign to malignant tumor (Loft and Poulsen, 1996). The process of initiation further proceeds through oxidative stress-induced Ca^{2+} changes leading to increase in intracellular free calcium as a result of its release from intracellular Ca^{2+} stores and through the influx of extracellular Ca^{2+} (Dreher and Junod, 1996). The promotion stage is characterised by the clonal expansion of initiated cells by the induction of cell proliferation and/or inhibition of programmed cell death (apoptosis). This process results in the formation of an identifiable focal lesion. This stage dose-dependently requires the continuous presence of the tumor promotion stimulus and therefore it is a reversible process (Loft and Poulsen, 1996). Many tumor promoters have a strong inhibiting effect on cellular antioxidant defence systems such as SOD, catalase, glutathione, etc. While a high level of oxidative stress is cytotoxic to the cell and halts proliferation by inducing apoptosis or even necrosis, a low level of oxidative stress can in fact stimulate the cell division in the promotion stage and thus stimulate the promotion of tumor growth (Dreher and Junod, 1996). This implies that production of ROS during this stage of carcinogenesis is the main line of ROS-related tumor promotion. Progression is the third and final stage of the carcinogenic process (Klaunig and Kamendulis, 2004). This stage involves cellular and molecular changes that occur from the preneoplastic to the neoplastic state. This stage is irreversible and is characterized by accumulation of additional genetic damage, leading to the

transition of the cell from benign to malignant. This stage is characterised by genetic instability and disruption of chromosome integrity.

2.4 Programmed cell death (PCD)

Programmed cell death may balance cell death with survival of normal cells; the equilibrium becomes disturbed and PCD plays key roles in ultimate decisions of cancer cell fate. Three types of programmed cell death have been identified. Apoptosis, or type I PCD, is characterized by specific morphological and biochemical changes of dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to neighbours or to extracellular matrix (Nishida et al., 2008). Biochemical changes include chromosomal DNA cleavage into internucleosomal fragments, phosphatidylserine externalization and a number of intracellular substrate cleavages by specific proteolysis (Martin and Green, 1995). Autophagy, or type II PCD, is an evolutionarily conserved catabolic process beginning with formation of autophagosomes. In general, autophagy plays a crucial pro-survival role in cell homeostasis, required during periods of starvation or stress due to growth factor deprivation (He and Klionsky, 2009). Autophagy controls a myriad of physiological processes including starvation, cell differentiation, cell survival and death (Liu et al., 2010). However, there is accumulating evidence that autophagic cells may commit suicide by undergoing cell death and coping with excessive stress, which differs from apoptosis and necrosis. Type III PCD termed programmed necrosis, which involves cell swelling, destruction of organelles, and disruption of the plasma membrane, leading to the release of intracellular components and inflammation (Wu et al., 2012). Different features of apoptotic, necrosis and

autophagy are showed in Figure 2.2. Thus, PCD may play an important role during preservation of tissue homeostasis and elimination of damaged cells, this has profound effects on malignant tissues.

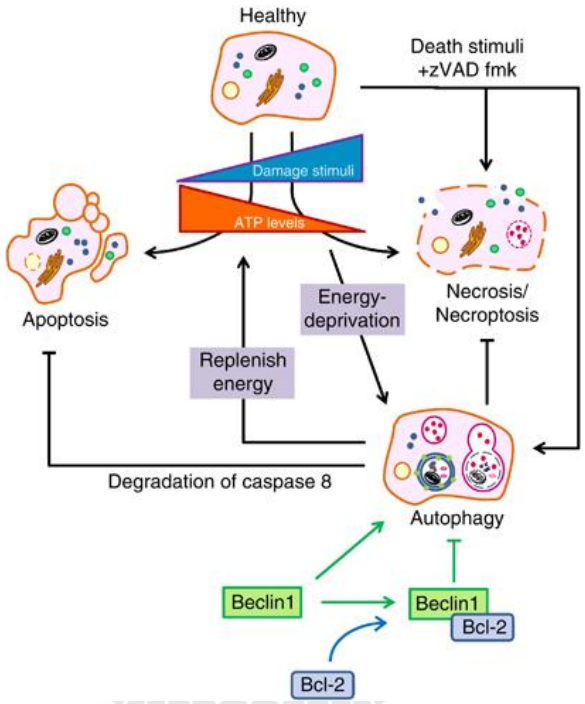

Figure 2.2 The interplay between apoptosis, necrosis and autophagy (Long and Ryan, 2012).

2.4.1 Apoptosis

Apoptosis occurs normally during development, aging, and as a homeostatic mechanism to maintain cell population in tissues. It is used to contrast necrosis which a cell actively pursues a course toward death upon receiving certain stimuli. Apoptosis was remained one of the most investigated processes in biologic research.

2.4.1.1 Morphological changes in apoptosis

Morphological alterations of apoptotic cell death that concern both the nucleus and the cytoplasm are remarkably similar across cell types and species (Hacker, 2000). During the early process of apoptosis, cell shrinkage, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding." Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages and degraded within phagolysosomes. There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines (Kurosaka et al., 2003).

2.4.1.2 Biochemical changes in apoptosis

Three main types of biochemical changes can be observed in apoptosis: (1) activation of caspases, (2) DNA and breakdown of protein, and (3) membrane changes and recognition by phagocytic cells (Kumar et al., 2010). Early in apoptosis, there is expression of phosphatidylserine (PS) in the outer layers of the cell membrane, which has been "flipped out" from the inner layers. This is recognized of

dead cells by macrophages, resulting in phagocytosis without the release of pro-inflammatory cellular components (Hengartner, 2000). Later, there is internucleosomal cleavage of DNA into oligonucleosomes in multiples of 180 to 200 base pairs by endonucleases. Although this feature is characteristics of apoptosis, it is not specific as the typical DNA ladder in agarose gel electrophoresis (McCarthy and Evan, 1998). Another specific feature of apoptosis is the activation of a group of enzymes belonging to the cysteine protease family named caspases. Activated caspases cleave many vital cellular proteins and break up the nuclear scaffold and cytoskeleton. They also activate DNAase, which further degrade nuclear DNA. Although the biochemical changes explain in part some of the morphological changes in apoptosis, it is important to note that biochemical analyses of DNA fragmentation or caspase activation should not be used to define apoptosis, as apoptosis can occur without oligonucleosomal DNA fragmentation and can be caspase-independent (Galluzi et al., 2010).

2.4.2 Molecular mechanisms of apoptotic cell death

Understanding the mechanisms of apoptosis is crucial and helps to understand the pathogenesis of conditions as a result of disordered apoptosis. This in turn, may help in the development of drugs that target certain apoptotic genes or pathways. Caspases are central of the mechanism of apoptosis as they are both the initiators and executioners. There are three pathways which caspases can be activated. The two commonly described initiation pathways are the intrinsic (or mitochondrial) and extrinsic (or death receptor) pathways of apoptosis (Figure 2.3). Both pathways eventually lead to a common pathway or the execution phase of apoptosis. A third

less well-known initiation pathway is the intrinsic endoplasmic reticulum pathway (O'Brien and Kirby, 2008).

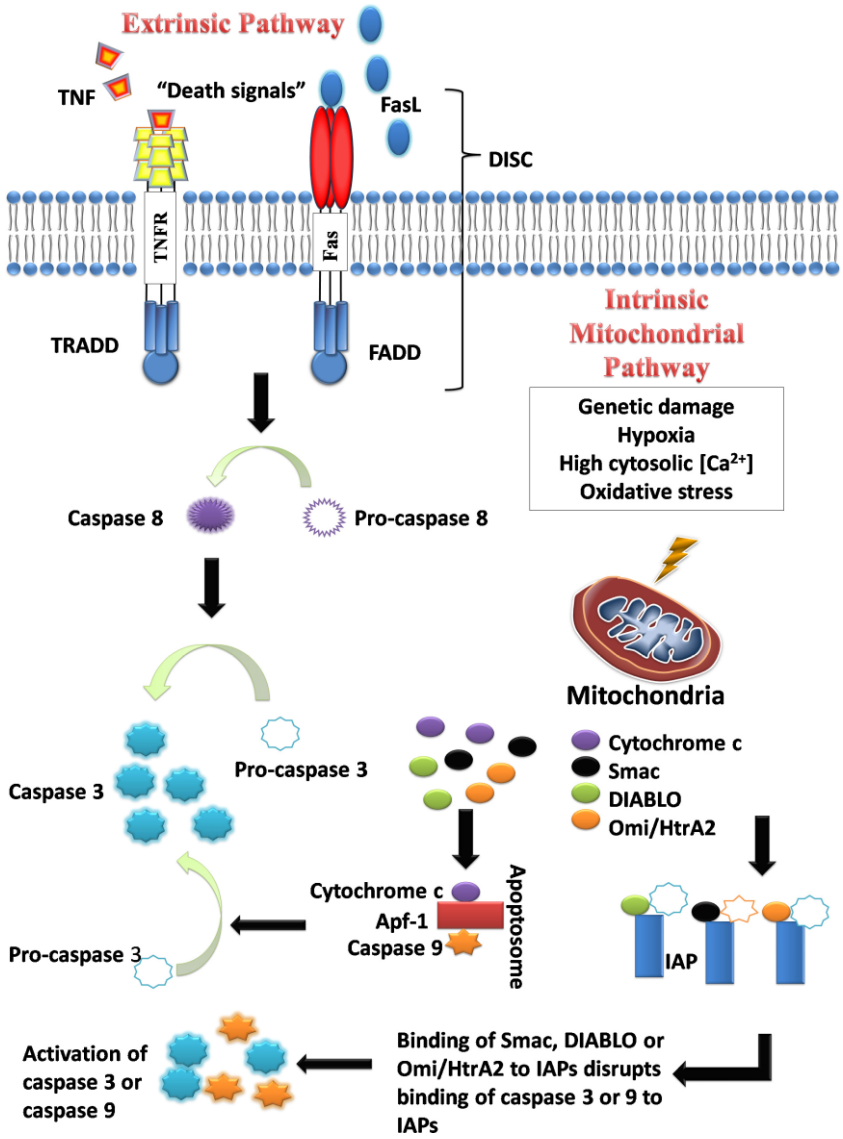

Figure 2.3 The extrinsic and intrinsic pathway of apoptosis (Wong, 2011).

2.4.2.1 The extrinsic death receptor pathway

The extrinsic death receptor pathway begins when death ligands bind to a death receptor (Figure 2.3). The death receptor (DR) family which includes tumor necrosis factor receptor TNF-R1, Fas, DR3, TRAIL-R1/2 (DR4/5) and DR6 can initiate the extrinsic pathway leading to apoptosis (Sayers, 2011). Although several death receptors have been described, the best known death receptors are the type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95) and their ligands called TNF and Fas ligand (FasL), respectively (Hengartner, 2000). These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases like caspase 8. Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein and the whole ligand-receptor-adaptor protein complex is known as the death-inducing signalling complex (DISC). DISC then initiates the assembly and activation of pro-caspase 8. The activated form of the enzyme, caspase 8, is an initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases (Karp, 2008). However, it can activate the intrinsic mitochondrial pathway by truncating Bid, a kind of proapoptotic protein in the cytosol, into its active form, tBid (Figure 2.4). tBid can trigger the activation of the mitochondrial pathway: cytochrome c, apoptosis-inducing factor (AIF) and other molecules are released from mitochondria, and apoptosis will be induced (Wang et al., 2005).

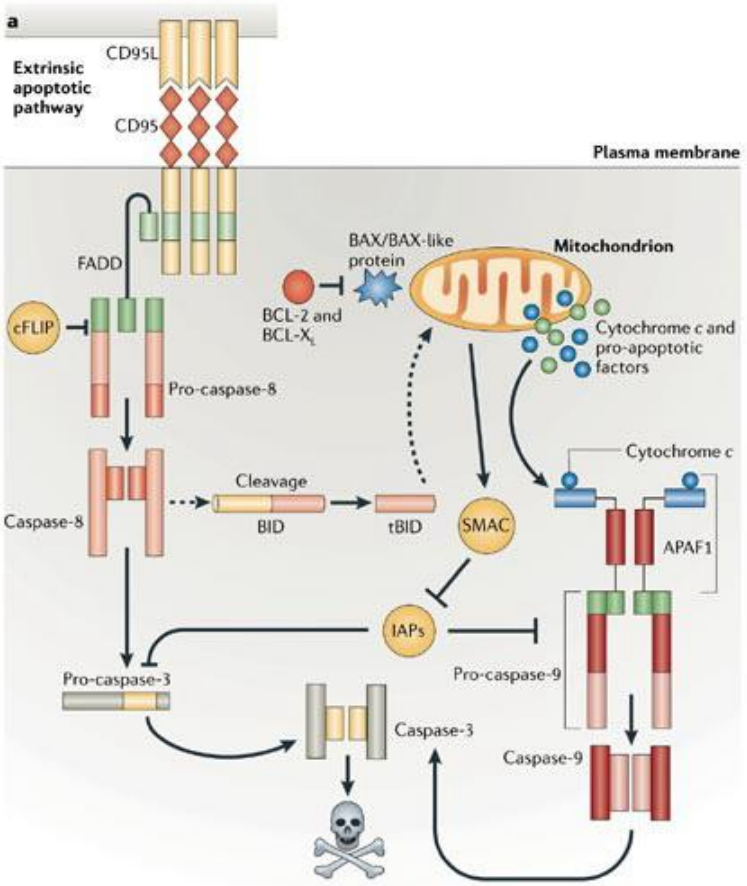

Figure 2.4 The extrinsic and intrinsic apoptosis pathway are interconnected by truncated BID (tBID) (adapted from Richard et al., 2006).

2.4.2.2 The intrinsic mitochondrial pathway

The intrinsic pathway is initiated within the cell and stimulated by internal stimuli such as irreparable genetic damages, hypoxia, extremely high concentrations of cytosolic Ca^{2+} and severe oxidative stress are some triggers of the initiation of the intrinsic mitochondrial pathway (Karp, 2008). This pathway is the result of increased mitochondrial permeability leading to the release of pro-apoptotic molecules such as cytochrome c into the cytoplasm and regulating of the Bcl-2 family protein (Figure 2.3). There are two main groups of the Bcl-2 proteins, namely the

pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1). While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release. It is not the absolute quantity but rather the balance between the pro- and anti-apoptotic proteins that determines whether apoptosis would be initiated (Reed, 1997). Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2). Cytoplasmic release of cytochrome c activates caspase 3 via the formation of a complex known as apoptosome which is made up of cytochrome c, Apaf-1 and procaspase-9 and in the presence of ATP/dATP. On the other hand, Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs) which subsequently leads to disruption in the interaction of IAPs with caspase-3 or -9 (Kroemer et al., 2007).

2.4.2.3 The intrinsic endoplasmic reticulum pathway

This intrinsic endoplasmic reticulum (ER) pathway is a third pathway and less well known. It is believed to be caspase 12-dependent and mitochondria-independent (Szegezdi et al., 2003). When the ER is injured by cellular stresses like hypoxia, free radicals or glucose starvation, there is unfolding proteins and reduced protein synthesis in the cell, and an adaptor protein known as TNF

receptor associated factor 2 (TRAF2) dissociates from procaspase-12 (O'Brien and Kirby, 2008).

2.4.3 Bcl-2 family

B cell lymphoma 2 (Bcl-2) protein was found in the mitochondrial membrane. It was a key regulators of apoptosis and the molecule is over-expressed in many types of cancer cell (Llambi and Green, 2011). While reduced Bcl-2 expression may promote apoptotic responses to anticancer drugs, increased expression of Bcl-2 leading to resistance to chemotherapeutic drugs and radiation therapy. Normally, these protein reside in the intermembrane space of mitochondria. They responsed to a variety of apoptotic stimuli then they are released to the cytosol and lead to the execution of apoptosis. These pro-apoptotic proteins may act either in a caspase dependent form such as cytochrome-c, Omi/HtrA2, and second mitochondrial activator of caspases (SMAC), or in a caspase independent form such as apoptosis-inducing factor (AIF) and endonuclease G. The proteins members of this family regulate apoptosis, promoting or inhibiting the permeabilization and disruption of the outer mitochondrial membrane, after apoptotic process trigger (Mohamad et al., 2005). The Bcl-2 family is comprised of a number of pro-apoptotic and anti-apoptotic members, pro-apoptotic members such as Bax, Bak, Bad, Bcl-XS, Bid, Bik, Bim and Hrk, anti-apoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1 (Engel and Henshall, 2009). Following a death signal, pro-apoptotic proteins are able to undergo post-translational modifications that include dephosphorylation and cleavage leading to their activation and translocation to mitochondria, from which apoptosis can be initiated (Shamas-Din et al., 2011). All BH3-only molecules require multi-

domain BH3 proteins to apply their intrinsic pro-apoptotic activities. These lead to release of cytochrome c and secondly mitochondria derived activator of caspases; the outer mitochondrial membrane becomes permeable in response to apoptotic stimuli, while cytochrome c can interact with Apaf-1 once released into the cytosol, leading to activation of caspase-9. Activated caspase-9 activates caspase-3, subsequently activating the downstream caspase cascade finally generating apoptosis (Wen et al., 2012).

2.4.4 Caspases classification and structure

The caspases (cysteinyl aspartate-specific protease) are a family of proteins that are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis. Caspases involved in cell death and comprised of 12 proteins in human. Phylogenetic analyses and studied of substrate specificity, indicate that caspase can be subdivided into three subfamily, as shown in Table 2.2. Subfamily I includes caspases 1, 4, 5 and 12, which are involved in inflammation. The prototype of this subfamily is caspase 1, previously known as interleukin-1β conversion enzyme, or ICE (Yuan et al., 1993). Subfamily II includes caspases 2, 8, 9 and 10. These caspases have a very large prodomain and they need molecular adaptors to be enzymatically activated. Caspase 8 and 10 are part of the signal transduction mechanism of apoptosis receptor such as CD95; in this case, the adapter molecular is FADD. Caspase 9 is activated in the apoptosome, and the necessary adaptor molecule is Apaf-1 (apoptosis-activating factor-1). Caspase 2 can be activated

in the PIDDosome complex, so name because of the adapter molecule p53-induced protein with a death domain. However, while these caspases have a long prodomain they can also be cleaved and activated by other caspases, allowing them to be both regulator or effector caspases, depending on the context of activation. Subfamily III consists of caspases 3, 6 and 7. The prototype of this subfamily is caspase 3, the homologue of ced-3 in *Caenorhabditis elegans*. Subfamily III caspases are directly involved in apoptosis in the terminal effector phase. Subfamily III caspases have a very small prodomain, indicating a simple regulation of their enzymatic activation. These caspases are in general activated directly by another caspase in a cascade of enzymatic amplification (Hernandez et al., 2009).

Table 2.2 Subfamily member of caspase family.

Subfamily	Role	Members
I	Inflammatory mediator	Caspase 1 (ICE)
		Caspase 4
		Caspase 5
		Caspase 12
II	Apoptosis activator	Caspase 2
		Caspase 8
		Caspase 9
		Caspase 10
III	Apoptosis executioner	Caspase 3
		Caspase 6
		Caspase 7

Structurally, all caspases are composed of a *N*-terminal pro-domain followed by a large and small subunit. The length of the *N*-terminal pro-domain is an important to determine caspase function and its position in the caspase activation cascade. Caspase with long N-terminal domain are often referred to as initiator, whereas those with short N-terminal domain are termed executioners. Among the

initiator caspases, there are two related yet distinct N-terminal domain types known as the caspase recruitment domain (CARD) and the death effector domain (DED). Through these domains, initiator caspases are able to interface with upstream signaling platforms and undergo activation. Executioner caspases do not possess these domain; therefore, their activation in response to a cell death stimulus must be mediated through an initiator caspase (Walsh and Martin, 2009).

2.4.5 Role of caspases

A hallmark of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The caspases play an important role in this process by activating DNases, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus. The cell inevitably undergoes apoptosis. The role of the caspases in the breakdown of chromatin is illustrated in Figure 2.5.

a) Inactivation of enzymes involved in DNA repair

The enzyme poly (ADP-ribose) polymerase, or PARP, is an important DNA repair enzyme and was one of the first proteins identified as a substrate for caspases. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3 (Tewari et al., 1995).

b) Breakdown of structural nuclear proteins

Lamin A and fodrin are essential components of the nuclear skeleton and cytosolic skeleton, respectively. The degradation of lamin by caspases 6 lead to the chromatins condensation and decomposition of the nuclear membrane. The cleavage of fodrin by caspases result in apoptotic body formation (Fan et al., 2005).

c) Fragmentation of DNA

The fragmentation of DNA into nucleosomal units is caused by an enzyme known as CAD, or caspase activated DNase. In normal cells, CAD resides in the nucleus, binding with its specific inhibitor, ICAD, to form a complex. ICAD is not only the inhibitor but also the molecular chaperone of CAD, essential for the proper folding of CAD. In apoptosis, caspase-9 damages the nuclear pores in an unknown fashion so that caspase-3 can enter the nucleus to cleave ICAD. This releases the CAD from the complex, which can result in DNA degradation (Enari et al., 1998).

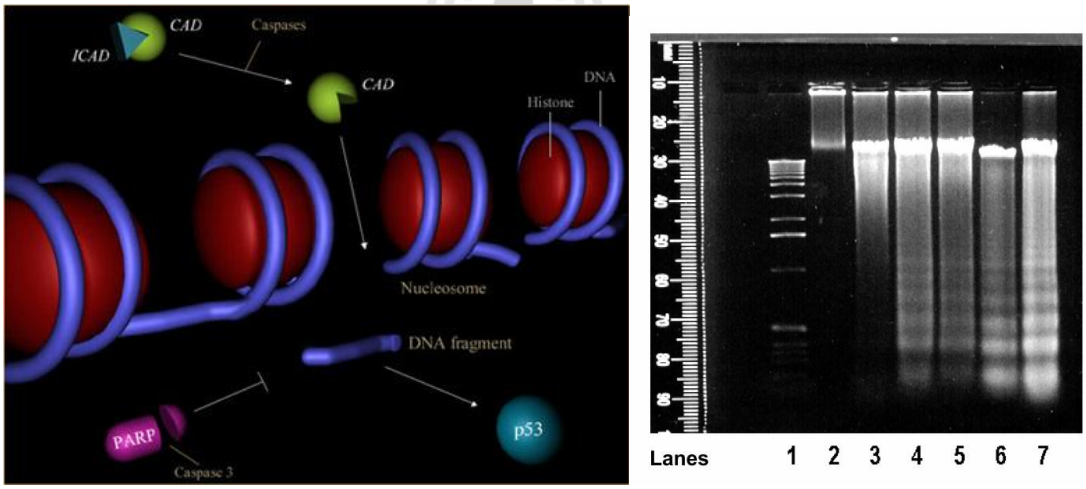

Figure 2.5 Breakdown of chromatin during apoptosis result in DNA fragmentation (Zhang and Bhavnani, 2005).

2.5 Antioxidant defence in carcinogenesis

The effect of reactive oxygen and nitrogen species is balanced by the antioxidant action of non-enzymatic antioxidants, as well as by antioxidant enzymes.

Such antioxidant defences are extremely important as they represent the direct removal of free radicals (pro-oxidants), thus providing maximal protection for biological sites. A good antioxidant should: (i) specifically quench free radicals; (ii) chelate redox metals; (iii) interact with (regenerate) other antioxidants within the antioxidant network; (iv) have a positive effect on gene expression; (v) be readily absorbed; (vi) have a concentration in tissues and biofluids at a physiologically relevant level; (vii) work in both the aqueous and/or membrane domains (Valko et al., 2006). The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase (Mates et al., 1999). Non-enzymatic antioxidants involve vitamin C, vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, a hormonal product of the pineal gland, melatonin and other compounds (McCall and Frei, 1999). Moreover, recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. Besides, epidemiological studies suggest that consumption of fruits and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer (Reddy et al., 1997). Phytochemical products from plants are known to induce apoptosis in neoplastic cells but not in normal cells. It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as isolated components. Evidence has emerged from various studies suggest that products derived from plants are useful in the treatment as well as in the prevention of cancer. Chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention. Understanding the modes of action of these compounds should provide useful

information for their possible application in cancer prevention and perhaps also in cancer therapy.

2.6 *Hyptis suaveolens* (L.) Poit

Hyptis is a genus of Lamiaceae with about 400 species (Willis, 1973). One of the species from genus, *Hyptis suaveolens* (Linn.) Poit belongs to the family Lamiaceae or the mint family. It is a medium aromatic shrub or woody herb distributed in the tropical and subtropical regions. Hyptis is a rigid annual herb of aggressive nature (Mudgal et al., 1997). It starts vegetative phase either from perrenating rootstock or viable seeds that either from persistent seed bank or from fresh stock with the onset of monsoon rains. It attains height of approximately 2.5 meters within a growing season. Its stem is quadrate and bears hair. Leaves are either ovate or obovate. Generally 3-5 cm long and 2-4 cm wide with serrulate margins and a long petiole (Figure 2.6A). Lower surface of the leaves bears hairs, petioles up to 3 cm long. Flowering starts at an early age of two to three months. It produces copious blue flowers in small cymes along branch that ends with reduced leaves. Calyx is hairy in nature and is nearly 5 mm long in flower while it enlarges to 10 mm long in fruit and become ribbed. Corolla is blue, strongly zygomorphic and bilabiate, declinate, and about 8 mm long, with a limb 5 mm in diameter. The flower has 4 stamens (Figure 2.6B). Hyptis flowers are pollinated by a large number of pollinators leading to enormous seed production. Hyptis fruits (nutlets) are about 1.2-1.5 mm long and seeds are protected in spined burr which help in its dispersal and are slightly notched at the end (Figure 2.6C) (Stone, 1970). Seeds of Hyptis are characteristically dimorphic. This dimorphism is reflected in the seed size (Wulff, 1973) and an inverse

relationship of seed size with light requirement has been observed which confers a good spectrum for the seed germination across a range of germination temperature, so seeds are capable of germinating across a range of temperature within 10 ºC to 40 ºC but 25 ºC to 30 ºC seems to be optimum for its growth. Seeds produce mucilage after getting in contact with water. Population expansion takes place by heavy sprouting after rains and through autogamic and allogamic reproduction (Aluri and Reddi, 1989).

Figure 2.6 *Hyptis suaveolens* (A), *Hyptis suaveolens*'s flower (B), *Hyptis suaveolens* 's seed (C) (Xiang, 2012).

Among the species in the genus Hyptis, *H. suaveolens* is a fast-growing perennial herb found in dense clumps along roadsides, in over-grazed pastures and around stockyards in the tropics. The plant gives off a characteristic minty smell when crushed. Originally native to tropical America, it is now considered a worldwide weed (Chukwujekwu et al., 2005). Almost all parts of *H. suaveolens* are being used in traditional medicine to treat various diseases such as gastrointestinal disorders, respiratory tract infections, colds, pain, fever, cramps, and skin diseases (Grassi et al.,

2006). Leaves of of *H. suaveolens* have been utilized as a stimulant, carminative, sudorific, galactogogue, and as a cure for parasitic cutaneous disease (Mandal et al., 2007). Crude leaf extract is also used as a relief to colic and stomachache. Leaves and twigs are considered to be antispasmodic and use in antirheumatic and antisuporific baths (Kirtikar and Basu, 1991). The decoction of the roots is highly valued as appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor (Chatterjee and Pakrashi, 1997). The fumes of *Hyptis suaveolens* dried leaves are used as an insectifuge to repel mosquitoes and control insect pests of stored grains (Ijeh et al., 2007) and possesses larvicidal activity against *Aedes aegypti* (Yongkhamcha and Indrapichate, 2012) and *Culex quinquefasciatus* (Arivoli and Tennyson, 2011). *H. suaveolens* also control the aflatoxin B1 production (Krishnamurthy and Shashikala, 2006). The ethanol extracts of leaves of *Hyptis suaveolens* revealed the presence of alkaloids, glycoside, saponin, tannins and flavonoids as major active constituents (Shaikat et al., 2012). Phytochemical studies of this genus have shown that the constituents include hyptadienic acid, suaveolic acid, suaveolol, methyl suaveolate, β-sitosterol, oleanolic acid, ursolic acid, rosamarinic acid, dehydroabietinol, 3β-hydroxylup-12-en-28-oic acid, 3β-hydroxylup-20(29)-en-27-oic acid, and essential oils (Satish et al., 2010). Suaveolol, isolated from *H. suaveolens*, have shown gastroprotective activity (Vera-Arzave, 2012). The major chemical compositions in *H. sauveolens* oil, 1,8-cineole and β-caryophyllene, are shown in Table 3 (Luz et al., 1984; Peerzada, 1997; Tripathi et al., 2009). Azevedo et al. (2001) reported that the essential oils of *H. sauveolens* are sabinene, limonene, biclyclogermacrene, β-phellandrene and 1,8-cineole. Oliveira et al. (2005) also reported that the compositions of essential oil of *H. sauveolens* is 1,8-cineole, (E)-caryophyllene, spathulenol.

Table 2.3 Components of essential oil of *Hyptis suaveolens* (L.) Poit identified by GC-MS analysis (Tripathi et al., 2009).

Peak no.	Components	Percentage in total oil
1	α-Thujene	0.8
2	β-Carene	1.1
3	α-Pinene	2.5
4	β-Pinene	11.7
5	β-Myreene	5.3
6	**1,8-Cineole**	**44.4**
7	Linalool	1.7
8	Camphor	1.1
9	Camphene	5.7
10	Terpinen-4-ol	2.2
11	α-Cubebene	0.3
12	β-Gurjunene	1.6
13	β-Elemene	1.2
14	β-Caryophyllene	10
15	α-Farnesene	2.8
16	β-Selinene	0.9
17	Longifolene	0.4
18	γ-Humulene	0.5
19	α-Copaene	0.6
20	γ-Codinene	0.3
21	α-Bergamolene	1.7
22	Unidentified	1.6
23	Unidentified	0.8
24	Unidentified	0.7

Moreover, the plant has been reported to possess antimicrobial (Asekun et al., 1999), antifungal (Zollo et al., 1997), antiplasmodial (Chukwujekwu, 2005), antimalarial (Ziegler et al., 2002), antinociceptive (Santos et al., 2007), antidiarrhoeal (Shaikat et al., 2012), anti-hyperglycemic (Mishra et al., 2011) and anti-inflammatory activities (Mahesh, 2001). The plant also possesses antirheumatic, antifertility, antiseptic (Chitra et al., 2009), antiparasitic (Dalziel, 1937), antifungal (Pandey et al., 1982; Singh et al., 1992), anticancer (Gurunagarajan and Pemaiah, 2011),

antibacterial, antioxidant and antimicrobial properties (Iwu et al., 1990; Asekun et al., 1999; Annie et al., 2003; Nantitanon et al., 2007). The plant shows insect repellent and insecticidal activities (Tripathia and Upadhyay, 2009; Buatone and Indrapichate, 2011; Benelli et al., 2012). *H. suaveolens* extracts are used effectively to control cowpea borer, *Maruca testulalis* (Gbehounou, 2007) and *Trogoderma granarium* (Musa et al., 2009). Additionally, the aqueous extract of *H. suaveolens* obtained from leaves exhibits low acute or chronic toxicity (Attawish et al., 2005).

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CHAPTER III

PHYTOCHEMICAL STUDIES AND ANTIOXIDANT PROPERTIES OF MINTWEED (*HYPTIS SAUVEOLENS* (L.) POIT) EXTRACTS

3.1 Abstract

Mintweed leaf ethanolic and water extracts (MLE/e, MLE/w), mintweed seed ethanolic and water extracts (MSE/e, MSE/w) were determined for total phenolic compounds (TPC), flavonoid content (TFC) and phytochemical availability by thin layer chromatography (TLC) and specific detecting reagents. The antioxidant activities were assayed by ferric reducing power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and thiobarbituric acid reactive substance (TBARS) methods. The results demonstrated that the MLE/e contained high TPC and TFC of 370.07 ± 7.10 mg GAE/g dried extract and 278 ± 3.40 mg CAE/g dried extract, respectively. Phytochemical analysis of the mintweed extracts showed that phenolics, antioxidants, terpenoids, essential oils, tannins and alkaloids were presented in the mintweed extracts whereas coumarins, cardiac glycosides and antraquinones were not detected. MLE/e exhibited ferric reducing activity of 8.52 ± 0.44 µmol $FeSO_4$/mg dried extract and MLE/e also possessed radical scavenging activity with IC_{50} of 9.26 ± 0.08 µg/mL. Moreover, MLE/e inhibited lipid peroxidation with IC_{50} of 5.42 ± 0.14 µg/mL. The

present work gives additional evidence that this plant is a potential source of many biologically active compounds.

3.2 Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. At low to moderate concentrations, they possess various physiological roles ranging from cellular signal transduction to defence against pathogens (Valko et al., 2007). However, during oxidative stress, there is an overproduction of ROS and RNS on one side and a deficiency of enzymatic and nonenzymatic antioxidant defence system on the other, resulting in degradation of cellular components such as DNA, carbohydrates, proteins and lipids. This will eventually lead to cellular dysfunction and cell death (Valko et al., 2007). Free radicals and oxidants can trigger lipid peroxidation, as well as the oxidation of proteins and DNA, causing extensive damages to cells. Oxidative stress resulted from an imbalance of oxidising species and natural antioxidants in the body has been thought to contribute to aging, cell apoptosis, and severe diseases such as cancer, Parkinson's disease, Alzheimer's disease, and even cardiovascular disorders (Giasson et al., 2002). Epidemiological studies and intervention trials on prevention of cancers and cardiovascular diseases in people taking antioxidant supplements suggested that dietary intake of antioxidants can help scavenge free radicals and oxidants and protect the body against diseases (Frei, 1994). Plants and their products are long used as sources of medicine. Recently, the medicinal properties of plants have been scientifically investigated throughout the world, due to their potent antioxidant activities, no side effects and economic a viability (Auddy, 2003). Many, medicinal

plants have become importance for the treatment of different disease conditions, such as diabetes, malaria, and anaemia (Fola, 1993). There is increasing interest in the biochemical function from natural antioxidant extracts from vegetables, fruits and medicinal plants, which can become candidates to prevent oxidative damage, and promoting health. Many medicinal plants contain large amounts of antioxidants other than vitamin C and E and carotinoids (Javanmardi et al., 2003). Antioxidants are molecules that can delay or prevent an oxidative reaction (Velioglu et al., 1998) catalysed by free radicals. This antioxidant effect is mainly due to the presence of phenolic components such as flavonoids (Pietta, 1998), phenolic acids and phenolic diterpenes (Shahidi et al., 1992). Antioxidants such as BHA (Butylated Hydroxy-Anisol), BHT (Butylated Hydroxy-Toluene) protect plants against oxidative assault (Dziezak, 1986) either by binding to metallic ions, eliminating free radicals or decomposing peroxides (Matook, 2005). However, BHA and BHT are now doubted for their safety (Grice, 1986). Thus, attention is now increasingly paid to the development and utilization of more effective and non-toxic antioxidants, of natural origin. A great number of natural medicinal plants have been tested for their antioxidant activities. It shown that raw extracts or isolated pure compounds from them were more effective antioxidants *in vitro* than BHT or vitamin E (Gu and Weng, 2001; Pyo et al., 2004). So, medicinal plants can be a potential source of natural antioxidants.

Mintweed, *Hyptis sauveolens* (L.) Poit, commonly known as “maeng lak kha” in Thailand, is used for traditional medicine for the treatment of various illness such as respiratory track, infection, colds, pain, fever, cramps, and skin diseases (Chukwjekwu et al., 2005). In addition, the leaves of *H. sauveolens* are used as a

stimulant, carminative, sudorific, galactogogue, and as a cure for parasitic cutaneous disease (Mandal et al., 2007). The phytoconstituents isolated from the plant are hyptadienic acid (Raja et al., 1990), suaveolic acid, suaveolol, methyl suaveolate, β-sitosterol, oleanolic acid, ursolic acid, rosamarinic acid (Manchard et al., 1974), dehydroabietinol (Ziegler et al., 2002), 3β-hydroxy lup-12-en-28-oic acid (Misra et al., 1983a), 3β-hydroxyl lup-20(29)-en-27-oic acid (Misra et al., 1983b) and essential oil (Peerzada, 1997). Moreover, the plant has been reported to possess antimicrobial (Asekun et al., 1999), antifungal (Zollo et al., 1997), antiplasmodial (Chukwujekwu, 2005), antimalarial (Ziegler et al., 2002), antinociceptive (Santos et al., 2007), antidiarrhoeal (Shaikat et al., 2012), anti-hyperglycemic (Mishra et al., 2011) and anti-inflammatory activities (Mahesh, 2001). The present study investigated the phytochemical properties and antioxidant activities of mintweed (*Hyptis sauveolens*) extracts by using ferric reducing ability power (FRAP), the stable DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical and the thiobarbituric acid reactive substance (TBARS) assay.

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 ($DPPH^{\bullet}$), and thiobarbituric acid 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 Collection of plant materials

Mintweed leaves were collected from the plants grown on the grounds of Suranaree University of Technology (SUT) in Nakhon Ratchasima, Thailand in October 2008. The leaves were washed under running tap water and sun dried. Mintweed seeds were collected in January 2009 at the same place. The seed mucilage was sloughed off and then the seeds were air dried. This plant was identified by a plant taxonomist, Professor Dr. Pranom Chantaranothai.

3.3.3 Sample preparations

The dried leaves and seeds were ground into fine powder by electric blender and then 50 grams of each plant powder was extracted in 500 ml water or 70% ethanol (v/v) for 24 h by Soxhlet extraction apparatus. 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 powder extracts were stored at -20°C until used. The water and the

ethanolic extracts were dissolved in RPMI 1640 culture medium and 0.2% DMSO, final concentration for cell culture.

3.3.4 Determination of phytochemical properties

3.3.4.1 Total phenolic compounds

The total phenolic compounds (TPC) of mintweed extracts were quantified by the folin-ciocalteu assay and followed the method described by Matthaus (2002). One hundred microliters of samples were mixed with 2 ml of 2% Na_2CO_3 and incubated at room temperature for 2 min. After the addition of 100 µl of folin- ciocalteu reagent (diluted with methanol, 1:1 v/v), the reaction was further incubated for 30 min at room temperature. The absorbance was measured at 750 nm using a spectrophotometer (CE1011, Cecil Instruments, Cambridge, England). The concentration of samples was calculated using gallic acid as a standard with a concentration rang of 50-300 µg/mL. The results were expressed as mg gallic acid equivalent (mg GAE) per mg dried extracts. All experiments were performed in triplicate.

3.3.4.2 Total flavonoid contents

The total flavonoid content of mintweed extracts was evaluated by reacting with aluminium chloride ($AlCl_3$) using the method described by Liu et al. (2002). Briefly, samples with a concentration of 5 mg/mL were dissolved in their original solvents. Two hundred and fifty microliters of sample were mixed with 1.25 mL of distilled water and then 75 µL of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150 µL of a 10% $AlCl_3.6H_2O$ solution was added and the

mixture was allowed to stand for 5 min. Five hundred microliters of 1M NaOH were added and the total volume was made up to 2.5 mL with distilled water. The absorbance was read at 510 nm using spectrophotometer. Catechin was used as standard. Flavonoid content was expressed as mg of catechin equivalent (mg CA) per mg dried extracts. The experiment was done in triplicate. Data are reported as mean ± SD.

3.3.4.3 Phytochemical screening of the extracts

a) Thin layer chomatography (TLC)

TLC was performed using silica gel 60 F_{254} 0.25 mm precoated plates (Merck, Darmstadt, Germany). The mintweed extracts were redissolved in original solvents of 70% (v/v) ethanol and water, respectively. The volume of 0.5 µL of each extract with the concentration of 20 mg/mL was spotted onto the TLC plate (10 times/spot). The TLC plate was conducted in mobile phases of ethyl acetate: methanol: water (81:11:8), depending on polarity of detected compounds. After a separation is complete, individual compounds appear as spots separated vertically. Each spot has a retention factor (R_f) which is the migration of compounds in a given TLC system was described as the following. The TLC plates with the extracts were observed under UV lamps at 254 and 366 nm and sprayed with specific reagents to detect some phytochemicals (Woraratphoka et al., 2012); (a) potassium hydroxide (KOH) reagent for an anthraquinones (red), anthones (yellow, UV-366 nm) and coumarins group (blue-green, UV-366 nm), coumarins was used as a standard, (b) Dragendorff's reagent for an alkaloids group, (c) anisaldehyde/sulfuric acid (H_2SO_4) reagent, eugenol was used as a standard, (d) vanillin/sulfuric (H_2SO_4) reagent for essential oils, steroid, terpenoids and phenols, estradiol was used as a standard, (e)

iron (III) chloride reagent for phenolic compounds including tannins, catechin was used as a standard, (f) DPPH reagent for an antioxidants, trolox was used as a standard, and (g) Kedde reagent for cardic glycosides. The detail protocols of sprayed reagents preparation were summarized in Appendix A.

$$R_f = \frac{Compound\ distance\ from\ origin\ (cm)}{Sovent\ front\ distance\ from\ origin\ (cm)}$$

b) Testing with specific reagents

A qualitative phytochemical test to detect the presence of terpenoids, tannins, alkaloids, and saponins was carried out using the method described by Sofowora, 1993; Trease and Evans, 1989; and Harborne, 1973. The intensity of the coloration determines the abundance of the compounds present.

1) Terpenoids test by Salkowski test

Mintweed leaf ethanolic and water extracts (0.05 g) and mintweed seed ethanolic and water extracts (0.2 g) were mixed with 2 mL of chloroform ($CHCl_3$). Three milliliters of concentrated H_2SO_4 were carefully layered on. A reddish brown coloration of the interface was formed indicating positive results of terpenoids.

2) Tannins test by Ferric chloride test

The powder extracts (0.2 g) were boiled in 4 mL of deionized water for 10 min. The mixture was filtered and few drops of 5% ferric chloride were added to the filtrate. A brownish green or blue-black coloration indicates the presence of tannins.

3) Alkaloids test by Dragendorff reagent

The powder extracts (0.2 g) was mixed with 3 mL in 2% H_2SO_4. Then, the mixture was filtered and few drops of Dragendorff reagent were added to the filtrate. Orange red precipitate indicates the presence of alkaloids.

4) Saponins test by Frothting test

The powder extracts (0.2 g) were mixed with 4 mL of deionized water and warmed for 10 min. The mixture was filtered and vigourously shaken for a stable persistent froth. To confirm the presence of saponins, 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).

3.3.5 Determination of antioxidant activities

3.3.5.1 Ferric reducing ability power (FRAP)

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain (1996). The FRAP assay was used to measure the total concentration of antioxidants. The principle of this method is based on the reduction of a ferric-2,4,6-tri(2-pyridyl)-striazine [Fe(III)-TPTZ] complex to its ferrous 2,4,6-tri (2-pyridyl) -s-triazine [Fe(II)-TPTZ] complex color formed in the presence of antioxidants (Figure 3.1).

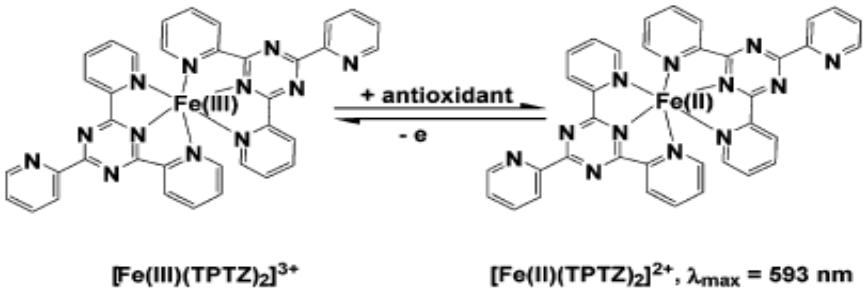

Figure 3.1 Chemical reaction of FRAP assay (Prior et al., 2005).

The FRAP reagent was freshly prepared by mixing of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) dissolved in 40 mM HCl, 20 mM $FeCl_3$ solution and 0.1 M acetate buffer (pH 3.6) in proportion 1:1:10 (v/v/v). Fifty microliters of sample were mixed with 1.5 mL of FRAP reagent for 10 min. The absorbance was measured at 593 nm. FRAP values were obtained by comparing with standard curve of 100 - 1000 µmol $FeSO_4.7H_2O$ and reported as µmol $FeSO_4.7H_2O$ equivalent per mg dried extract.

3.3.5.2 Free radical scavenging capacity

The stability of 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical is widely used as the model system to investigate the scavenging activities of several natural compounds or crude extracts of plants. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent (Nikhat et al., 2009). The scavenging reaction between DPPH and an antioxidant (H-A) are shown in Figure 3.2.

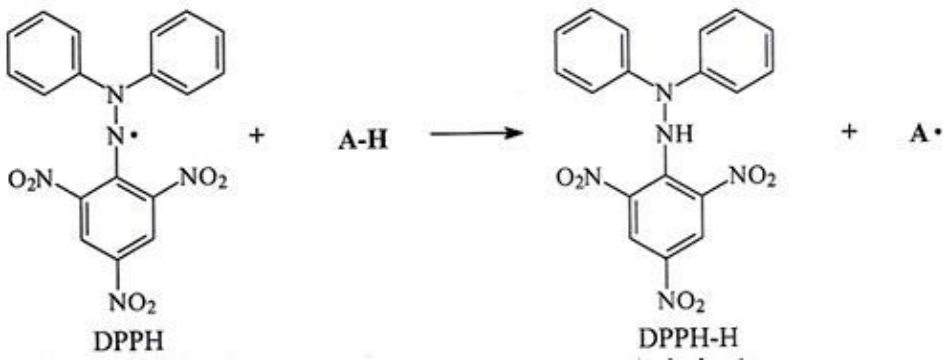

Figure 3.2 Structure of DPPH before and after reaction with antioxidant (A-H) (Halliwell and Gutteridge, 2007).

Antioxidants react with DPPH•, which is a stable free radical, and DPPH• is reduced to the DPPH-H and as a consequence, decreasing the absorbance of the DPPH• radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability (Oktay et al., 2003). The effect of sample on the DPPH radicals was estimated according to the method of Sanchez-Moreno et al. (1999) with some modification. The various concentrations of samples in 50 µL were mixed with 1.95 mL of 40 mg/L methanolic DPPH• solution. The mixtures were vigorously shaken and left to stand at room temperature for 45 min in the dark. The absorbance was read at 515 nm. Methanol was used as blank while the DPPH• solution in methanol was used as control. L-ascorbic acid and catechin were used as standard controls. The experiments were carried out in triplicate. The results were expressed as percentage inhibition as the following formula:

$$\%\ inhibition = \left(\frac{A\ control - A\ sample}{A\ control} \right) \times 100$$

Where $A_{control}$ and A_{sample} are the absorbance values of the control and test sample, respectively. The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50}) was obtained and expressed as µg dried extract per mL of a reaction assay.

3.3.5.3 Measurement of the antioxidant activity by inhibition of lipid peroxidation (LPO)

The thiobarbituric acid reactive substances (TBARS) assay is used to quantify oxidative stress by measuring the peroxidative damage to lipids that occurs with free radical generation. Free radical damage to lipids results in the production of malondialdehyde (MDA), which reacts with TBA under conditions of high temperature (90°C to 100°C) and acidity generating a chromogen that can be measured either spectrophotometrically at 530-540 nm or spectrofluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm (Figure 3.3) (Oakes and Van Der Kraak, 2003).

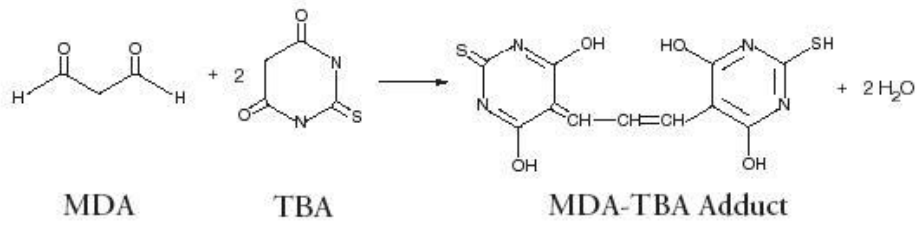

Figure 3.3 Thiobarbituric acid reaction (Gerard-Monnier, 1997).

The TBARS assay using phosphatidylcholine liposome was determined by the method described by Gonzalez-Paramas et al. (2004) with some modification. The extracts at different concentrations were added into the reaction mixture containing 150 mM KCl, 0.2 mM $FeCl_3$, and 1 mg/mL of phosphatidylcholine liposome (final concentration). Peroxidation was started by adding sodium L-ascorbate at a final concentration of 0.05 mM in a final volume of 0.4 mL. Samples were incubated at 37 °C for 60 min and the reaction was stopped by addition of 0.4 mL of 20% trichloroacetic acid (TCA), 0.01% of butylated hydroxytoluene (BHT) and 0.25 N HCl and 0.4 mL of 0.8% thiobarbituric acid (TBA). The mixtures were then incubated at 80°C for 30 min for pink color development, cooled with tap water and centrifuged for 10 min at 5000 rpm. The production of TBARS was measured at 535 nm. LPO inhibition was calculated from the malondialdehyde (MDA) formed in the presence of the mintweed extract with relative to the control as following formula:

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

Where $A_{control}$ and A_{sample} are the absorbance values of the control and test sample, respectively. The Fe^{2+} and L-ascorbate induced liposome without sample was used as control, while un-induced liposome was used as a blank. LPO inhibition (%) against various concentrations of the mintweed extract was plotted and calculated for $IC_{50.}$

3.3.6 Statistical analysis

All experiments were carried out in triplicates and the data were analyzed by ANOVA followed by the Duncan's Multiple Range Test to determine significant differences between groups at $p < 0.05$.

3.4 Results and Discussion

3.4.1 Total phenolic and flavonoid contents of mintweed extracts

Total phenolic compounds and flavonoid contents of mintweed extracts are shown in Table 3.1. Total phenolic compounds of the extracts were from 370.02 ± 7.10 to 77.02 ± 2.05 mg GAE/g dried extract. This study showed that total phenolic compounds in the mintweed extracts were ranged as MLE/e (370.02 ± 7.10 mg GAE/g dried extract) > MLE/w (319.45 ± 8.67 mg GAE/g dried extract) > MSE/e (135.92 ± 2.17 mg GAE/g dried extract) > MSE/w (77.02 ± 2.05 mg GAE/g dried extract). The highest amount of total phenolic compounds was observed in MLE/e and the lowest amount was observed in MSE/w. Total flavonoid contents of the extracts were ranged as MLE/e (278.81 ± 3.40 mg CAE/g dried extract) > MLE/w (240.81 ± 5.01 mg CAE/g dried extract) > MSE/e (86.28 ± 0.67 mg CAE/g dried extract) > MSE/w (15.38 ± 0.21 mg CAE/g dried extract) (Table 3.1). There were significant difference ($p < 0.05$) amount of all the extracts in total phenolic and flavonoid contents.

Both leaf ethanolic and water extracts contained much significantly higher amounts of both phenolic compounds and flavonoid contents than seed extract. Polyphenols are the principal antioxidant agents in natural products from plants (Kahkonen et al., 1999). They are potent free radical terminators (Shahidi et al.,

1992). They donate hydrogens to free radicals, hence, break the reaction of lipid oxidation at the initiation step (Gulçin et al., 2004). Thus, high polyphenolic content will mean a strong antioxidant power and a strong radical scavenging activity. However, this is not always the case since plant tissues are often made up of different matrix that may react differently with changes of chemicals/reagents or reaction mechanisms.

Table 3.1 Total phenolic compounds and total flavonoid contents of mintweed (*H. sauveolens)* extracts. Data were mean ± SD., (n = 3).

Extract	Total phenolic mg GAE/g dried extract	Total flavonoid mg CAE/g dried extract
MLE/e	370.07 ± 7.10^{a}	278.81 ± 3.40^{a}
MLE/w	319.45 ± 8.67^{b}	240.81 ± 5.01^{b}
MSE/e	135.92 ± 2.17^{c}	86.28 ± 0.67^{c}
MSE/w	77.02 ± 2.05^{d}	15.38 ± 0.21^{d}

Different letters within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

3.4.2 Phytochemical screening of mintweed extracts

3.4.2.1 Thin layer chromatography (TLC) fingerprinting

TLC plate was used for determining of phytochemical availability in the mintweed extracts. After TLC plate was developed with the mobile phase, EtOAc: MeOH: H_2O (81:11:8), the plate was completely dried visualized under UV lamp or daylight. Compounds with native fluorescence were viewed as bright zones on a dark background under UV light at short wavelength (254 nm) and long

wavelength (366 nm). TLC fingerprints of the mintweed extracts were visualized under UV-254 nm and UV-366 nm. Their R_f values were shown in Figure 3.4 and Table 3.2. Compounds that absorbed at 254 nm, including most compounds with aromatic rings and conjugated double bonds and some unsaturated compounds, such as anthraquinones, coumarins, flavonoids and polyphenols, were detected as dark violet spots on a bright green background (Janchen, 1991). TLC profiles of the mintweed extracts under UV-254 nm (Figure 3.4A) demonstrated that all extracts contained compound with conjugated double bonds, polyphenolic compounds. In addition, all mintweed extracts present blue fluorescence under UV-366 nm, while red fluorescence was observed only in MLE/e with R_f of value 0.79 (Figure 3.4B).

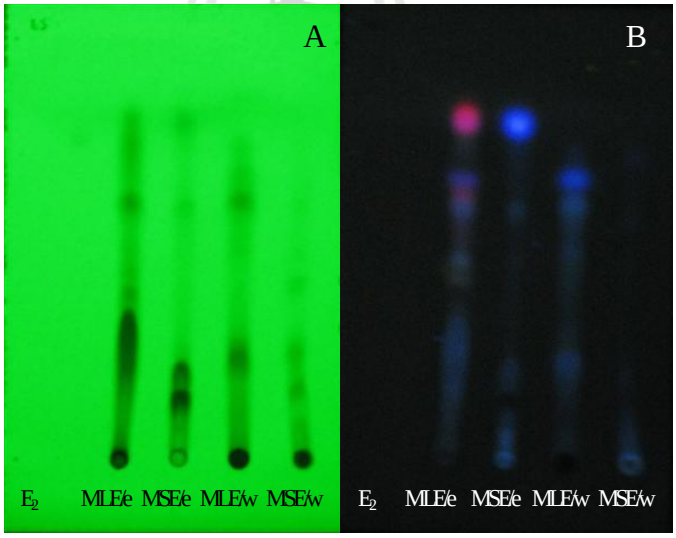

Figure 3.4 TLC fingerprints of mintweed extracts were developed in ethyl acetate: methanol: water (81:11:8) and visualized under UV light: A, 254 nm; B, 366 nm. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract; E_2, estradiol.

Table 3.2 R_f values of TLC fingerprints of mintweed extracts visualized under UV-254 and UV-366 nm.

TLC Detection	R_f of extracts			
	MLE/e	MSE/e	MLE/w	MSE/w
UV 254 nm	0.79 (LD)	0.79 (LD)	0.60 (B)	0.42 (LD)
	0.74 (LD)	0.60 (LD)	0.23 (B)	0.25 (LD)
	0.72 (B)	0.21 (B)		0.16 (LD)
	0.44 (LD)	0.14 (B)		
	0.39 (LD)			
UV 366 nm	0.79 (LR)	0.79 (LBl)	0.65 (LBl)	0.06 (Bl)
	0.65 (Bl)	0.58 (Bl)	0.58 (Bl)	
	0.62 (P)	0.21 (Bl)	0.47 (Bl)	
	0.58 (Bl)	0.06 (Bl)	0.23 (Bl)	
	0.45 (Bl)			
	0.42 (G)			
	0.32 (Bl)			

(LD), light dark; (B), black; (LR), light red; (Bl), blue; (R), red; (G), grey; (LBl), light blue. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract.

TLC plates of the mintweed extracts were sprayed with KOH reagent and Dragendorff's reagent as showed in Figure 3.5 and the R_f values were demonstrated in Table 3.3. KOH reagent was used to detect the anthraquinones (red and fluorescense intent red under UV-366 nm), anthrones (yellow and more bright fluorescence yellow under UV-366 nm) and coumarins (bright blue fluorescence). The coumarins was used as standard (green) with R_f values of 0.75 (Figure 3.5A and Table 3.3). No green or yellow spot appeared in the TLC profile of the extracts (Figure 3.5A). These could be all of the mintweed extracts did not have anthraquinones, anthrones, and coumarins. The TLC plate of the mintweed extracts after being sprayed with Dragendorff reagent, there was no brown or orange spot on

the yellow background in visible light (Figure 3.5B and Table 3.3), inducating the absence of alkaloids.

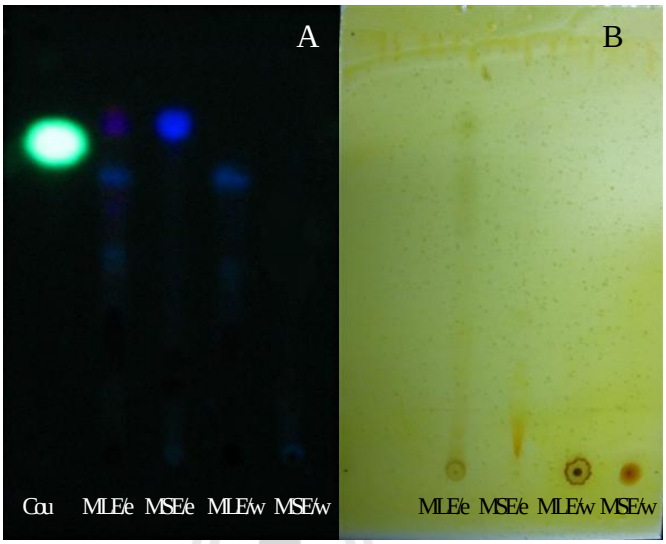

Figure 3.5 TLC fingerprints of mintweed extracts were developed in ethyl acetate: methanol: water (81:11:8) and sprayed with specific reagents: A, KOH reagent under UV-366 nm; B, Dragendorff's reagent. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract; Cou; coumarin.

Table 3.3 R_f values of TLC fingerprints of mintweed extracts detected by KOH and Dragendorff reagent.

TLC Detection	R_f of extracts			
	MLE/e	MSE/e	MLE/w	MSE/w
KOH	0.79 (P) 0.65 (Bl)	0.79 (Bl)	0.65 (Bl) 0.43 (Bl)	-
Coumarin 0.75 (LGr)	0.58 (DBl) 0.47 (LBl)			
Dragendorff	-	0.05 (CYL)	-	0.00 (CYD)

(P), purple; (Bl), blue; (DBl), dark blue; (LBl), light blue; (Bl), blue; (LGr), light green; (CYL), cadmium yellow light; (CYD), cadmium yellow deep. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract.

To determine hydrophobic compounds such as essential oils and terpenoids, the system of ethyl acetate: methanol: water (81:11:8) was used as a mobile phase. TLC plate of the mintweed extracts was sprayed with anisaldehyde/ H_2SO_4 reagent and vanillin/H_2SO_4 reagent as showed in Figure 3.6A and the R_f values were demonstrated in Table 3.4. Anisaldehyde/ H_2SO_4 reagent was used to detect the essential oils (purple or red). Eugenol was used as the standards of phenyl essential oils with R_f value of 0.83 (Figure 3.6A and Table 3.4). Essential oils was found in MLE/e, MSE/e and MLE/w with different levels. Vanillin/H_2SO_4 reagent was used to detect the terpenes (red and blue). Estradiol was used as the standards of sterol triterpenoid with R_f value of 0.79. Terpenoids was found in all the mintweed extracts with different levels after spraying with vanillin/H_2SO_4 reagent (Figure 3.6B and Table 3.4).

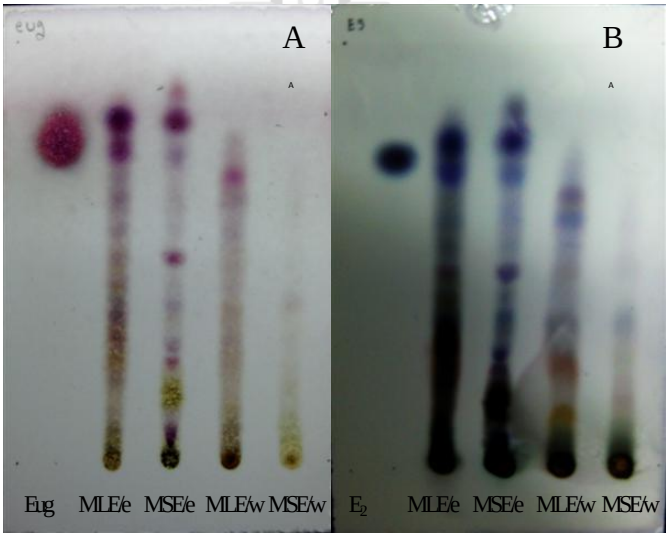

Figure 3.6 TLC fingerprints of mintweed extracts were developed in ethyl acetate: methanol:water (81:11:8) and sprayed with specific reagents: A, anisaldehyde/H_2SO_4 reagent; B, vanillin/H_2SO_4 reagent. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract; Eug, eugenol; E_2, estradiol.

Table 3.4 R_f values of TLC fingerprints of mintweed extracts detected by anisaldehyde/ H_2SO_4 and vanillin/H_2SO_4 reagent.

TLC Detection	R_f of extract			
	MLE/e	MSE/e	MLE/w	MSE/w
Anisaldehyde	0.79 (V)	0.88 (P)	0.69 (LPu)	-
	0.74 (Pu)	0.79 (Pu)	0.63 (LPu)	
	0.63 (LPu)	0.71 (LPu)	0.54 (LPu)	
	0.56 (LPu)	0.48 (Pu)		
	0.50 (LPu)	0.38 (LPu)		
	0.04 (Br)	0.28 (LPu)		
	0.38 (Br)	0.24 (Pu)		
	0.35 (PGr)	0.16 (Y)		
Eugenol	0.31 (Bl)	0.06 (Pu)		
0.83 (Pu)	0.25 (Br)			
Vanillin	0.79 (NBl)	0.86 (DBl)	0.65 (LV)	0.33 (LBl)
	0.70 (DBl)	0.79 (NBl)	0.58 (Bl)	0.25 (PG)
	0.60 (G)	0.70 (Bl)	0.47 (PG)	0.23 (LP)
	0.54 (G)	0.48 (Pu)	0.36 (G)	0.05 (Gr)
	0.47 (DG)	0.40 (G)	0.52 (Br)	
	0.43 (DG)	0.35 (Bl)	0.14 (Y)	
		0.31 (G)		
Estradiol		0.27(NBl)		
0.77 (NBl)		0.23 (LV)		

(V), violet; (LV), light violet; (Pu), purple; (LPu), light purple; (Bl), blue; (NBl), nevy blue; (DBl), dark blue; (LBl), light blue; (Br), brown; (Gr), green; (PGr), pale green; (P). pink; (LP), light pink; (Y), yellow; (G),grey; (DG), dark grey; (PG), pale grey. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract.

TLC plates of the mintweed extracts were sprayed with $FeCl_3$ reagent, DPPH reagent and Kedde reagent as showed in Figure 3.7 and the R_f values were demonstrated in Table 3.5. $FeCl_3$ reagent, catechin, was used as standard reference for phenolic compound with R_f value of 0.67 (Figure 3.7A and Table 3.5). All mintweed extracts showed dark spots when sprayed with $FeCl_3$ reagent, representing the present of the phenolic compounds and tannins (Figure 3.7A).

DPPH reagent, torlox, was used as a standard reference for antioxidant compound with R_f value of 0.60 (Figure 3.7B and Table 3.5). All mintweed extracts showed yellow spots on the purple background when sprayed with DPPH reagent representing the antioxidant compounds. Moreover, there was three extracts which were MLE/e, MSE/e and MLE/w, showed the same R_f value of trolox with R_f 0.60 (Figure 3.7B). Cardiac glycosides, appeared pink, red or purple in visible light after sprayed with Kedde reagent, was also not detected in all mintweed extracts (Figure 3.7C and Table 3.5). When comparing two different compounds ran under identical chromatography conditions, polar compounds had a higher affinity for the sorbent, and slowly moved up on the plate as the solvent migrates. These compounds had relatively small R_f values. Conversely, nonpolar compounds had less affinity for the stationary phase, they comparatively moved up quickly in the plate, and therefore had relatively larger R_f values (Gibbons and Gray, 1998). The R_f can provide corroborative evidence as to the identity of a compound. For DPPH reagent, trolox was used as a standard and ran on a TLC plate side by side with the extracts. It was noticed that three extracts, MLE/e, MSE/e and MLE/w, had the same R_f values of trolox with 0.60 (Figure 3.7B and Table 3.5). If two substances have the same R_f value, they are likely to be the same compound. If they have different R_f values, they are definitely different compounds.

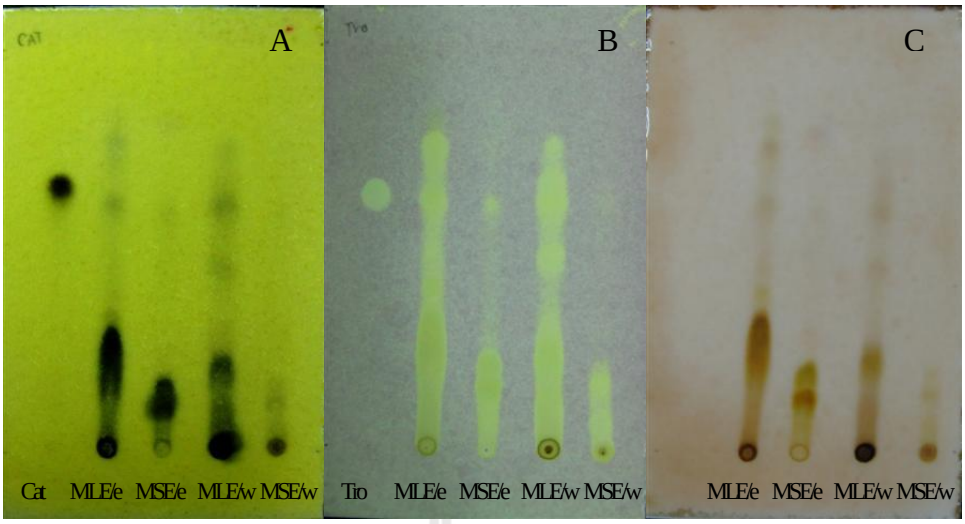

Figure 3.7 TLC fingerprints of mintweed extracts were developed in ethyl acetate: methanol: water (81:11:8) and sprayed with specific reagents: A, FeCl3 reagent; B, DPPH reagent; C, Kedde reagent. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract; Cat, catechin; Tro, trolox.

Table 3.5 R_f values of TLC fingerprints of mintweed extracts detected by $FeCl_3$, DPPH and Kedde reagents.

TLC Detection	R_f of extracts			
	MLE/e	MSE/e	MLE/w	MSE/w
$FeCl_3$	0.77 (DB)	0.23 (B)	0.60 (DB)	0.21 (DB)
Catechin	0.60 (DB)	0.12 (DB)	0.46 (DB)	
0.67 (B)			0.21 (B)	
DPPH	0.73 (Y)	0.60 (Y)	0.75 (Y)	0.07 (Y)
	0.60 (Y)	0.20 (Y)	0.66 (Y)	0.13 (Y)
Trolox		0.14 (Y)	0.60 (Y)	
0.60 (Y)			0.46 (Y)	
Kedde	0.79 (PBr)	0.25 (Y)	0.65 (PBr)	0.15 (Br)
	0.73 (PBr)	0.13 (Br)	0.44 (PBr)	
	0.60 (PBr)		0.21 (Br)	
	0.44 (PBr)			
	0.37 (Br)			

(DB), diluted black; (B), black. (Y), yellow; (Br), brown; (PBr), pale brown. MLE/e, mintweed leaf ethanolic extract; MSE/e, mintweed seed ethanolic extract; MLE/w, mintweed leaf water extract; MSE/w, mintweed seed water extract.

Isolation of pure, pharmacologically active constituents from plants takes a long and tedious process. For this reason, it is necessary to have methods available which eliminates unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential biological activities. This procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is thus economically very important. Thin layer chromatography (TLC) is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment (Peteros and Uy, 2010).

3.4.2.2 Specific reagent testing of mintweed extracts

1) Terpenoids by Salkowski test

Salkowski test was used for qualitative detection of terpenoids in the extracts. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids. All mintweed extracts, MLE/e, MLE/w, MSE/e and MSE/w showed a reddish brown coloration of the interface (Figure 3.8).

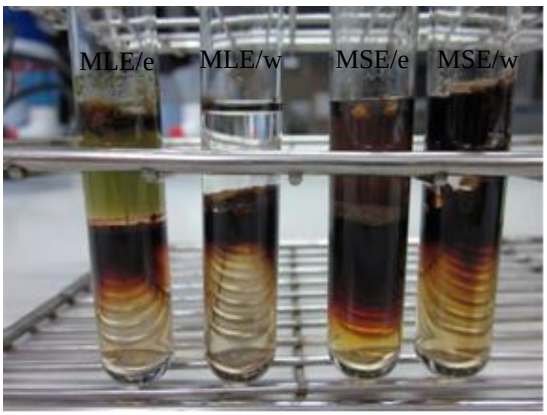

Figure 3.8 Terpenoids detection in mintweed extracts by Salkowski test. MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

2) Tannins by Ferric chloride test

A few drops of 5% ferric chloride was added to the mintweed extracts, blue-black coloration was observed in MLE/e, MLE/w and MSE/e while, no blue-black coloration in the MSE/w. A browrish green or a blue-black coloration showed for the presence of tannins (Figure 3.9).

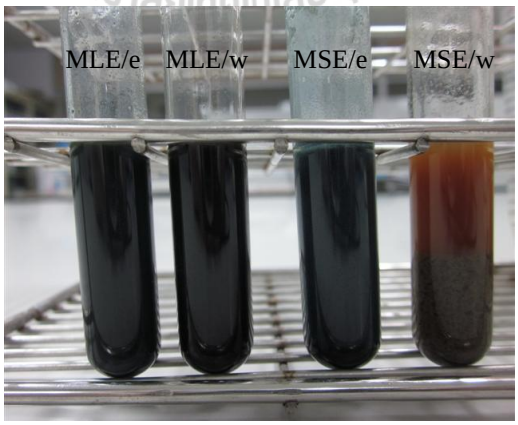

Figure 3.9 Tannins detection in mintweed extracts by Ferric chloride test. MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

3) Alkaloids by Dragendorff reagent test

A few drops of Dragendorff reagent was added to the mintweed extracts. Orange red precipitation was the only one found in MSE/w. Orange red precipitate indicates the presence of alkaloids (Figure 3.10).

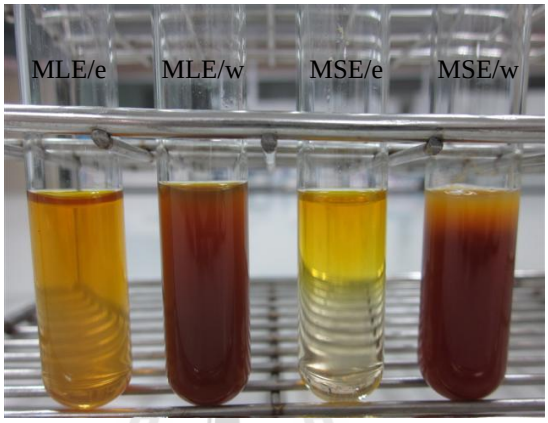

Figure 3.10 Alkaloids detection in mintweed extracts by Dragendorff reagent test. MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

4) Saponins by Frothing test

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources. More specifically, they are in an amphipathic glycosides group, they produce soap-like foaming when shaken vigorously in aqueous solutions (Silva et al., 1998). Thick persistent froth indicates saponins. The presence of saponins was confirmed by adding NaOH to basify the extracts and eliminates the interference of some proteins and fatty acids which was able to form froths. Furthermore, saponins was also confirmed by adding HCl. Saponin glycoside can be hydrolyzed into aglycone sapogenin which cannot or is less ability to form stable frothing when shaken (Trease and Evans, 1983). The extacts

were boiled and transferred into a test tube and then shaked for 10 min. It was noticed that MSE/w showed honeycomb froth which was greater than 2 cm height from the surface of solution and stable for 10 min. The ability to foam was also detected in MLE/e and MLE/w but low level in honeycomb froth approximately 1 cm height (Figure 3.11A). After adding NaOH (Figure 3.11B), the higher froth was detected in MSE/e, while froth was lower in MSE/w, MLE/e and MLE/w. After adding HCl (Figure 3.11C), no froth was observed in all the mintweed extracts indicating that all mintweed extracts might contain different amount of saponins.

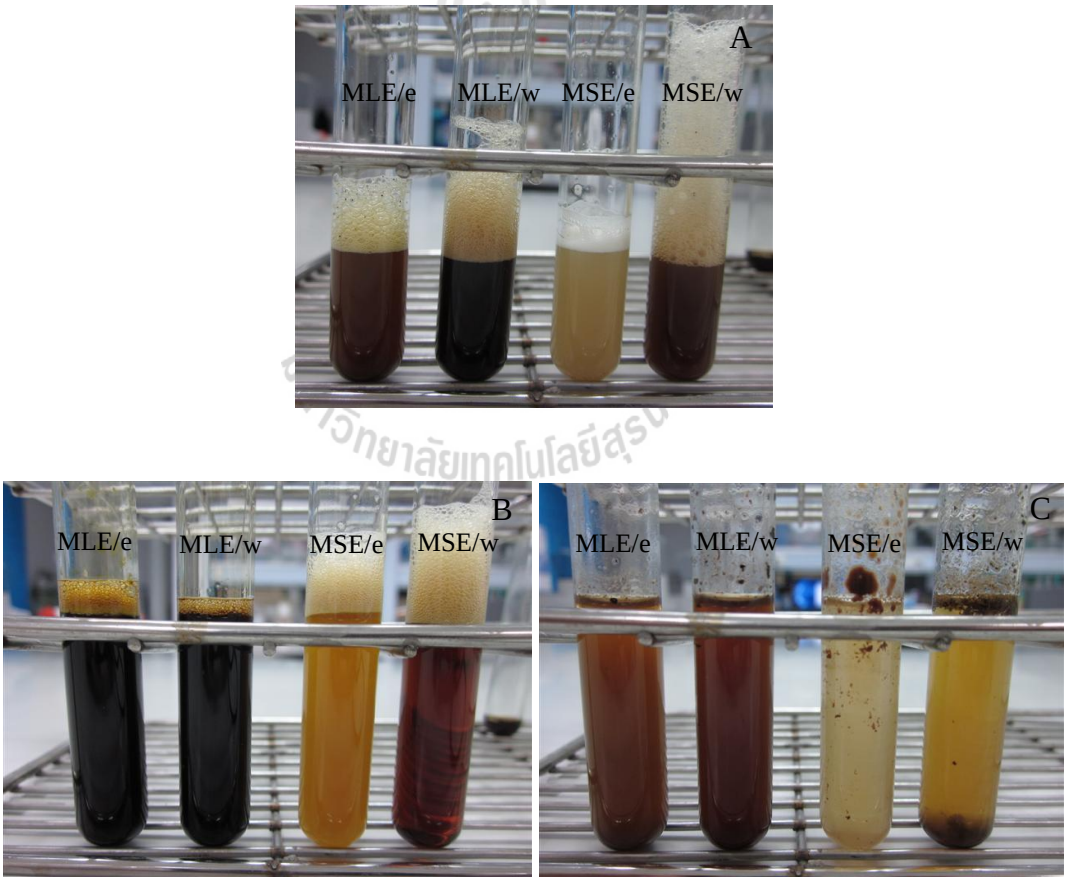

Figure 3.11 Frothting test of mintweed extracts. A, after shaking, B, after adding NaOH and shaking, C, after adding HCl, boiling for 10 min and shaking. MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

The phytochemical characteristics of the mintweed extracts investigated are summarized in Table 3.6. Phenolics, antioxidants, and terpenoids were presented in all mintweed extracts, but coumarins, cardiac glycosides and antraquinones were not detected. Essentail oils was presented in MLE/e, MLE/w and MSE/e. Saponins were presented only in mintweed seed extracts, MSE/w and MSE/w. Alkaloids was detected only in MSE/w, but essential oils and tannins were not found in MSE/w.

Table 3.6 Summary of phytochemical screening of mintweed extracts detected by TLC and specific tests.

Phytochemical category	MLE/e	MLE/w	MSE/e	MSE/w
Coumarins [a]	-	-	-	-
Antraquinones [a]	-	-	-	-
Cardiac glycosides [a]	-	-	-	-
Essential oils [a]	+	+	+	-
Phenolic [a]	+	+	+	+
Antioxidants [a]	+	+	+	+
Alkaloids [b]	-	-	-	+
Terpenoids [b]	+	+	+	+
Saponins [b]	-	-	+	+
Tannins [b]	+	+	+	-

[a] Detected by TLC, [b]detected by TLC and tests with specific reagents. MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

The phytochemical screening and quantitative estimation of chemical constituents of the mintweed extracts showed that the plant extracts were rich in phenolics, antioxidants, terpenoids, saponins, tannins and essential oils, which were in agreement with Mbatchou et al. (2010), who reported that *Hyptis suaveolens* LAM extracts presence of alkaloids, flavonoids, terpenoids, tannins, aldehydes and ketones.

Preliminary phytochemical screening of ethanolic leaf extracts of *Hyptis suaveolens* revealed the presence of alkaloids, glycosides, saponins, tannins and flavonoids as major active constituents (Shaikat et al., 2012). Moreover, leaves of *Hyptis suaveolens* had flavonoids, alkaloids, tannins and soponins (Ijeh et al., 2007). As a report of Danmalam et al., 2009, alkaloids, flavonoids, tannins, steroids and/or terpenes presented in the methanolic extract of *H. suaveolens* leaves. The major chemical compositions in *H. sauveolens* oil is a terpenoid group. It contains 1,8-cineole and β-caryophyllene from distillation (Luz et al., 1984; Peerzada, 1997; Tripathi et al., 2009). Oliveira et al. (2005) reported that the compositions of essential oils of *H. sauveolens* are 1,8-cineole, (*E*)-caryophyllene, spathulenol. Azevedo et al. (2001) also reported that the essential oils of *H. sauveolens* are sabinene, limonene, biclyclogermacrene, β-phellandrene and 1,8-cineole. The essential oils of *H. sauveolens* was reported to possess antimicrobial (Asekun et al., 1999), antifungal (Singha and Handiquea, 1997), anti-inflammatory activities (Grassia, 2006) and repellent activity against *Sitophilus granarius* (Conti et al., 2011). In addition, the essential oils had antimalarial, anticancer, antimicrobial activities (Khamsan et al., 2011) anti-proliferative activity on KB and P388 cell lines (Manosroi et al., 2006) and induced apoptosis (Cha and Kim, 2012). Terpenes was reported to possess antimicrobial (Joshi et al., 2008), anti-inflammatory (Qi et al., 2010), antiproliferative activities (de Almeida et al., 2010) and terpenoids induced cell cycle arrest and apoptosis (Chen et al., 2010). Tannins had antibacterial (Akiyama, 2001), antiproliferative (de Melo, 2010) activities and tannic acid-induced apoptosis (Chen et al., 2009). Saponins had antiproliferative (Liu et al., 2000), antimicrobial (Soetan et al., 2006), antiviral (Amoros et al., 1987), antinociceptive and anti-inflammatory

effects (Choi, 2005). Moreover, astragalus saponins induced growth inhibition and apoptosis (Tin et al., 2007). The alkaloids had antimicrobial (Karou, 2005), antitumor (Zupko et al., 2009) activities and induced apoptosis (Kun et al., 2008). The presence of bioactive compounds in the mintweed extracts is an indication of their many possible therapeutic uses.

3.4.3 Antioxidant activities of mintweed extracts

3.4.3.1 Ferric reducing ability power (FRAP)

The ability of the plant extracts to reduce ferric ions was determined using the FRAP assay developed by Benzie and Strain (1996). An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe(III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous- TPTZ (Fe(II)-TPTZ) complex which strongly absorbs light at 593 nm. The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. A higher absorbance indicates a higher ferric reducing power (Yim et al., 2010). Antioxidant activity of mintweed extracts, which was determined by FRAP assay, is shown in Table 3.7. The results showed that MLE/e possessed the highest ferric reducing activity of 8.52 ± 0.44 μM $FeSO_4$/mg dried extract followed by, MLE/w (6.27 ± 0.03 μM $FeSO_4$/mg dried extract), and MSE/e (2.01 ± 0.35 μM $FeSO_4$/mg dried extract), while MSE/w (1.36 ± 0.00 μM $FeSO_4$/mg dried extract) showed the lowest antioxidant activity. The standards, catechin and ascorbic acid had ferric reducing activity of 16.26 ± 0.65 and 19.31 ± 0.22 μM $FeSO_4$/mg dried extract, respectively. There were significant differences ($p < 0.05$) between the mintweed extracts and standards, catechin and ascorbic acid, in antioxidant activity. Results showed that

the activities of the mintweed extracts were not comparable to catechin and ascorbic acid. This is understandable, since catechin and ascorbic acid are pure compounds, while the plant crude extracts still need to be isolated for active compounds responsible for antioxidant activities.

Table 3.7 Ferric reducing ability power (FRAP) values of mintweed (*H. sauveolens*) extracts, catechin and ascorbic acid. Data were mean ± SD., (n = 3).

Sample/standard	FRAP (μM $FeSO_4$/mg dried extracts)
MLE/e	8.52 ± 0.44^{c}
MLE/w	6.27 ± 0.03^{d}
MSE/e	2.01 ± 0.35^{e}
MSE/w	1.36 ± 0.00^{f}
Catechin	16.26 ± 0.65^{b}
Ascorbic acid	19.31 ± 0.22^{a}

Different letters) within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

Thaiponga et al. (2006) reported that the comparison of ABTS, DPPH, FRAP and ORAC assays for the antioxidant activity in methanolic extract of guava fruits. The FRAP technique showed high reproducibility, was simple, rapidly performed and showed the highest correlation with both ascorbic acid and total phenolics. Therefore, it would be an appropriate technique for determining antioxidant activity in the mintweed extracts.

3.4.3.2 DPPH radical scavenging activity

The DPPH radical was widely used in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude mixtures such as ethanolic extract of plants. DPPH radical is scavenged by antioxidants through the donation of a hydrogen, forming the reduced DPPH-H. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 515 nm. The radical scavenging activity (%) and IC_{50} values of the mintweed extracts which were measured by DPPH assays are shown in Table 3.8A and 3.8B. The antioxidant potential determined by DPPH reagent, MLE/e showed the highest antioxidant activity with an IC_{50} value of 9.26 ± 0.08 µg/mL, followed by MLE/w (10.89 ± 0.07µg/mL), and MSE/e (32.85 ± 0.05 µg/mL), while MSE/w (147.17± 1.67 µg/mL) showed the lowest antioxidant activity.

Table 3.8A DPPH free radical scavenging activity of standard antioxidants, catechin and ascorbic acid. Data were mean ± SD., (n = 3).

Sample	Conc. (µg/mL)	% inhibition	IC_{50} (µg/mL)
Catechin	2	40.83 ± 1.42	
	3	55.42 ± 0.43	
	4	66.06 ± 1.25	2.67±0.06[a]
	5	79.96 ± 1.18	
	6	91.95 ± 0.51	
Ascorbic acid	2	25.02 ± 1.33	
	3	35.53 ± 0.48	
	4	47.14 ± 0.10	4.22±0.01[b]
	5	58.81 ± 0.37	
	6	70.74 ± 0.55	

Different letters within the same column were significantly different ($p < 0.05$)

Table 3.8B DPPH free radical scavenging activity of mintweed (*H. sauveolens*) extracts. Data were mean ± SD., (n = 3).

Sample	Conc. (µg/mL)	% inhibition	IC_{50} (µg/mL)
MLE/e	5	28.56 ± 0.66	
	7.5	41.04 ± 0.35	
	10	53.39 ± 0.50	9.26 ± 0.08^{c}
	12.5	66.89 ± 0.50	
	15	78.85 ± 1.10	
MLE/w	5	24.01 ± 0.28	
	7.5	33.64 ± 0.56	
	10	46.06 ± 0.93	10.89 ± 0.70^{d}
	12.5	58.10 ± 0.36	
	15	68.17 ± 0.58	
MSE/e	2.5	5.93 ± 0.10	
	12.5	23.85 ± 0.78	
	25	40.58 ± 0.51	32.85 ± 0.05^{e}
	37.5	57.16 ± 0.65	
	50	71.94 ± 0.50	
MSE/w	50	18.21 ± 0.52	
	100	34.84 ± 1.71	
	150	53.22 ± 0.77	147.17 ± 1.67^{f}
	200	66.83 ± 1.62	
	250	81.33 ± 0.31	

Different letters within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

As reported by Gavani and Paarkh (2008) the methanolic extract of leaves of *H. suaveolens* exhibited antioxidant radical scavenging activity with IC_{50} value of 14.04 µg/mL. The antioxidant potential of *H. suaveolens* oil determined by DPPH method expressed as IC_{50} was 3.72 mg/mL (Nantitanon et al., 2007). The variability of IC_{50} values among the *H. suaveolens* could apparently be due to the influences of different varieties, locations, harvest seasons, and solvents for extraction etc., which would affect the level of antioxidant capacity present in this plant.

DPPH is a free radical that forms a stable molecule on accepting an electron or a hydrogen atom. Free radicals induce oxidative stress *in vivo* that may lead to oxidative modification or damage of some biological structures such as lipids, proteins, DNA and may give rise to degenerative diseases. There is need for antioxidant intervention which one of the plants studied may be of importance. *In vitro* study sounds encouraging as all plants studied have some radical scavenging effects (Biapa et al., 2007).

3.4.3.3 Inhibition of liposomal lipid peroxidation

Oxidative stresses in the cellular environment result in the formation of highly reactive and unstable lipid hydroperoxides. Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which can be colorimetrically quantified following its controlled reaction with thiobarbituric acid. The measurement of thiobarbituric acid reactive substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation (Yagi, 1998). The results of antioxidant capacity of mintweed extracts, using the TBARS method, percentage of lipid peroxidation inhibition and IC_{50} values are presented in Table 3.9. MLE/e exhibited the highest antioxidant activity with IC_{50} of 5.42 ± 0.14 µg/mL followed by MLE/w (17.46 ± 0.23 µg/mL), and catechin (17.92 ± 0.18 µg/mL). MSE/e had antioxidant activity of 39.77 ± 0.23 µg/mL, while the lowest antioxidant activity presented in MSE/w with IC_{50} of 725 ± 15.40 µg/mL. There were significant difference between the MSE/e and MSE/w in antioxidant activity ($p < 0.05$). When compared to pure component,

catechin, IC_{50} value of MLE/w and MLE/w were no significant differences ($p < 0.05$). So the mintweed extracts may be useful in preventing lipid peroxidation.

Table 3.9 Antioxidant capacity of mintweed (*H. sauveolens*) extracts and catechin by TBARS method. Data were mean ± SD., (n = 3).

Sample	Conc.(µg/mL)	% inhibition	IC_{50} (µg/mL)
MLE/e	2	5.95 ± 5.51	
	4	27.02 ± 5.30	5.43 ± 0.37[a]
	6	56.23 ± 9.17	
	8	87.08 ± 1.26	
MLE/w	5	10.84 ± 0.92	17.45 ± 0.21[a]
	10	22.90 ± 0.65	
	15	41.33 ± 0.17	
	20	59.63 ± 1.08	
MSE/e	10	9.59 ± 6.50	35.95 ± 1.78[b]
	20	27.79 ± 4.13	
	40	51.98 ± 3.21	
	60	73.58 ± 1.82	
MSE/w	200	10.54 ± 1.13	725.48 ± 17.81[c]
	400	28.97 ± 0.99	
	600	39.08 ± 0.99	
	800	57.07 ± 2.37	
Catechin	10	7.76 ± 2.50	17.92 ± 0.18[a]
	15	25.12 ± 2.00	
	20	59.25 ± 0.84	
	25	97.48 ± 0.37	

Different letters within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

Pradeep et al. (2011) reported that *H. suaveolens* showed a protective effect on the antioxidant status of the animal which was evident from the low lipid peroxidation level. So the mintweed extracts may be useful in preventing lipid

peroxidation. The sensitivity of measuring thiobarbituric acid reactive substances (TBARS) has made this assay as the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stresses (Yagi, 1998). The assay has provided important information regarding free radical activity in disease states and has been used for measurement of antioxidant activity of several compounds (Hunnisett, 1995). Although much controversy has appeared in the literature regarding the specificity of TBARS toward compounds other than MDA, it remains the most widely employed assay used to determine lipid peroxidation (Armstrong and Browne, 1994).

The summary of the antioxidant capacity of the mintweed extracts as determined by FRAP, DPHH and TBARS assays are shown in Table 3.10. These different methods could be explained by different mechanisms of analytical method. FRAP assay measures the ability to reduce a ferric tripyridyltrizaine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}-TPTZ) of sample. DPPH assay are based on the reduction of DPPH free radical of samples. TBARS assay estimates MDA for estimating oxidative stress effects on lipids. MLE/e exhibited the highest antioxidant activity in the TBARS, DPHH, and FRAP assays, but the IC_{50} value from TBARS might be lower than from DPPH assay which was high antioxidant activity.

Table 3.10 Antioxidant activity of mintweed extract, *H. sauveolens*, with FRAP, DPPH and TBARS assay. Data were mean ± SD., (n = 3).

Sample	FRAP ($\mu M FeSO_4$/mg extracts)	DPPH IC_{50} (µg/mL)	TBARS IC_{50} (µg/mL)
MLE/e	8.52 ± 0.44^{c}	9.26 ± 0.08^{c}	5.42 ± 0.37^{a}
MLE/w	6.27 ± 0.03^{d}	10.89 ± 0.70^{d}	17.46 ± 0.21^{a}
MSE/e	2.01 ± 0.35^{e}	32.85 ± 0.05^{e}	39.77 ± 1.78^{b}
MSE/w	1.36 ± 0.00^{f}	147.17 ± 1.67^{f}	725.75 ± 17.81^{c}
Catechin	16.26 ± 0.65^{b}	2.67 ± 0.06^{a}	17.92 ± 0.18^{a}
Ascorbic acid	19.31 ± 0.22^{a}	4.22 ± 0.01^{b}	NP

Different letters) within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract; NP, not performed.

Various methods available for the assessment of the antioxidant capacity of samples differ in terms of their assay principles and experimental conditions. Consequently, different methods and antioxidants have varying contributions to total antioxidant potential (Katalinic et al., 2006). However, three antioxidant assays, FRAP, DPPH and TBARS give same antioxidant activity in MLE/e. The high scavenging property of MLE/e may be due to hydroxyl groups existing in the chemical structure of phenolic compounds that provide the necessary component as a radical scavenger. Free radicals are often generated as by-products of biological reactions or from exogenous factors. The involvement of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for diseases (Gyamfi et al., 1999).

3.5 Conclusions

The present study showed that the MLE/e contained the highest amount of phenolic and flavonoid contents followed by MLE/w, MSE/e and MSE/w. All the mintweed extracts exhibited different extent of antioxidant activity, MLE/e showed the greatest antioxidant activity estimated by, FRAP and DPPH assays. In TBARS assay, MLE/e, LME/w and catechin showed similarly effect to inhibit lipid peroxidation of phosphatidyl choline, but no significant differences ($p < 0.05$) among of them. This may be related to a rich source of antioxidants of flavonoids and phenolic compounds, terpenoids, essential oils, and tannins in mintweed extracts. Further study made clear that MLE/e possessed the highest potent antioxidant activity, so it can be used in health protective or therapeutic agents.

3.6 References

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CHAPTER IV

CYTOTOXICITY AND PROLIFERATIVE PROPERTIES OF MINTWEED (*HYPTIS SAUVEOLENT* (L.) POIT) EXTRACTS ON NORMAL PBMCS AND JURKAT CELLS

4.1 Abstract

Mintweed leaf ethanolic and water extracts (MLE/e, MLE/w), mintweed seed ethanolic and water extracts (MSE/e, MSE/w) were investigated for their toxicity by brine shrimp lethality assay (BSLA). The proliferation effect of the mintweed extracts determined on Jurkat cells and normal peripheral blood mononuclear cells (PBMCs) by Alamar blue assay. The results showed that MLE/e had the highest lethality and followed by MSE/w, MSE/w and MLE/w with LC_{50} values of 360.48, 470.60, 708.26, and 1,282.47 µg/mL, respectively. Moreover, MLE/e had the highest inhibition on the proliferation of Jurkat cells with IC_{50} value of 553.52 ± 14.07 µg/mL, while the lowest was MSE/w with IC_{50} value of 5,813.45 ± 111.25 µg/mL. MLE/w and MSE/e had cytotoxicity with IC_{50} values of 912.06 ± 16.86 and 2,385.95 ± 81.28 µg/mL, respectively. The results indicated that MLE/e showed the highest cytotoxicity on *A. salina* and inhibited the growth of Jurkat human cancer cells, induced the proliferation of normal PBMCs at low concentration.

4.2 Introduction

A number of extracts or compounds have been tested and determined whether they contain biochemical and cellular properties. This is usually one of the first step in discovery of bioactive compounds. In principle, *in vitro* or *in vivo* bioassay systems are used to detect the biological activities of the extracts or pure substances from living organism. At present, a large number of bioassays are available differing in degrees of sophistication. Simple and inexpensive assays suitable for the rapid screening of the extracts in the typical laboratory have been developed. Thus, cytotoxicity screening models provide important preliminary data in helping further studies for selection of plant extracts with potential antitumor properties. The methods for detection of biological activity of plant extracts can be divided into two groups for screening purposes: primary screening assays and specific screening assays (Ghisalberti, 1993).

Primary screening assay, brine shrimp lethality assay (BSLA), developed by Meyer et al. (1982) might be used as a simple tool for primary screening and fractionating of physiologically active plant extracts. The lethality of the tested brine shrimps was simply monitored. This bioassay detects a broad range of biological activities and a diversity of chemical structures. Brine shrimps have been previously utilized in various bioassay systems, such as environmental studies (Sorgeloos et al., 1978), natural toxins (Harwig and Scott, 1971) and bioactive substances in plant extracts (Meyer et al., 1982). The assay has been widely utilized as a tool for screening of the isolation of bioactive compounds from plant extracts (Sam, 1993). The assay is inexpensive, uncomplicated to establish, simple to handle and not required particular equipment or aseptic techniques.

The specific screening assays are the *in vitro* tests which are more sophisticated than primary screening bioassays. Many biological assays are designed to quantitate cell proliferation and/or cytotoxicity on various cell types. They are important tools for identification of molecules and mechanisms involved. A number of bioassay systems have been developed for quantitation of cell numbers, cell survival, or cell proliferation. There are several commonly used cytotoxicity screening assays that are currently being used, including ATP measurement, MTT, neutral red, membrane integrity/LDH release, macromolecular synthesis, glutathione depletion and more (Hamid et al., 2004). All assays are afflicted with certain disadvantages, e.g. time consuming procedures, the use of radioactive material or the use of expensive equipment and reagents. Therefore, the development of *in vitro* cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds (Barile et al., 1994). The one-step Alamar blue is an oxidation-reduction indicator that changes fluorescence color from blue to red. It has been used to study the proliferation of tumor cell lines and the effects of chemotherapeutic drugs, which found to provide a rapid, sensitive and non-toxic fluorescence assay of cell viability (Page et al., 1993). There are a few reports that ethanolic extract of *Hyptis suaveolens* possess potent anticancer activity against Ehrlich ascites carcinoma by activating apoptotic pathway (Gurunagarajan and Pemaiah, 2011). A major compound of *H. suaveolens*, di-terpenoid, induced apoptosis of Jurkat cells (Liu et al., 2004). Pentacyclic lupine-type triterpene could significantly inhibit the proliferation of Jurkat cells by arresting cells in G_0/G_1 phase, and inducing apoptosis (Chen et al., 2008).

Therefore, the aim of this study is to investigate the cytotoxicity of mintweed (*H. suaveolens*) extracts by brine shrimp lethality assay and *in vitro* anti-proliferative activity against normal peripheral blood mononuclear cells (PBMCs) and Jurkat cells, human T leukemia cells.

4.3 Materials and methods

4.3.1 Cell line and culture reagents

Jurkat E6.1 (human leukemic T) cell line was purchased from Cell Line Services (CLS, Germany). RPMI 1640, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco (New York, USA). Alamar blue was purchased from Invitrogen (California, USA). Catechin, ascorbic acid, Histopaque-1077 were purchased from Sigma-Aldrich Company Ltd. (St. Louis MO, USA). Dimethylsulfoxide (DMSO) was obtained from Amresco (Ohio, USA).

4.3.2 Collection of plant materials

Leaves of mintweed were collected from the plants grown on the grounds of Suranaree University of Technology (SUT) in the Nakhon Ratchasima, Thailand, in October 2008. The leaves were collected, washed under running tap water and then sun dried. The seeds were collected in January 2009 from the same place. The seed mucilage coat was sloughed off and then the seeds were air dried. *H. suaveolens* was kindly identified by a plant taxonomist, Professor Dr. Pranom Chantaranothai.

4.3.3 Sample preparations

The dried leaves and seeds were ground into fine powder by electric blender and then 50 grams of each plant powder was extracted in 500 ml water or 70% ethanol (v/v) for 24 h by Soxhlet extraction apparatus. The extracts were filtered, evaporated (Buchi instruments, Switzerland) and lyophilized into powder (Freeze-zone 12 plus, Labconco Corporation, Missouri, USA). The powder extracts were stored at -20°C until used. The water and ethanolic extracts were dissolved in RPMI 1640 culture medium and 0.2% DMSO, respectively.

4.3.4 Brine shrimp lethality assay

4.3.4.1 Brine shrimp hatching

Brine shrimp eggs (*Artemia salina*) was obtained from a local pet shop and hatched in artificial seawater prepared by dissolving 38 g of sea salt in 1 litter of distilled water. The artificial seawater was filtered and autoclaved. The two-unequal compartment plastic chamber with several holes on divider was used for hatching. The eggs were put into the larger compartment which was darken. While the smaller compartment was illuminated under a 20 Watt lamp (Figure 4.1). After 24 h of incubation at room temperature (25°C ± 2°C), the larvae (nauphii) were attracted by light source so that they moved to the smaller compartment and pipetted into a 24 well plate (SPL Life Sciences, Gyeonggi-do, Korea), whereas their shells were left in the other side. (Wanyoike et al., 2004).

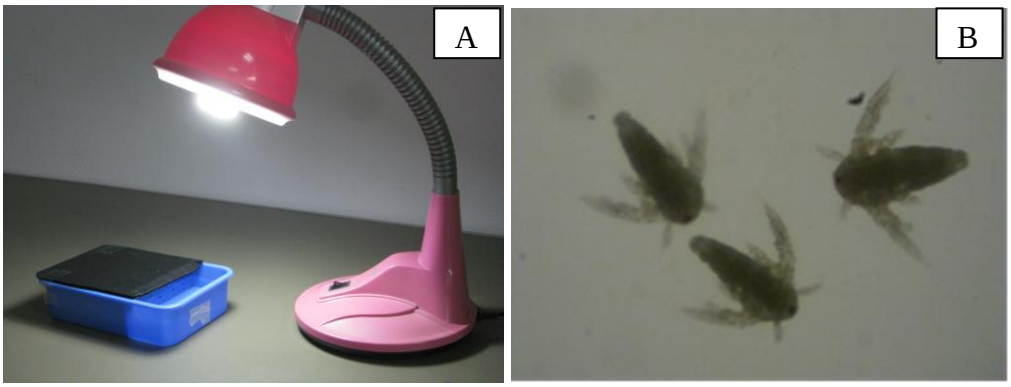


Figure 4.1 Brine shrimp lethality assay. (A) *Artemia salina* cysts hatched in the large compartment and the light was applied to the small compartment. (B) The first stage of *Artemia salina* under stereomicroscope.

4.3.4.2 Brine shrimp lethality assay

The brine shrimp bioassay was found to be reliable, inexpensive and convenient for assessment of biologically active medicinal plants (Massele and Nshimo, 1995). Moreover, Solis et al. (1993), supported that this assay may be a useful testing for biologically activity compounds since the brine shrimp responds similarly to mammalian system. For example, the DNA-dependent-RNA-polymerase of *A. salina* has been shown to be similar to the mammalian type and the organism also has an ouabaine sensitive Na^+ and K^+ dependent ATPase. The cytotoxicity of the plant extracts was monitored by the brine shrimp lethality test (Meyer et al., 1982). Samples were dissolved in deionized water and dimethylsulphoxide (DMSO) and diluted with artificial sea salt water, to obtain 1% DMSO. Two hundred microliters of suspension of nauplii containing 10 larvae were added onto a 24 well plate (10 nauplii/well) (Nunc, Denmark). Then, various concentrations of samples in 800 µL were added into each well. The plates were covered and incubated at room temperature for 24 h. The plates were then examined

under a binocular stereomicroscope (Olympus SZ61, Olympus Corporation, Tokyo, Japan) and the dead nauplii in each well were counted and recorded. The experiment was performed in six replicates. Percentage mortality of nauplii was calculated as following:

$$\%\ Mortality = \left(\frac{no.\ of\ \ dead\ nauplii}{initial\ no.\ of\ \ live\ nauplii} \right) \times 100$$

Then, data were analyzed using Probit analysis, a readily available personal computer program to estimated lethality concentration that kill 50% (LC_{50}) values with 95% confidence intervals.

4.3.5 Cytotoxic effect against normal human lymphocytes and Jurkat cells

4.3.5.1 Preparation for normal lymphocytes

Whole blood from healthy donors was kindly taken from Blood Bank, the Thai Red Cross Society, Nakhon Ratchasima. Peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation in Histopaque-1077 (Figure 4.2). The whole blood, diluted with PBS 1:1 (v/v), was carefully pipetted onto the Histopaque-1077 and centrifuged at 400×g for 30 min at room temperature. The upper layer was carefully aspirated off until about 0.5 cm above the opaque interface which contained mononuclear cells. Then, the opaque interface was transferred into a clean conical centrifuge tube. The PBMCs were washed three times with phosphate buffered saline (PBS), pH7.4 and centrifuged at 250×g for 10 min. The cells were cultured in complete RPMI 1640 medium overnight

to allow the monocytes and platelets attach to the culture flask. The floating lymphocytes were collected and used for assay.

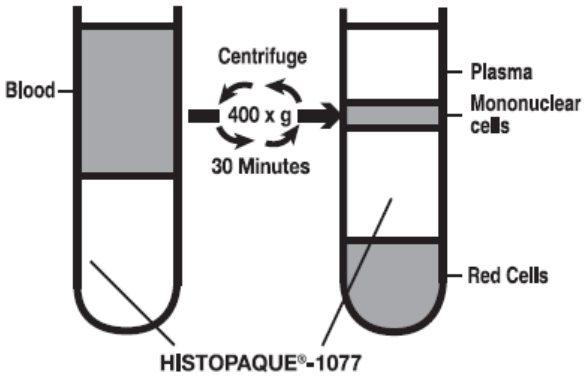


Figure 4.2 Normal lymphocyte separation by Histopaque-1077 (Hofman et al., 1982).

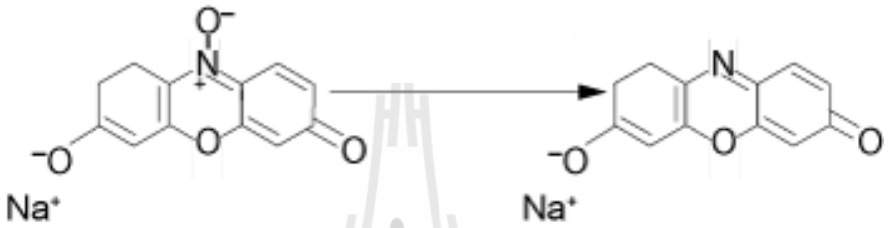
4.3.5.2 Cell culture

Human T lymphocyte leukemia cells, Jurkat E6-1, were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin and incubated in humidified atmosphere of 95% air and 5% CO_2 at 37°C incubator.

4.3.5.3 Cell viability by Alamar blue assay

Alamar blue cell viability reagent functions as a cell health indicator by using the reducing power of living cells to quantitatively measure the proliferation of various human and animal cell lines, bacteria, plants, and fungi allowing ones to establish relative cytotoxicity of agents within various chemical

classes. When cells are alive they maintain a reducing environment within their cytosol. Resazurin, the active ingredient of Alamar blue reagent, is a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent (Figure 4.3).



Resazurin sodium salt
weakly fluorescent

Resorufin sodium salt
strongly fluorescent

Figure 4.3 Resazurin and resorufin structures. Nonreduced Alamar blue correspond to resazurin and reduced Alamar blue to resorufin (O'Brien et al., 2000).

Cell viability was determined by Alamar blue (AB), the method described by Berntsen et al. (2010) with slight modification. PBMCs ($1x10^5$ cells/well) and Jurkat cells ($2.5x10^4$ cells/well) were cultured in a 96-well black clear bottom plate (Costar, Corning Incorporated, NY, USA) and treated with various concentrations of the extracts in a final volume of 100 µL/well. After incubation for 24 h, 10 µL of AB was added and incubated for 4 h (giving a final AB concentration of 10% v/v). The fluorescence of the assay solution was measured at an excitation wavelength at 540 nm and emission wavelength at 590 nm in spectrofluorometer (Spectra MAX Gemini EM, Molecular devices, California, USA). The standard

antioxidants, catechin, and ascorbic acid, were also evaluated for their cytotoxicity on normal and Jurkat cells. Vehicle control, 0.2% (v/v) DMSO in medium, was used as a negative control. Blank was set using wells without cells and subtracted as background from each sample. Three replicate were carried out and the percentage of cell viability was calculated as following:

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

The percentage of cell viability was plotted against various concentrations of extracts. The concentration of extracts effectively inhibits 50% of cell viability (IC_{50})

4.3.6 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 data were analyzed by one-way ANOVA, followed by Duncan's multiple range tests using SPSS program and results were presented as mean ± SD. The differences between the means of treated and control groups are considered by unpaired student t' test and the difference was considered to be statistically significant when $p < 0.05$. All the experiments were repeated three independent sets and in triplicate each.

4.4 Results and discussion

4.4.1 Cytotoxic effects of mintweed extracts on *Artemia salina*

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of cytotoxicity. It has also been suggested for screening of pharmacological activities in plant extracts (Carballo et al., 2002). The cytotoxic effects of various concentrations of mintweed extracts on *A. salina* nauplii for 24 h, and the IC_{50} values are shown in Table 4.1. The cytotoxicity values of the extracts on *A. salina* ranged as following: MLE/e > MSE/w > MSE/w > MLE/w. The highest of lethality on brine shrimps was observed in MLE/e with LC_{50} value of 360.48 µg/mL. MSE/w, MSE/w, and MLE/w showed the cytotoxicity with LC_{50} values of 470.60, 708.26, and 1,282.47 µg/mL, respectively. The magnitude of lethality was directly proportional to the concentration of the extracts.

According to Rieser et al. (1996) crude extracts with LC_{50} values less than 250 µg/ml were considered significantly active and had the potential for further investigation. However, crude extracts giving the LC_{50} values less than 1000 µg/mL were considered low cytotoxic activity and were not toxic on *A. salina* (Mayer et al., 1982). Therefore, MLE/e, MSE/w, and MSE/w exhibited low cytotoxic activity and MLE/w was not toxic on *A. salina*. The standard antioxidant, ascorbic acid showed the LC_{50} value of 13.77 µg/mL. While catechin had LC_{50} value of 2,332 µg/mL on *A. salina* nauplii (Table 4.1). These could consider that ascorbic acid might induce some toxic whereas catechin was less toxic for living organisms.

Table 4.1 Cytotoxic effects of mintweed extracts assayed by brine shrimp lethality assay at 24 h. Data were mean ± S.D., (n = 60).

Extract	Conc.(µg/mL)	% Mortality	LC_{50} (µg/mL)
MLE/e	250	1.67 ± 0.40	
	300	11.67 ± 0.75	360.48 (344.02-381.05)
	350	35.00 ± 1.87	
	400	83.33 ± 1.03	
MLE/w	500	3.33 ± 0.51	
	1000	23.33 ± 1.50	1282.47 (1118.26-1457.79)
	1500	75.00 ± 0.54	
	2000	83.33 ± 1.86	
MSE/e	250	8.33 ± 0.98	
	500	18.33 ± 0.75	708.26 (594.32-874.67)
	750	55.00 ± 1.87	
	1000	80.00 ± 2.45	
MSE/w	100	1.67 ± 0.40	
	250	8.33 ± 0.98	470.60 (294.94-808.63)
	500	45.00 ± 1.38	
	1000	100.00 ± 0.00	
Catechin	2000	43.33 ± 1.21	
	4000	65.00 ± 1.04	2332 (1848.36-2736.94)
	6000	85.00 ± 0.83	
	8000	100.00 ± 0.00	
Ascorbic acid	5	11.67 ± 1.16	
	10	20.00 ± 1.41	13.77 (11.44-16.70)
	20	73.33 ± 1.50	
	40	100.00 ± 0.00	

MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

The cytotoxic efficacy of the extracts and antioxidant standards, catechin and ascorbic acid are showed in Table 4.2. It obviously indicated that ascorbic acid

might induce higher cytotoxicity on *A. salina* than the extracts whereas, catechin much more less cytotoxicity than the extracts. MLE/e showed more cytotoxic effect than catechin 6.47 fold, but less than ascorbic acid 26.18 fold. MLE/w possessed more cytotoxic effect than catechin 1.8 fold, but less than ascorbic acid 93 fold. MsE/e showed more cytotoxic efficacy than catechin 3.29 fold, but less than ascorbic acid 51.44 fold. MLE/e had more cytotoxic effect than catechin 4.96 fold, but less than ascorbic acid 34 fold. The result indicated that mintweed extracts showed higher cytotoxicity effect than catechin but, much lesser than ascorbic acid.

Table 4.2 The comparison of cytotoxic efficacy (LC_{50}) between the extracts and the antioxidant standards, catechin and ascorbic acid.

Extract- Standard	Cytotoxic efficacy (fold)
MLE/e > CA	6.47
< AA	26.18
MLE/w > CA	1.8
< AA	93
MSE/e > CA	3.29
< AA	51.44
MSE/w > CA	4.96
< AA	34

MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract; CA, catechin; AA, ascorbic acid.

Ahmed et al. (2010) reported that the cytotoxicity against brine shrimps of the essential oil obtained from the leaves of *Annona senegalensis* Pers. was mild to moderate with LC_{50} of 27.3 μg/mL. The cytotoxic activity of three terpenes, ZB-1, ZB-2 and ZB-3, isolated from the bark of *Zanthoxylum budrunga* Wall. was evaluated

by brine shrimp lethality bioassay. They had LC_{50} values of 11.49, 19.99 and 13.19 µg/mL, respectively (Md Anwar-Ul Islam, et al., 2003). The crude ethanol extract from the stem bark of *Acacia senegal* partitioned in n-hexane, chloroform, ethylacetate and methanol soluble fractions. n-hexane soluble fraction showed highest toxicity in with LC_{50} value of 6.7674 µg/mL. Phytochemical analysis of this fractions revealed the presence of alkaloids, steroids, cardiac glycosides, Tannins, reducing sugars and flavonosides (Mudi and Salisu, 2009).

4.4.2 Proliferative effects of mintweed extracts on Jurkat cells (human T lymphocyte leukemia)

Jurkat cells are an immortal line of human T lymphocyte cells which produce interleukin 2 and useful for study human T lymphocyte leukemia. The viability of Jurkat cells when treated with various concentrations of the mintweed extracts and the proliferative effects, IC_{50} values, is shown in Table 4.3. It was observed that mintweed leaf extracts possessed higher anti-proliferative effects on human leukemia T cells than the seed extracts. The anti-proliferative effect values of the extracts on Jurkat cells were ranged as: MLE/e > MLE/w > MSE/e > MSE/w. The highest anti-proliferative effect on Jurkat cells was MLE/e with IC_{50} value of 553.52 ± 14.07 µg/mL, while the lowest one was MSE/w with IC_{50} value of 5,813.45 ± 111.25 µg/mL. MLE/w and MSE/e had anti-proliferative effect with IC_{50} values of 912.06 ± 16.86 and 2,385.95 ± 81.28 µg/mL, respectively. Both antioxidant standards, catechin and ascorbic acid, exhibited proliferative effects on Jurkat cells with IC_{50} values of 339.74 ± 14.55 and 211.29 ± 13.40 µg/mL, respectively.

Doll-Boscardin et al. (2012) studied the *in vitro* cytotoxic potential of the essential oils from young and adult leaves of *E. benthamii* and some related terpenes (α-pinene, terpinen-4-ol, and γ-terpinene) on Jurkat, J774A.1 and HeLa cells lines. The essential oils of *E. benthamii* showed more cytotoxicity than α-pinene and γ-terpinene, and presented cytotoxicity against for Jurkat and HeLa cell lines based on MTT assay. Jenkins et al. (2001) reported that 17-β-Estradiol (estrogen) inhibited Jurkat T cell proliferation by accumulation of cells in S and G_2/M phases of the cell cycle, and induced apoptosis over 72 h in a dose-dependent manner.

Interestingly, the mintweed seed extracts, MSE/e and MSE/w, possessed lower anti-proliferative efficacy than mintweed leaf extracts. These unique inhibitory proliferative effects at high doses of seed extracts may indicate that the cells had the ability to enter the recovery phase at a certain state.

Table 4.3 Proliferative effects of mintweed leaf and seed extracts on Jurkat cells, human T lymphocyte leukemia by Alarma blue assay at 24 h. Data were mean ± S.D., (n= 3).

Extract	Conc. (µg/mL)	Jurkat % viability	Jurkat IC_{50} (µg/mL)
MLE/e	200	92.45 ± 2.00	
	400	79.59 ± 3.25	553.52 ± 14.07[c]
	600	49.15 ± 3.00	
	800	8.95 ± 1.30	
MLE/w	600	66.08 ± 2.10	
	800	53.99 ± 7.40	912.06 ± 16.86[d]
	1000	49.35 ± 7.70	
	1200	32.54 ± 2.43	
MSE/e	1500	66.17 ± 0.90	
	2000	55.19 ± 1.80	2,385.95 ± 81.28[e]
	2500	47.55 ± 0.72	
	3000	39.99 ± 3.70	
MSE/w	2000	72.01 ± 0.31	
	4000	62.93 ± 0.60	5,813.45 ± 111.25[f]
	6000	48.42 ± 0.70	
	8000	35.52 ± 0.80	
Catechin	200	65.48 ± 2.30	
	300	54.63 ± 1.50	339.74 ± 14.55[b]
	400	42.18 ± 3.25	
	500	33.46 ± 1.23	
Ascorbic acid	100	86.12 ± 4.00	
	200	40.45 ± 6.40	211.29 ± 13.40[a]
	300	24.62 ± 5.80	
	400	12.04 ± 0.70	

Statistical analysis was performed by ANOVA, different letters within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

4.4.3 Proliferative effects of mintweed extracts on human normal lymphocyte cells

The proliferative effects of mintweed leaf and seed extracts were performed by Alarmar blue assay are shown in Table 4.4. It appeared that the effects of mintweed on the proliferation of normal lymphocytes were higher than those of Jurkat cells (Table 4.3). At the same treatment concentration, the viability and the IC_{50} of the normal cells were higher. The mintweed leaf extracts were more potent than the seed extracts and ethanolic extracts were more potent than the water extracts. The highest effect on normal lymphocytes was MLE/w with IC_{50} value of 1,140.52 ± 6.05 µg/mL which was followed by MLE/e with IC_{50} value of 1,356.17 ± 136.78 µg/mL. The lowest was MSE/w with IC_{50} value of 11,366.26 ± 266.28 µg/mL. MSE/e effect showed IC_{50} value of 2,920.68 ± 155.38 µg/mL. Both antioxidant standards, catechin and ascorbic acid, exhibited high proliferative effect on normal lymphocytes with IC_{50} values of 647 ± 12.76 and 772.47 ± 79.13 µg/mL, respectively. MLE/e and MLE/w were most significant effects among all extracts ($p < 0.05$). This study obviously indicated that the mintweed leaf extracts (MLE) possessed proliferative property on normal human lymphocytes as opposed to the anti-proliferative property on human leukemia cell line (Table 4.3). The mintweed seed extracts (MSE) were much less effects of proliferation on both normal lymphocytes and lymphocyte cell line. In addition, MLE were required less low concentration to induce either the proliferation of the normal lymphocytes or the anti-proliferation of the lymphocyte cell line. Miksusanti (2012) found that essential oil from *Curcuma xanthorrhiza* had effects to stimulate lymphocyte proliferation by increasing

concentration of essential oil resulted in greater activation of lymphocytes. Lymphocyte proliferation by essential oil were in a range of 146,55-356,66%.

Philippi et al. (2010) evaluated methanol extracts of three Brazilian medicinal plants, *Calophyllum brasiliense* (roots), *Ipomoea pes-caprae* (whole plant) and *Matayba elaeagnoides* (bark). The results suggested that the extracts of all three studied plants induced T lymphocyte proliferation. *I. pes-caprae* showed immunostimulatory activity three fold higher than the *C. brasiliense* extract, while the *M. elaeagnoides* extract was 1.5 fold higher.

Alamar blue (AB) is a sensitive oxidation-reduction indicator that fluorescence and changes color upon reduction by living cells. The reduction of alamar blue is mediated by mitochondrial enzymes (O'Brien et al., 2000). Moreover, AB has been used as a viability indicator in studies of cornical and granule cell cultures (White et al., 1996), mammalian cell cytotoxicity (O'Brien et al., 2000), oligodendrocyte death (Back et al., 1999) tomato cells (Byth et al., 2001).) and amyloid b toxicity (Zhang et al., 1996).

Table 4.4 Proliferative effects of mintweed leaf extracts on PBMCs (peripheral blood mononuclear cells) by Alarma blue assay at 24 h. Data were mean ± S.D., (n= 3).

Extract	Conc.	PBMCs	
	(µg/mL)	% viability	IC_{50} (µg/mL)
MLE/e	200	140.50 ± 9.50	
	400	125.36 ± 0.70	1356.17 ± 136.78[a]
	600	115.92 ± 2.50	
	800	89.93 ± 0.43	
MLE/w	600	93.09 ± 2.00	
	800	81.90 ± 3.30	1140.52 ± 6.05[a]
	1000	61.18 ± 0.30	
	1200	44.24 ± 1.34	
MSE/e	1500	142.34 ± 4.80	
	2000	133.50 ± 3.00	2920.68 ± 155.38[b]
	2500	128.47 ± 3.40	
	3000	119.69 ± 5.00	
MSE/w	2000	107.23 ± 2.00	
	4000	86.34 ± 0.50	11366.26 ± 266.28[c]
	6000	79.72 ± 2.00	
	8000	71.31 ± 0.80	
Catechin	200	106.00 ± 2.50	
	300	99.23 ± 6.50	647 ± 12.76[a]
	400	82.35 ± 0.60	
	500	67.91 ± 0.80	
Ascorbic acid	100	104.69 ± 2.50	
	200	103.61 ± 0.90	772.47 ± 79.13[a]
	300	90.40 ± 1.80	
	400	82.40 ± 2.00	

Statistical analysis was performed by ANOVA, different letters within the same column were significantly different ($p < 0.05$). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract.

The proliferative property of the extracts was evaluated using Jurkat cell line and normal PBMCs. The inhibitory concentration (IC_{50}) at which 50% of the cells died was determined. The IC_{50} values of Jurkat human leukemia cells and normal PBMCs treated with various concentrations of the mintweed extracts are shown in Figure 4.4. It was obvious that the proliferation of the Jurkat cells, the T lymphocyte leukemia, was reduced by all mintweed extracts as opposed to the proliferation of the normal human lymphocytes, PBMCs. The mintweed water extracts, MLE/w and MSE/w and catechin were able to induce the anti-proliferation of on Jurkat cells with different concentrations while, no significant cytotoxicity on normal lymphocytes ($p < 0.001$). The ethanolic extracts, MLE/e and MSE/e and ascorbic acid also induced cytotoxicity on Jurkat cells with various concentrations but they have no cytotoxic effects on normal lymphocyte cells treated with the same concentration ($p < 0.01$). It indicated that mintweed ethanolic extracts potentailly inhibited leukemia cell growth.

H. suaveolens significantly stimulated human lymphocyte proliferative responses and enhanced NK cells activity (Sriwanthana et al., 2007). Ahmad et al. (2010) reported that the aqueous extract of *C. roseus* induced cell death of Jurkat cells at 24, 48 and 72 hours in a time- and dose-dependent manner. However, the cells treated at 48 and 72 hours showed higher cytotoxic effects with IC_{50} values of 2.55 and 2.38 μg/mL, respectively. In contrast, the extract induced normal PBMCs proliferation. Moreover, the suspended cells exhibited distinctive morphological features of dendritic cells and gained the powerful capacity to stimulate proliferation of allogenic lymphocytes (Lin et al., 2006).

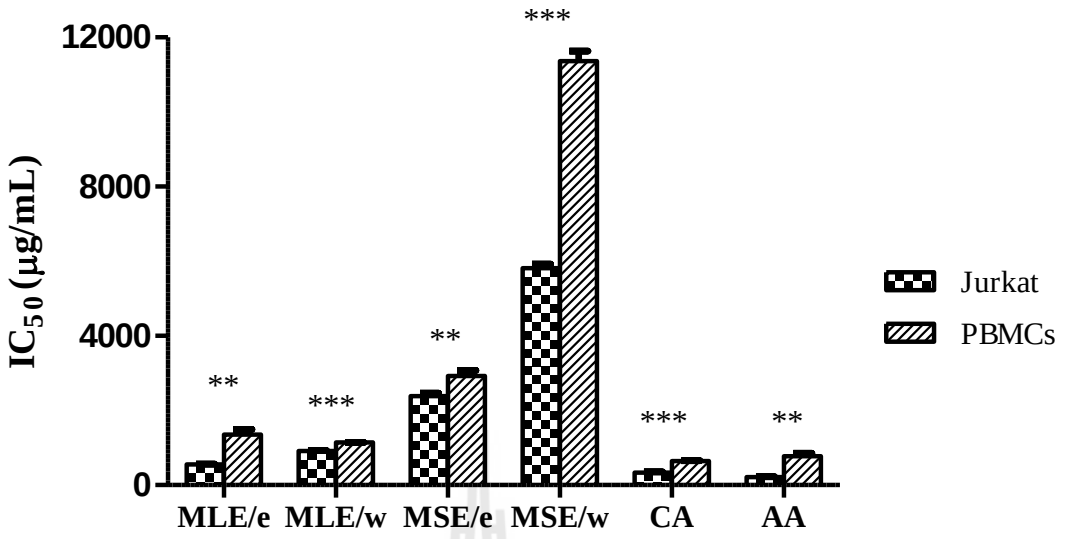


Figure 4.4 Proliferative effects of mintweed extracts on Jurkat cells and PBMCs (peripheral blood mononuclear cells) by Alarma blue assay at 24 h (n = 3). MLE/e, mintweed leaf ethanolic extract; MLE/w, mintweed leaf water extract; MSE/e, mintweed seed ethanolic extract; MSE/w, mintweed seed water extract; CA, catechin; AA, ascorbic acid., (** = $p < 0.01$, *** = $p < 0.001$).

The proliferative effect may be due to the phytochemical constituent of the plant extracts (chapter III). Phenolic compound, antioxidants, terpenoids, saponin essential oils and tannins are the major compounds that inhibited the growth of Jurkat cells and found in MLE/e, MLE/w, MSE/e and MSE/w. Chen et al. (2010) reported that terpenoids induced cell cycle arrest and apoptosis against bladder cancer cells. Moreover, plant-derived terpenoid ingredients suppressed nuclear factor-kappaB (NF-kappaB) signaling, the major regulator in the pathogenesis of inflammatory diseases and cancers (Salminena et al., 2008). Saponins was capable of inducing apoptosis in mammalian cells (Zhu et al., 2005). Essential oils and tannins could be the major

components in MLE/e, MLE/w, MSE/e. An essential oil and its major constituent induced apoptosis by increasing expression of mitochondrial cytochrome c and apical death receptors in human leukaemia HL-60 cells (Kumara et al., 2008), antimicrobial (Bassolé and Juliani, 2012) and anti-proliferative activity (Manosroia et al., 2006). In addition, tannins induced anti-inflammatory and antiulcer in rodent (Souza et al., 2007) and anti-proliferative activity (de Melo et al., 2010). MSE/w containing alkaloid, was toxic to *A. salina*, inhibited the Jurkat cells proliferation lesser than the other extracts. This results suggested that MLE/e contained many phytochemical components, phenolic, antioxidant, terpenoids, saponins, essential oils and tannins, therefore, MLE/e showed the highest cytotoxicity on *A. salina* and inhibited the growth of human lymphocyte cancer cells, but induced the proliferation of normal PBMCs at the low concentration. A number of studies documented that crude extract were more significant in inhibitory effects on cell growth as compared to the purified compounds (Kuete et al., 2008).

4.5 Conclusions

This study indicated that MLE/e was the most cytotoxicity on *Artemia salina*. MLE/e also inhibited the proliferation of Jurkat human leukemia cells, but promoted normal peripheral blood immune cells proliferation. It was noticed that the MLE/e may contain compounds that specifically induced cell death to Jurkat cells. For their merits further investigation as a potential therapeutic agent and may be applicable for modulating the normal and transformed immune cells in leukemia patients.

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CHAPTER V

EFFECTS OF MINTWEED (*HYPTIS SAUVEOLENS* (L.) POIT) EXTRACTS ON APOPTOTIC INDUCTION ON JURKAT CELLS

5.1 Abstract

Effects of mintweed leaf ethanolic and water extracts (MLE/e, MLE/w), mintweed seed ethanolic and water extracts (MSE/e, MSE/w) on apoptotic induction in cultured human T lymphocyte Jurkat cells were determined. The hallmarks of apoptotic cell death, nuclear morphological change and DNA fragmentation were observed by Hoechst 33258 fluorescence staining and agarose gel electrophoresis. The alteration on proteins involving in apoptosis including activation of caspase-9, Bax and Bcl-2 was monitored by Western blotting analysis. The results showed that mintweed extracts induced apoptotic cell death in Jurkat cells as indicated by chromatin condensation and DNA fragmentation. Apoptotic proteins were increased in Caspase-9 and Bax levels and decreased in Bcl-2 level. These indicated that mintweed extracts could be candidates for preventive and therapeutic application for cancer treatment.

5.2 Introduction

Cancer is a very complex disease, a disease of worldwide importance. It is being one of the leading causes of death globally. It has been proven that the occurrence and development of tumor cells is closely related to abnormality of the intracellular signal transduction system (Wolf et al., 2007). Although anticancer drugs have played a major role in the success stories in cancer treatments, there are still many types of cancers where effective molecular therapeutics are nonexistent. Therefore, there is an impetus to identify and develop more potent therapeutic agents for cancers (Fang et al., 2010). Apoptosis plays a vital role in controlling cell numbers in many physiological and developmental stages, tissue homeostasis, and regulation of immune system, while insufficient apoptosis is an integral part of cancer development. Activation of apoptotic pathways is a key method by which anticancer drugs kill tumor cells. It is well-known that anticancer drugs can stimulate apoptotic signaling through two major pathways. One is the death receptor (extrinsic) pathway involving death receptor and death ligand interaction, such as Fas receptor (Fas) and other members of the tumor-necrosis factor (TNF) receptor family. These receptors activate caspase-8 and subsequently caspase-3, the major caspases participating in the execution phase of apoptosis (Thorburn, 2004). Another apoptotic pathway is the mitochondrial (intrinsic) pathway, which is activated by the release of various pro-apoptotic factors from mitochondria intermembrane space (Kuwana and Newmeyer, 2003). The pro-apoptotic and anti-apoptotic members of Bcl-2 family regulate the release of cytochrome c. The released cytochrome c interacts with apoptotic protease activating factor 1 (Apaf-1) and activates caspase-9, which in turn proteolytically activates downstream caspase-3. Activated caspase-3 cleaves many substrates,

including poly ADP ribose polymerase (PARP), a DNA repair enzyme that leads to inevitable cell death (Bose et al., 2009). A good chemopreventive agent is a naturally occurring agent that can induce apoptosis in cancer cells without much side effects. Searching for new biological active compounds, novel chemotherapeutic agents derived from active phytochemicals, could be used to improve the anti-carcinogenicity of standard drug treatment. A variety of tropical plants have useful biological activities and some offer potential therapeutic applications (Watanapokasin et al., 2011). Mintweed, *Hyptis sauveolens* (L.) Poit, is used for traditional medicine for the treatment of various illness such as respiratory track, infection, colds, pain, fever, cramps, and skin diseases (Chukwjekwu et al., 2005). In addition, the leaves of *H. sauveolens* is used as a stimulant, carminative, sudorific, galactogogue, and as a cure for parasitic cutaneous disease (Mandal et al., 2007). The phytochemicals of *H. sauveolens* which revealed the present of alkaloids, flavonols, flavones, flavonones, terpenoids, tannins, aldehydes and ketones (Mbatchou et al., 2010). Their biological activities possessed antimicrobial (Joshi et al., 2008), anti-inflammatory (Qi et al., 2010), anti-proliferative activities (de Almeida et al., 2010) and induced cell cycle arrest and apoptosis (Chen et al., 2010). Moreover, the plant has been reported to exhibit antiplasmodial (Chukwujekwu, 2005), antimalarial (Ziegler et al., 2002), antinociceptive (Santos et al., 2007), antidiarrhoeal (Shaikat et al., 2012), anti-hyperglycemic (Mishra et al., 2011) and anti-inflammatory activities (Mahesh, 2001). The plant also possessed antirheumatic, antifertility, antiseptic (Chitra et al., 2009) and anticancer (Gurunagarajan and Pemaiah, 2011). Therefore, the present study investigated the effect of mintweed (*Hyptis sauveolens* (L.) poit) extracts on the apoptotic induction of human Jurkat, lymphocyte cancer cells.

5.3 Materials and methods

5.3.1 Chemicals and reagents

RPMI 1640, penicillin-streptomycin, Hepes and fetal bovine serum (FBS) were purchased from Gibco (New York, USA). Alamar blue and Hoechst 33258 and SeeBlue® Plus 2 prestained standard were purchased from Invitrogen (California, USA). Ribonuclease A from bovine pancreas, acrylamide, bis-acylamide were purchased from Sigma-Aldrich Company Ltd. (St. Louis MO, USA). Dimethylsulfoxide (DMSO) and ρ-formaldehyde 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, agarose, and Lambda DNA/ Hind III marker were purchased from Promega (Wisconsin, USA). 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). Nitrocellulose membranes was obtained from Amersham Biosciences (Vienna, Austria).

5.3.2 Collection of plant materials

Leaves of mintweed were collected from the plants grown on the grounds of Suranaree University of Technology (SUT) in the Nakhon Ratchasima, Thailand, in October 2008. The leaves were collected, washed under running tap water and then sun dried. The seeds were collected in January 2009 from the same place. The seed mucilage coat was sloughed off and then the seeds were air dried. *H.*

suaveolens was kindly identified by a plant taxonomist, Professor Dr. Pranom Chantaranothai.

5.3.3 Sample preparations

The dried leaves and seeds were ground into fine powder by electric blender and then 50 grams of each plant powder was extracted in 500 ml water or 70% ethanol (v/v) for 24 h by Soxhlet extraction apparatus. The extracts were filtered, evaporated (Buchi instruments, Switzerland) and lyophilized into powder (Freeze-zone 12 plus, Labconco Corporation, Missouri, USA). The powder extracts were stored at -20°C until used. The water and ethanolic extracts were dissolved in RPMI 1640 culture medium and 0.2% DMSO, respectively.

5.3.4 Cell culture

Human T lymphocyte leukemia cell lines, Jurkat E6-1, were purchased from the Korean cell line bank (Seoul, Korea). Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented (FBS), 1 mM sodium pyruvate, 100 IU/mL penicillin and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37°C incubator.

5.3.5 Analysis of nuclear morphology

Apoptotic cells were defined on the basis of nuclear morphology change such as chromatin condensation and DNA fragmentation. Nuclear morphology was examined using the method of Chen et al. (2008). The cells ($2.5x10^4$ cells/well) were cultured into a 96-wells plate in 100 μL media and incubated with various

concentrations of the extracts (100 µL/well). After incubation for 24 h, the medium was removed and washed twice with PBS, pH 7.4. The cells were fixed with 200 µL of ρ-formaldehyde (4%, v/v) for 20 min at room temperature and then washed with PBS. The cells were stained with 100 µL of Hoechst 33258 (10 µg/mL) in PBS containing 0.1% (v/v) triton x-100 solution in the dark at 37°C for 30 min. The cells were washed with PBS, and then observed and photographed immediately under the inverted fluorescence microscope (Olympus IX51 with Digital Camera DP50 + View Finderlite Program, Olympus corporation, Japan).

5.3.6 DNA fragmentation analysis

Apoptotic DNA fragmentation induced in Jurkat T cells following the extracts treatment was determined using the method described by Itoh et al., 1995 with slightly modification. The cells ($1x10^6$ cells/well) were cultured into a 6-well plate and incubated with various concentrations of the extracts for 0, 6, 12, and 24 h. After incubation, the cells were harvested by centrifugation at 2000 rpm (CT15RT versatile refrigerated centrifuge, Techcomp, HK) and washed with PBS, and then lysed with 180 µL lysis buffer (10 mM Tris-HCl, pH 8, 0.1M NaCl, 1 mM EDTA), 20 µL of 10% SDS was added, and the cells were incubated with 200 µL of Proteinase K (final concentration 100 µg/mL) at 56°C for 4 h in water bath. DNA was extracted twice with equal volume of phenol: chloroform: isoamyl (25:24:1) solution, the upper layer was collected, to which 3 M sodium acetate was added at a 1:10 (v/v) ratio and then precipitated with 2.5 volume of absolute ethanol and kept in -20°C overnight. DNA pelleted was collected by centrifugation at 12000 rpm 4°C for 30 min, washed with cold 70% ethanol twice times and then dried. The DNA was resuspended in

Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1mM EDTA). 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 5 µg DNA in TE buffer was mixed with ribonuclease A (RNase A), final concentration of 100 µg/mL and incubated at 37°C for 30 min. Loading dye was added to samples at 1:5 (v/v) ratio and the DNA fragments were separated on a 1.5% agarose gel with applying the voltage of 70 V for 90 min. The DNA gel was stained with ethidium bromide (0.5 µg/mL) and the DNA pattern was visualized under UV light, and photographed.

5.3.7 Western blot analysis for apoptotic protein

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. Among the proteins that play a key role in the regulation of apoptosis and that are affected by various apoptosis inducing agents are the members of the caspase and the Bcl-2 family. During apoptotic induction, the levels of protein in apoptotic cascade are changes. Therefore, increasing of the expression of pro-apoptotic proteins, Bax, and caspase-9 and also decreasing of the expression of anti-apoptotic protein, Bcl-2, are generally monitored.

5.3.7.1 Preparation of protein lysate

Jurkat cells, $4x10^6$ cells, were incubated with different concentrations of the extracts for difference time. The cells were harvested by centrifiguration at 2,000 rpm and washed twice with PBS then lysed with 180 µL lysis buffer (50 mM Tris, pH 8, 150 M Nacl, 5 mM EDTA, 0.5% NP-40, 0.5 mM PMSF,

Protease inhibitor cocktail) for 30 min on ice. The cell lysate was collected by centrifugation at 13,000 rpm (CT15RT versatile refrigerated centrifuge, Techcomp, HK) 4 °C for 30 min. The lysate was collected and kept in -80°C for further analysis and the aliquot part was used for protein determination.

5.3.7.2 Determination of protein concentration

Protein contents were measured by Bradford assay (Bradford, 1976). The protein lysate was diluted in lysis buffer (1:9). Ten microliters of diluted protein lysate was mixed with 200 µL of Bradford dye reagent (1:4 dilution) in a 96-wells plate. The reaction was incubated at room temperature for 10 min and then measured the absorbance at 595 nm. Bovine serum albumin (BSA) was used as a standard curve for protein determination.

5.3.7.3 SDS-PAGE gel electrophoresis and Western blotting

For western blot analysis, 40 µg of protein lysate was mixed with 5x SDS-gel loading buffer, and then boiled for 5 min. The protein lysates were loaded on 12% SDS-PAGE gels using the constant voltage of 120 V for 2.30 h and transferred onto nitrocellulose membrane by electroblotting with a constant 400 mA for 4 h at 4°C. Colored protein marker was used as a protein molecular weight standard. The nonspecific binding sites were blocked by incubating the blot with 3% (caspase-9) and 5% (Bcl-2, Bax and actin) (w/v) BSA in 0.1% (v/v) Tween-20 in Tris-buffered saline (TBST) for 1 h at room temperature. After blocking, the membrane was washed with TBS and incubated with the primary antibodies against anti-mouse Bcl-2 (1:1000 dilution with 5% BSA in TBST, 3 h), Bax (1:1000 dilution

with 5% BSA in TBST, 3 h), Caspase 9 (1:500 dilution with 1% BSA in TBST, 3 h) and actin (housekeeping protein) (1:1000 dilution with 5% BSA in TBST, 3 h) at room temperature. Then, the membrane was washed thrice in TBST for 5 min each and incubated with the diluted secondary goat anti-mouse IgG-horseradish peroxidase-conjugated antibody (1:5000 or 1:10000 dilution with 3% BSA in TBST) for 1 h at room temperature. The blotted membrane was washed thrice in TBST for 5 min each and then washed twice in TBS for 5 min each. The proteins were detected using a chemiluminescent detection solution for 5 min and autoradiographed on X-ray films.

5.4 Results and discussion

5.4.1 Analysis of nuclear morphology

The most common indicators of apoptosis include morphological changes examined under the inverted fluorescence microscope. All the extracts marked morphological changes in cell apoptosis after treated with different concentration of mintweed extracts for 24 h. The chromatin condensation and nuclear fragmentation found when the cells were stained with Hoechst 33258 staining (Figure 5.1 and 5.3). No apoptosis was found in the untreated group. MLE/e induced chromatin condensation and nuclear fragmentation in Jurkat cells at the concentrations of 400, 600 and 800 µg/mL. MLE/e at concentration of 800 µg/mL showed the highest chromatin condensation and nuclear fragmentation in Jurkat cells (Figure 5.1). MLE/w, MSE/e and MSE/w also exhibited the highest chromatin condensation and nuclear fragmentation in Jurkat cells at concentration of 1,200 (Figure 5.1), 3,000 and 8,000 µg/mL (Figure 5.2), respectively. Both of standard

control, catechin and ascorbic acid, possessed the highest chromatin condensation and nuclear fragmentation in Jurkat cells with the same concentration of 800 µg/mL (Figure 5.3). In this study, the results of morphological analyses showed that mintweed extract significantly killed Jurkat cells by changing nuclear morphology with dose dependent manner, apoptotic cell numbers increased with increasing concentration of the extracts. Liu et al., (2004) reported that Jurkat cells were exposed to oridonin, di-terpeniod compound, marked morphological changes in cell apoptosis, including condensation of chromatin and nuclear fragmentation, were found when Hoechst 33258 staining was used. Cha and Kim (2012) found that essential oil of *Cryptomeria japonica* induced morphological changes, most cells were detached from the dishes, and cell rounding and shrinking and induced cell death on KB cells after treated with essential oil with concentrations of 0.2 and 0.4 mg/mL for 12 h.

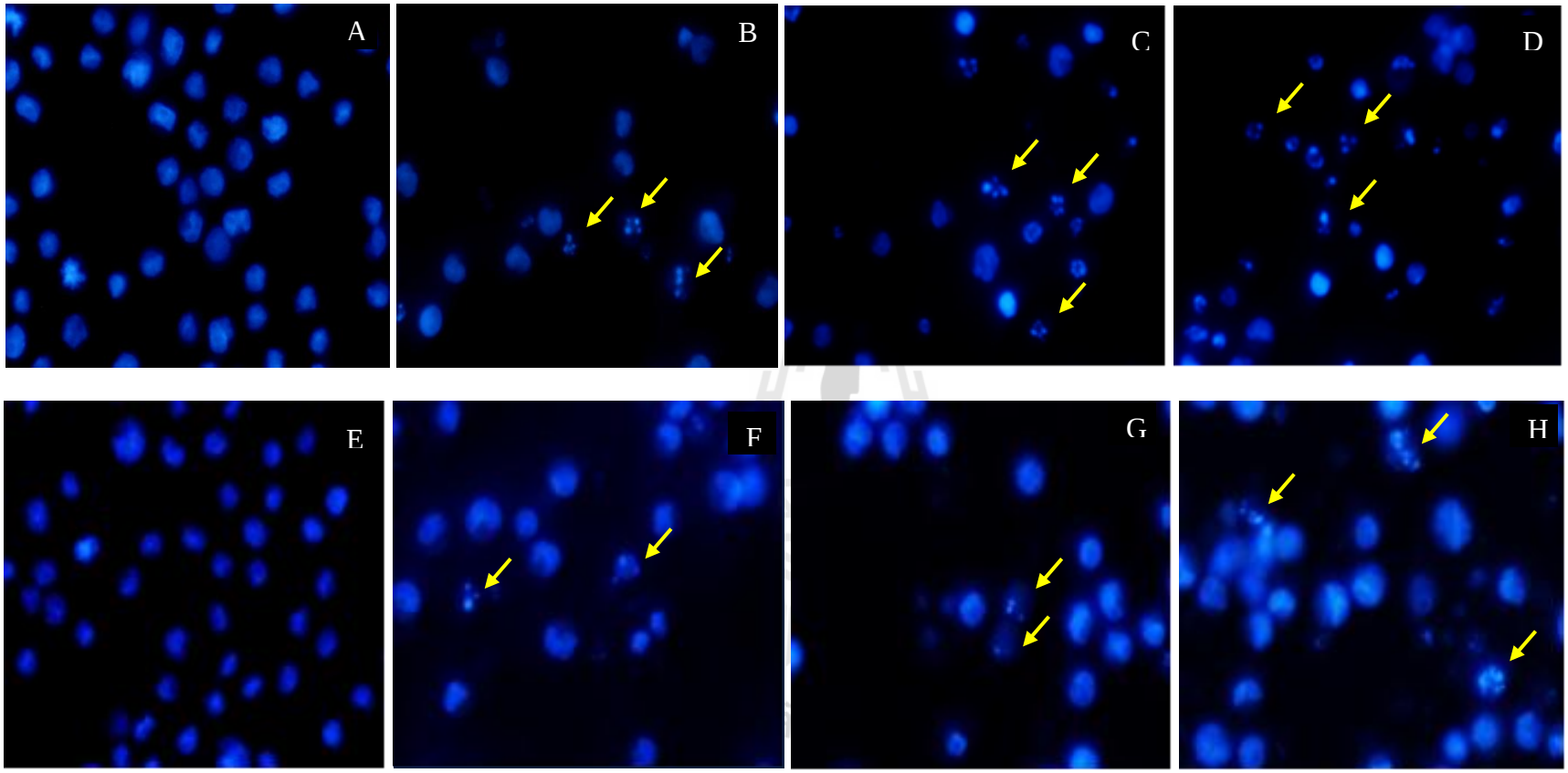

Figure 5.1 Apoptotic effects of mintweed leaf extracts on Jurkat cells, 24 h. A, MLE/e control; B, MLE/e 400 µg/mL; C, MLE/e 600 µg/mL; D, MLE/e 800 µg/mL; and E, MLE/w control; F, MLE/w 800 µg/mL; G, MLE/w 1000 µg/mL; H, MLE/w1200 µg/mL. The experiments were triplicate performed. (Hoechst 33258 staining, 400x)

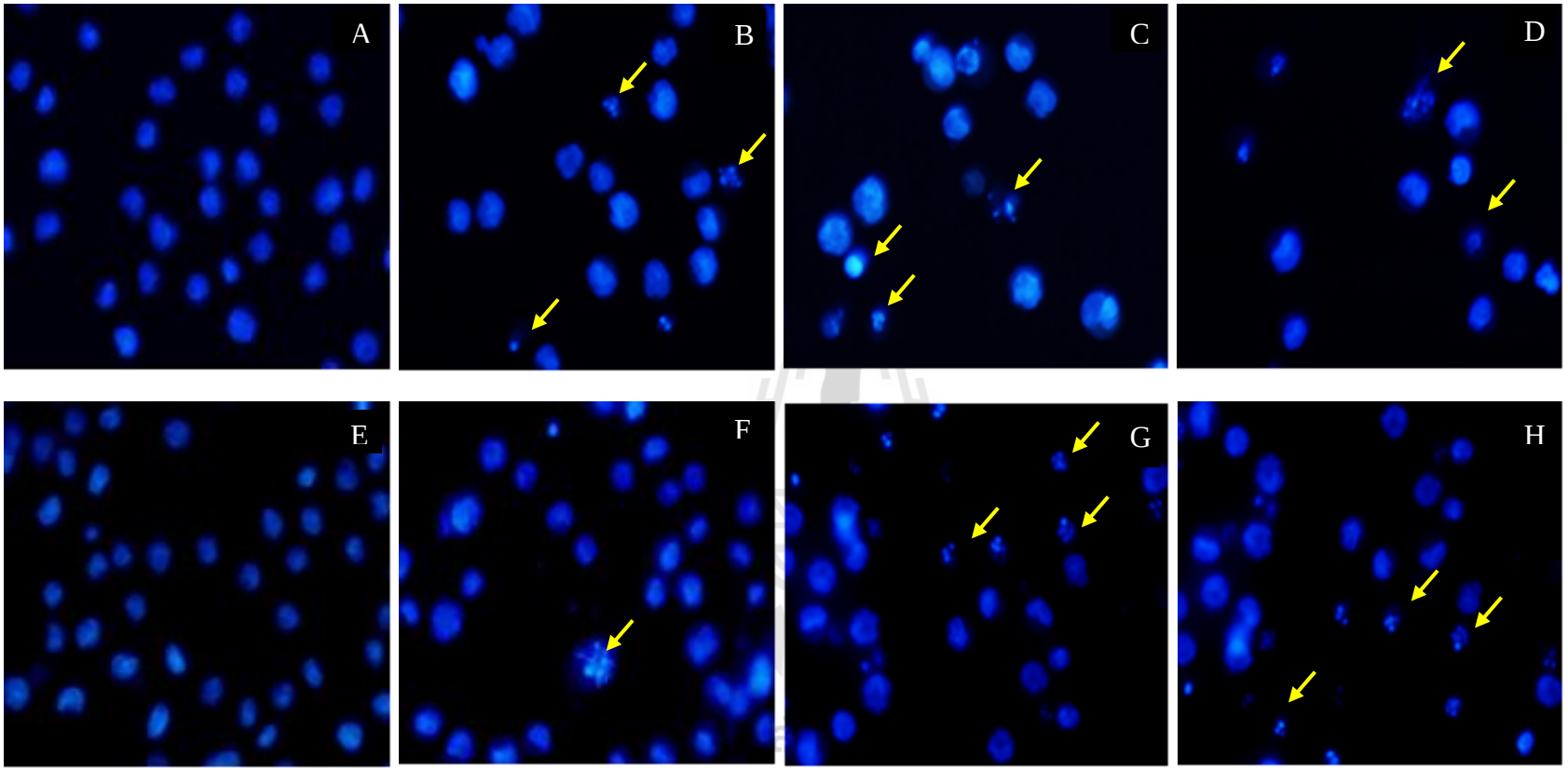

Figure 5.2 Apoptotic effects of mintweed seed extracts on Jurkat cells, 24 h. A, MSE/e control; B, MSE/e 2000 µg/mL; C, MSE/e 2500 µg/mL; D, MSE/e 3000 µg/mL; and E, MSE/w control; F, MSE/w 4000 µg/mL; G, MSE/w 6000 µg/mL; H, MSE/w 8000 µg/mL. The experiments were triplicate performed. (Hoechst 33258 staining, 400x)

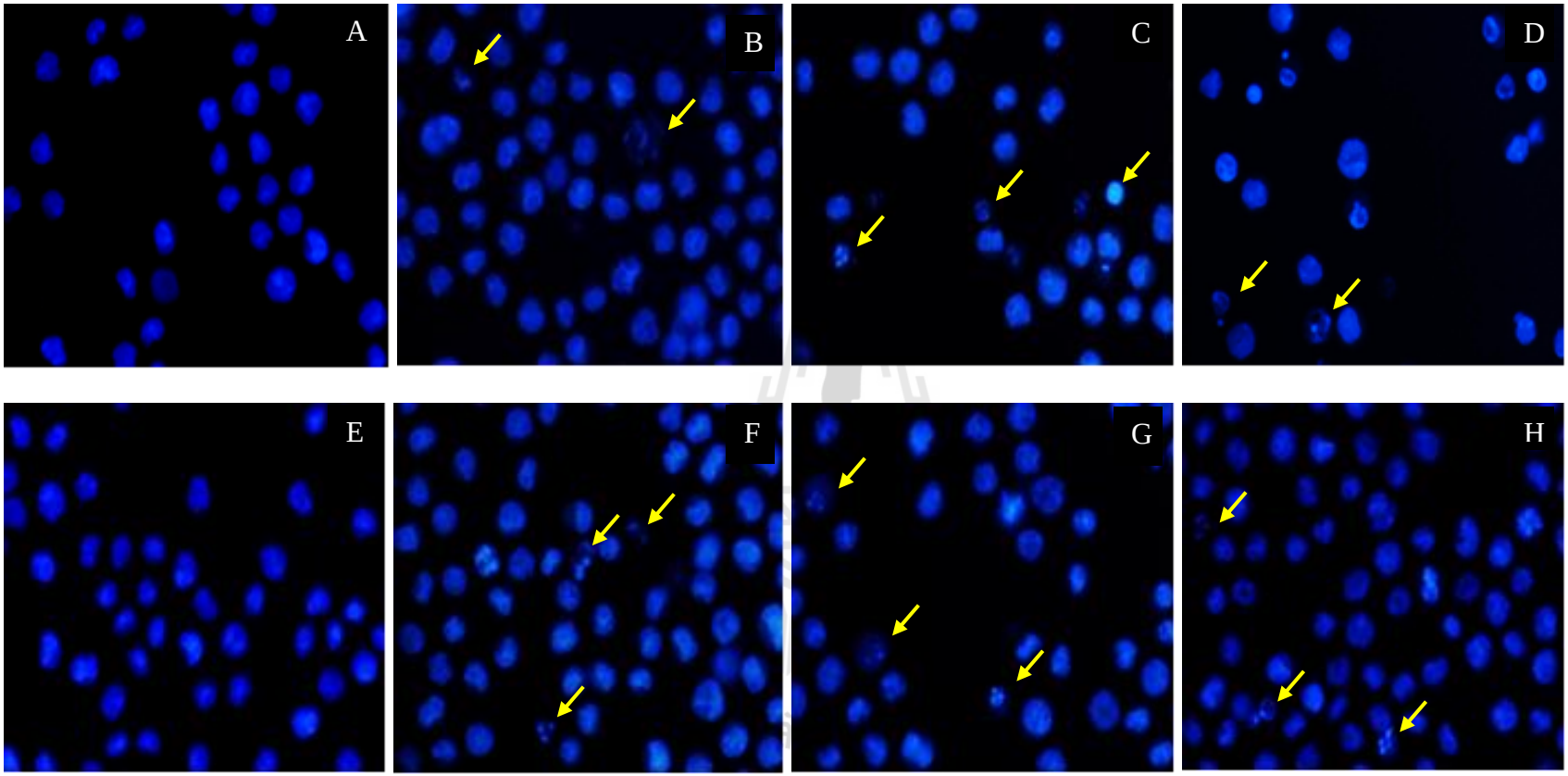

Figure 5.3 Apoptotic effects of catechin and ascorbic acid on Jurkat cells, 24 h. A, control; B, CA 400 µg/mL; C, CA 600 µg/mL; D, CA 800 µg/mL; and E, AA control; F, AA 400 µg/mL; G, AA 600 µg/mL; H, AA 800 µg/mL. CA, catechin and AA, ascorbic acid. The experiments were triplicate performed. (Hoechst 33258 staining, 400x)

5.4.2 DNA fragmentation of Jurkat treated cells

Agarose gel electrophoresis of DNA extracted from apoptotic cells, by revealing a characteristic pattern of preferential DNA cleavage at internucleosomal sections (DNA laddering), which is a hallmark of apoptosis, has become a common method used to confirm the apoptotic mechanism of cell death. Apoptotic DNA fragmentation of Jurkat cells was analyzed after treatment with various concentrations and times of the mintweed extracts for 24 h, DNA laddering was performed. As shown in Figure 5.4, the extracts could induce apoptosis in a dose- and time-dependent manner, as indicated by significant DNA fragmentation in treated cells, whereas no DNA fragmentation was seen in control cells. Jurkat cells treated with MLE/e induced DNA ladder, began to be detectable at concentration of 400 µg/mL, the concentration of 600 and 800 µg/mL induced an extensive DNA fragmentation (Figure 5.4A). In addition, cells treated with 600 µg/mL MLE/e, it began to occur oligonucleosomal DNA fragmentation at 6 h and more detected at 12 and 24 h for incubation times (Figure 5.4B). MLE/w also induced DNA fragmentation with concentrations of 1,000 and 1,200 µg/mL (Figure 5.5A) and cells treated with 1000 µg/mL MLE/w showed DNA fragmentation at 12 and 24 h (Figure 5.5B). Jurkat cells treated with 2,000, 2,500 and 3,000 µg/mL MSE/e exhibited significant DNA fragmentation, high concentration induced extensive DNA fragmentation (Figure 5.6A) and the DNA ladders began to detect at 12 and 24 h after the Jurkat cells were treated with 2000 µg/mL MSE/e (Figure 5.6B). MSE/w induced DNA fragmentation with concentrations of 6,000 and 8,000 µg/mL, high concentration induced extensive DNA fragmentation (Figure 5.7A), the DNA ladders began to be slightly detectable at 6 h and more detected at 12 and 24 h after the Jurkat cells were treated with 6000

μg/mL MSE/w (Figure 5.7, B). The results demonstrate that mintweed extracts is able to induce DNA ladder in a dose and time dependent manner. In each case, DNA is cleaved by an endonuclease that fragments the chromatin into nucleosomal units, size fragments of about 180-200 base pairs (bp), a well-known feature indicative of apoptosis and appear as a DNA ladder when run on an agarose gel (Compton, 1992). Gurunagarajan and Pemaiah (2011) reported that the ethanolic extract of *Hyptis suaveolens* exhibited extensive double strand breaks there by yielding a ladder appearance in EAC cells. Moreover, incubation of Jurkat cells with oridonin, di-terpeniod compound, 32-64 μmol/L for 48 h elicited a characteristic "ladder" of DNA fragmentation (Liu et al., 2004). In addition, Suhai et al. (2011) reported that *boswellia sacra* essential oil induced genomic DNA fragmentation in a time-dependent manner in three human breast cancer cell lines (T47D, MCF7, MDA-MB-231). The essential oil exhibited similar patterns and visible fragmented genomic DNA within 8 h after treatment. Fragmentation of DNA into nucleosomal unit is cause by a specific enzyme know as CAD (caspase activated DNase). Normally, CAD exists as an inactive complex with ICAD also known as DNA fragmentation. During the apoptosis ICAD cleaved by caspase-3 to release CAD leading to better DNA fragmentation (Nagata, 2000).

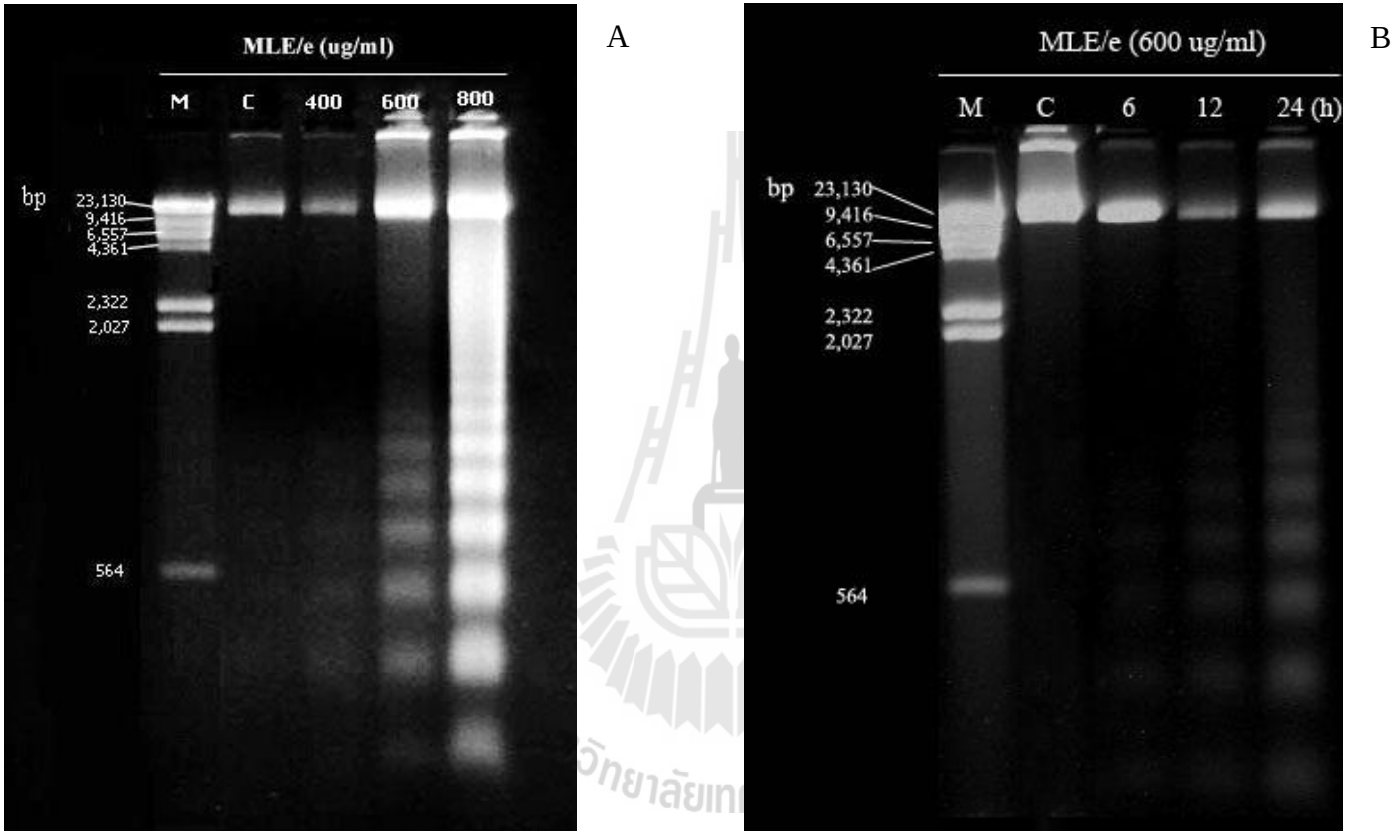

Figure 5.4 Induction of DNA fragmentation by mintweed extracts in Jurkat cells. (A) Cells were treated with different concentrations of MLE/e for 24 h. (B) Cells were treated with 600 µg/mL of MLE/e for indicated times. DNA fragmentation was analyzed by electrophoresis in 1.5% agarose gel. M, marker; C, control.

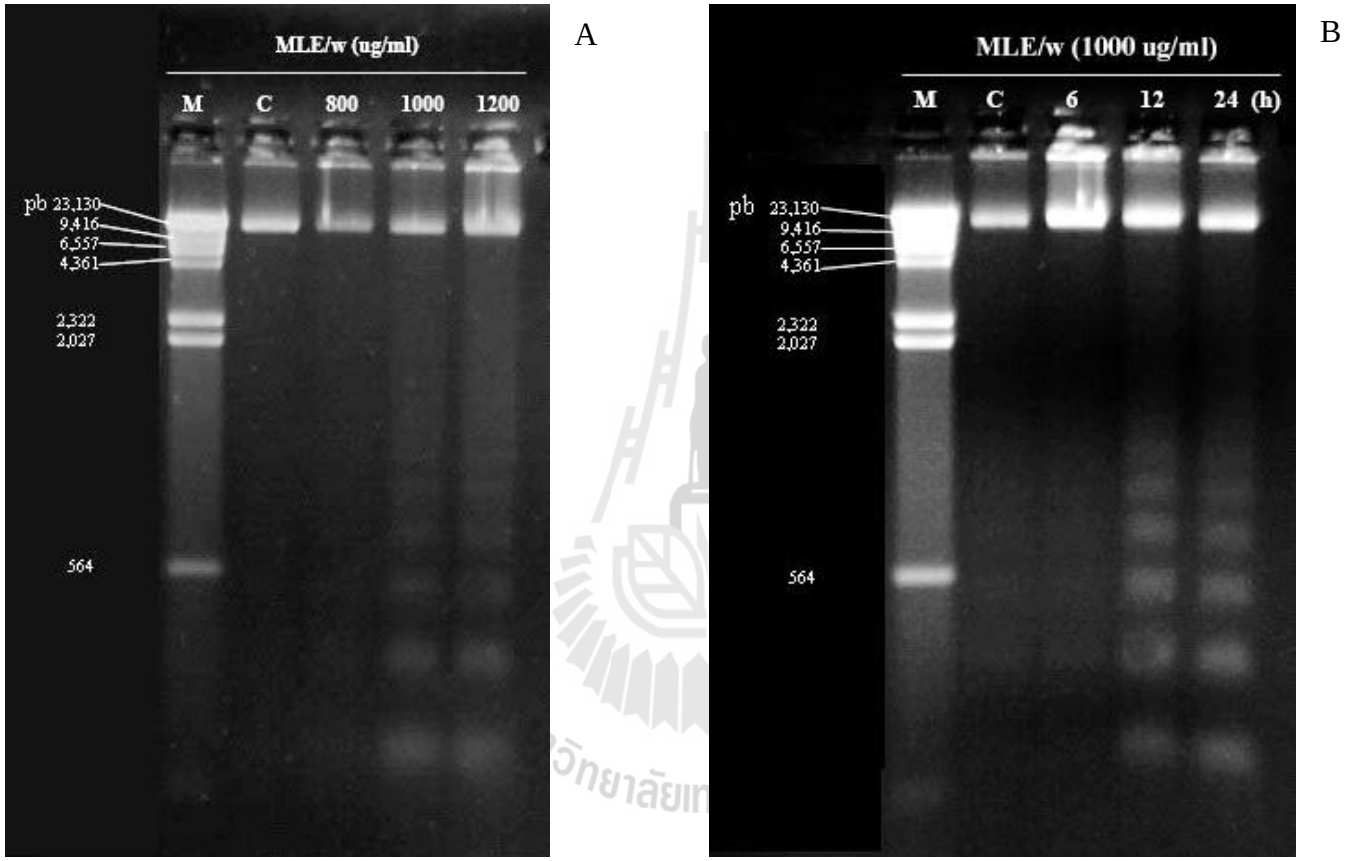

Figure 5.5 Induction of DNA fragmentation by mintweed extracts in Jurkat cells. (A) Cells were treated with different concentrations of MLE/w for 24 h. (B) Cells were treated with 1000 µg/mL of MLE/e for indicated times. DNA fragmentation was analyzed by electrophoresis in 1.5% agarose gel. M, marker; C, control.

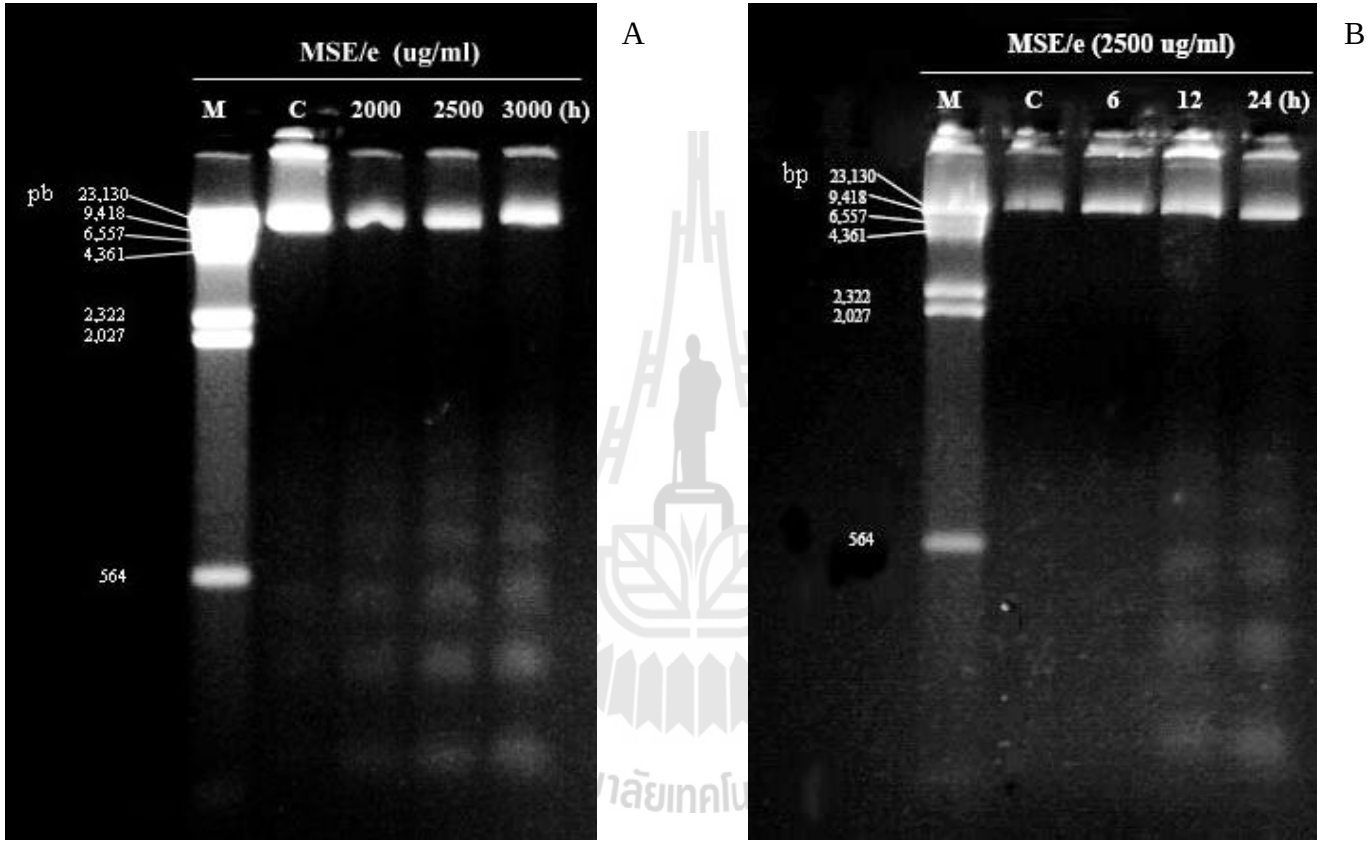

Figure 5.6 Induction of DNA fragmentation by mintweed extracts in Jurkat cells. (A) Cells were treated with different concentrations of MSE/e for 24 h. (B) Cells were treated with 2500 µg/mL of MLE/e for indicated times. DNA fragmentation was analyzed by electrophoresis in 1.5% agarose gel. M, marker; C, control.

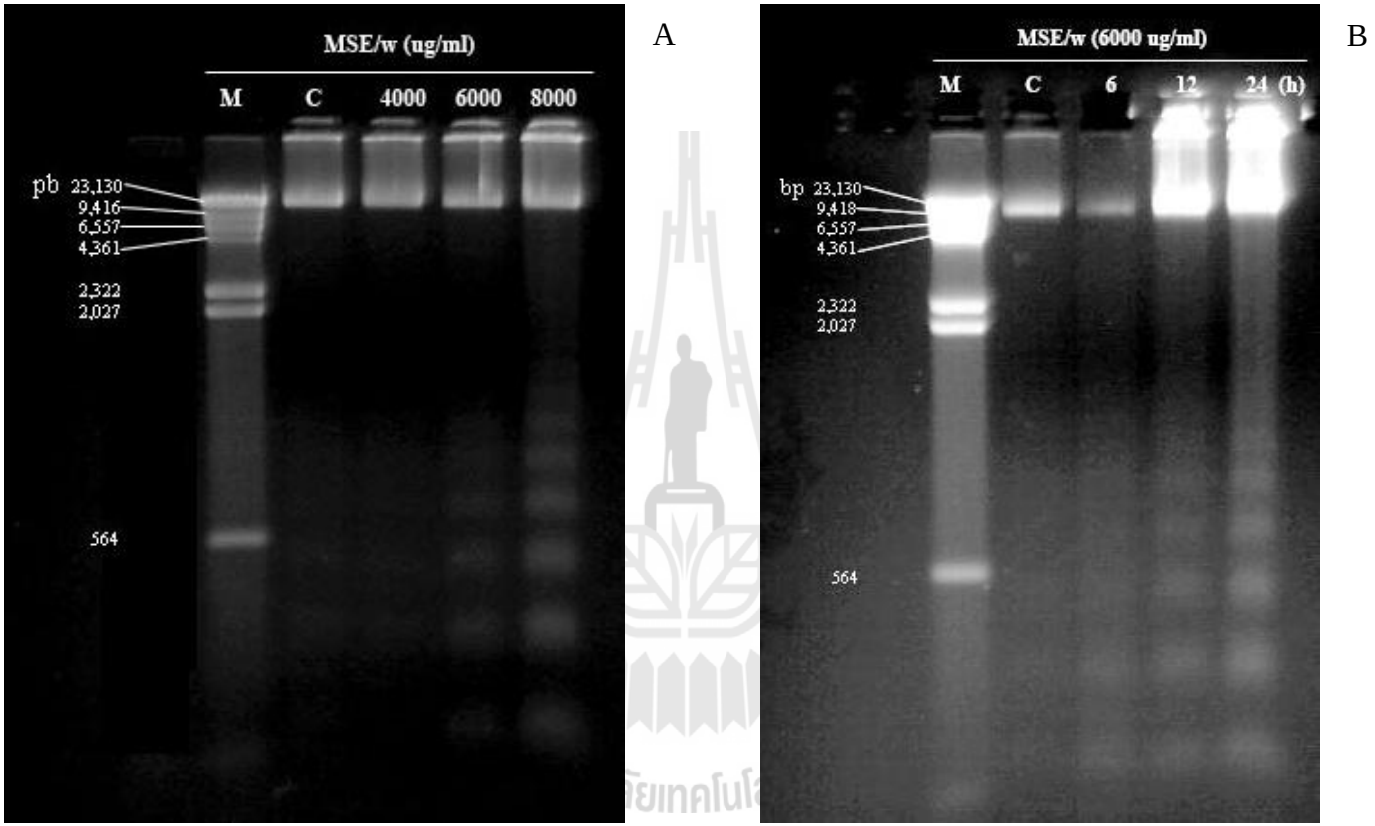

Figure 5.7 Induction of DNA fragmentation by mintweed extracts in Jurkat cells. (A) Cells were treated with different concentrations of MSE/w for 24 h. (B) Cells were treated with 6000 µg/mL of MLE/e for indicated times. DNA fragmentation was analyzed by electrophoresis in 1.5% agarose gel. M, marker; C, control.

Detection of DNA fragments by gel electrophoresis is a widely method to determine the mode of cell death. The internucleosomal DNA fragmentation, DNA laddering, is considered to be a very specific marker of apoptosis. The amount of the degraded DNA, especially within the range of mono- and oligonucleosomal DNA fragments, increases with progression of apoptosis. The intensity of DNA bands on the gels, therefore, reflects both the number of apoptotic cells and the degree of DNA cleavages per individual cell (Darzynkiewicz and Traganos, 1998).

5.4.3 Effect of mintweed extracts on caspase-9, apoptotic protein, Bax and Bcl-2

Among the proteins that play key roles in the regulation of apoptosis and are affected by various apoptosis inducing agents such as caspase-9 and the members of the Bcl-2 family. In order to see whether mintweed extracts affected the expression of caspase-9, Bcl-2 and Bax proteins, total proteins were extracted from untreated and mintweed-treated Jurkat cells and analyzed for the expression of proteins. The protein expression of caspase-9, Bax and Bcl-2 in Jurkat cells is shown in Figure 5.8 and 5.9. As shown in Figure 5.8A, MLE/e at 400 and 600 μg/mL induced a increase of Caspase-9 (46 kDa) in a concentration-dependent manner. Caspase-9 become apperent upon treatment of MLE/e at 400 and 600 μg/mL for 12 h. Activated caspase-9 was apparent upon treatment with 400 μg/mL MLE/e but not at 600 μg/mL MLE/e for 24 h, may due to cell death degraded at higher concentrations. Bcl-2 family proteins including Bcl-2 (26 kDa) and Bax (23 kDa) are the critical regulatory proteins for mitochondrial mediated cell death. The amount of anti-apoptotic protein, Bcl-2, dramatically decreased after treatment with 600 μg/mL

MLE/e for 12 h. In contrast, the expression of pro-apoptotic protein, Bax, began to dramatically increased in the presence of 400 μg/mL MLE/e for 12 h (Figure 5.8A). Activation of caspase-9 occurred at 800 and 1000 μg/mL MLE/w after 24 h of incubation. The amount of caspase-9 increased with concentration and time-dependent fashion. There was little change noted in the expression level of anti-apoptotic Bcl-2 after a treatment with MLE/w but the expression of Bax protein levels was dramatically increased after treatment with 800 and 1000 μg/mL MLE/w for 12 h (Figure 5.8B). As shown in Figure 5.9A, immunoblot analysis revealed that the MSE/e treatment incrased the caspase-9 protein level with concentration of 2500 μg/mL for 24 h. Moreover, MSE/e treatment began to dramatically decrease the Bcl-2 protein and increase the Bax protein level in the presence of 2000 μg/mL for 12 h. Caspase-9 expression apparently increased after treatment with 6000 and 8000 MSE/w for 24 h. The amount of Bcl-2 began to decrease after treatment with 6000 and 8000 μg/mL MSE/w for 12 h. In contrast, the expression of Bax began dramatical increased in the presence of 8000 μg/mL MSE/w for 24 h (Figure 5.9B). Cha and Kim (2012) reported that essential oil from *Cryptomeria japonica* induces apoptosis in human oral epidermoid carcinoma cells via mitochondrial stress and activation of caspases by approximately 50% and 70% degradation of procaspase-8 and -9 were observed when the cells were exposed to 0.2 and 0.4 mg/mL of the essential oil for 12 h, respectively. Moreover, essential oil treatment increased the intensity of the bands corresponding to Bax protein in mitochondrial fractions, but significantly reduced the level of Bcl-2 protein. Liu et al. (2012) reported that terpenoids from *Zingiber officinale* induced apoptosis in endometrial cancer cells

through the activation of p53, increase in p53 was associated with 90% decrease in Bcl2, whereas no effect was observed on Bax.

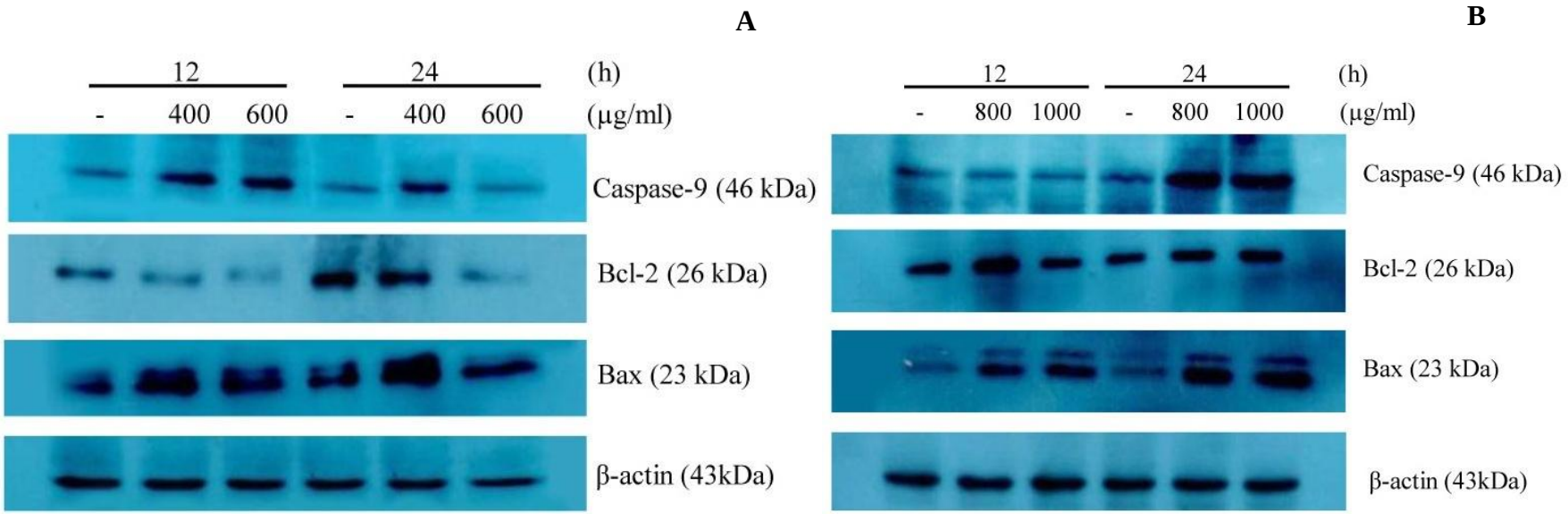

Figure 5.8 Effects of mintweed leaf extracts on caspase-9 and Bcl-2 family for 12 and 24 h. (A) Effect of MLE/e on caspase-9, Bax, and Bcl-2. Equal proteins of whole cell lysates from Jurkat cells with or without treatments of 400 and 600 μg/mL MLE/e were separated by 12% SDS-PAGE and were immunoblotted with antibodies against caspase-9, Bax, Bcl-2, and β-actin. (B) Effects of MLE/w on caspase-9, Bax, and Bcl-2. Equal proteins of whole cell lysates from Jurkat cells with or without treatments of 800 and 1000 μg/mL MLE/w were similarly performed. Two independent experiments were conducted and shown the same patterns of changes.

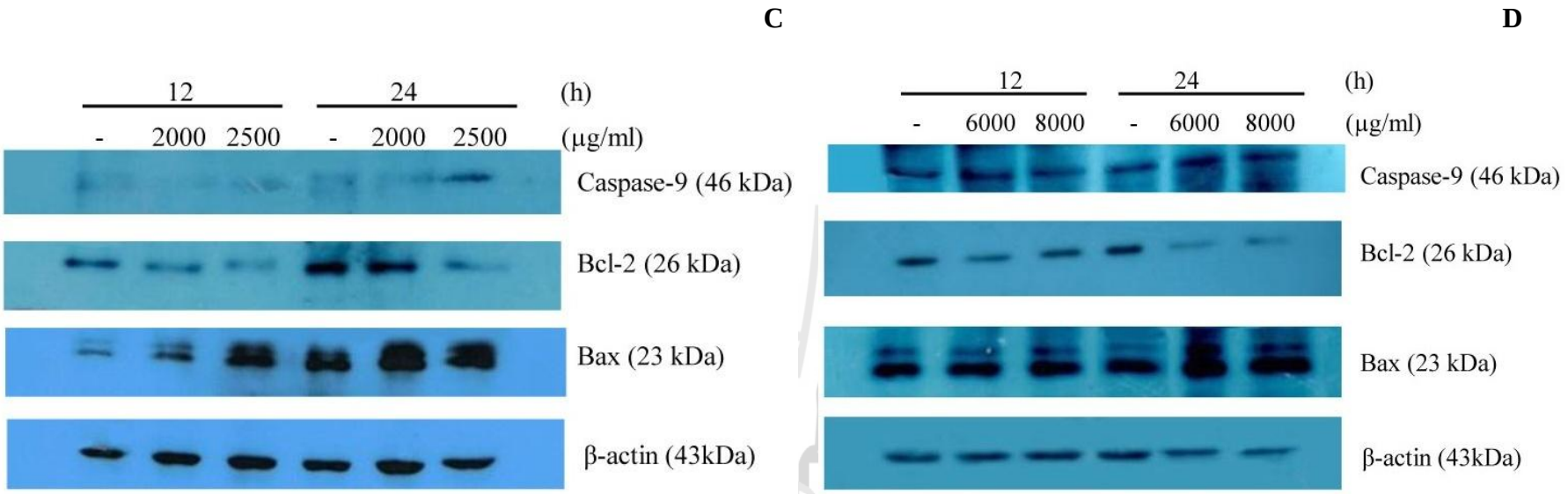

Figure 5.9 Effects of mintweed seed extracts on caspase-9 and Bcl-2 family for 12 and 24 h. (C) Effect of MSE/e on caspase-9, Bax, and Bcl-2. Equal proteins of whole cell lysates from Jurkat cells with or without treatments of 2000 and 2500 μg/mL MSE/e were separated by 12% SDS-PAGE and were immunoblotted with antibodies against caspase-9, Bax, Bcl-2, and β-actin. (D) Effects of MSE/w on caspase-9, Bax, and Bcl-2. Equal proteins of whole cell lysates from Jurkat cells with or without treatments of 6000 and 8000 μg/mL MSE/w were similarly performed. Two independent experiments were conducted and shown the same patterns of changes.

There are three families of Bcl-2 proteins that play an important roles in controlling cell life and death. The Bcl-2 family (e.g., Bcl-2 and Bcl-XL) functions to inhibit apoptosis, whereas the Bax subfamily (e.g., Bax, Bak, and Bcl-Xs) and the BH3-only family (Bid, Bad, and several others) promote apoptosis (Harris and Thompson, 2000). The pro-apoptotic Bcl-2 family members Bax and Bak have been observed to translocate from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form pores and mediate cytochrome c release (Nechushtan et al., 1999). Bax has been reported to induce the release of cytochrome c, which upon entry into the cytosol forms a complex with other molecules, Apaf-1 and the unprocessed proform of caspase-9 (Li et al., 1997). Several pathways involve p53-mediated apoptosis, and one of these is the Bcl-2 and Bax proteins. The Bax protein is a p53 target and known to promote cytochrome c release from mitochondria which in turn activates caspase-3. Regulation of Bax/Bcl-2 and caspases activity becomes important targets for cancer intervention. Caspase-3 the major executioner caspase (Cohen, 1997), thus activation of caspase-3, -8 and -9 is involved in apoptotic induction and found evident expression of activated caspase-3, -8 and -9 (Watanapokasin et al., 2011). These results suggested that the Bax induction and Bcl-2 down-regulation may be potentially involved in the mintweed extracts-induced apoptotic responds.

5.5 Conclusions

Induction of apoptosis is considered a possible mechanism of most of the chemotherapeutic agents. The target of the apoptosis signaling pathway is a promising strategy for the development of novel chemotherapeutic molecules. Some researches

have tried to develop potential chemotherapeutic agents from natural products. This study showed that all the mintweed extracts, especially MLE/e, induced apoptotic cell death in Jurkat cells by morphological changes, which were cell shrinkage, chromatin condensation and nuclear fragmentation, after treated with different concentrations of mintweed extracts for 24 h. MLE/e showed the highest activity in inducing the DNA fragmentation of Jurkat cells and DNA ladder formation with concentrations of 400 to 800 µg/mL. In addition, Western blot analysis showed that induction of apoptosis by mintweed extracts was accompanied by the increase of caspase-9, pro-apoptotic Bax and the decrease of Bcl-2 level. These data indicated that mintweed extracts were able to induce cell death through the mitochondrial death pathway. All the mintweed extracts, MLE/e could provide a molecular rationale for further therapeutic interventions in carcinogenesis. However, the cytotoxic effects on other cell lines and more detail to elaborate the mechanism of apoptotic action of mintweed extracts is need to be further studies.

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CHAPTER VI

CONCLUSIONS

In the present study, mintweed (*Hyptis suaveolens* (L.) Poit) extracts were investigated for the phytochemicals properties, antioxidant activities, cytotoxicity on Jurkat leukemia cells and normal PBMCs cells, and the apoptotic cell death pathway. Mintweed extracts contained a variety of phytochemicals which mostly were phenolic compounds, flavonoids and terpenoids. MLE/e had the highest total phenolic of 370.02 ± 7.10 mg GAE/g dried extract, followed by MLE/w, MSE/e and MSE/w of 319.45 ± 8.67 , 135.92 ± 2.17 and 77.02 ± 2.05 mg GAE/g dried extract, respectively. In addition, MLE/e also showed the highest total flavonoids of 278.81 ± 3.40 mg CAE/g dried extract followed by MLE/w, MSE/e and MSE/w of 240.81 ± 5.01, 86.28 ± 0.67 and 15.38 ± 0.21 mg CAE/g dried extract, respectively. Terpeniods was found in all extracts with different levels. Essential oils and tannin were contained in MLE/e, MLE/w and MSE/e. Saponins was found in MSE/e and MSE/w, whereas alkaloids was contained in only MSE/w. Antioxidant activity of mintweed extracts, which was determined by FRAP assay showed that MLE/e possessed the highest ferric reducing activity of 8.52 ± 0.44 µM $FeSO_4$/mg dried extract followed by MLE/w and MSE/e of 6.27 ± 0.03 and 2.01 ± 0.35 µM $FeSO_4$/mg dried extract, respectively. MSE/w showed the lowest antioxidant activity of 1.36 ± 0.00 µM $FeSO_4$/mg dried extract. DPPH assay, MLE/e showed the highest antioxidant activity and followed by MLE/w, MSE/e and MSE/w with IC_{50} values of 9.26 ± 0.08, 10.89 ± 0.07, 32.85 ±

0.05 and 147.17 ± 1.67 µg/mL, respectively. There were significant differences ($p < 0.05$) between the mintweed extracts and standards, catechin and ascorbic acid, in antioxidant activity. TBARS assay, MLE/e exhibited the highest antioxidant activity with IC_{50} value of 5.42 ± 0.14 µg/mL followed by MLE/w and catechin with IC_{50} values of 17.46 ± 0.23 and 17.92 ± 0.18 µg/mL, respectively. MSE/e had antioxidant activity with IC_{50} value of 39.77 ± 0.23 µg/mL, while the lowest antioxidant activity presented in MSE/w with IC_{50} value of 725 ± 15.40 µg/mL. When compared to a pure component, catechin, the IC_{50} value of MLE/e and MLE/w, revealed no significant differences ($p < 0.05$) between them in antioxidant activity. Mintweed extracts exhibited cytotoxicity on *A. salina* and an anti-proliferation on Jurkat leukemia cells and normal human PBMCs which could depend upon the different phytochemical components. MLE/e had the highest lethality on brine shrimp and was followed by MSE/w, MSE/w and MLE/w with LC_{50} values of 360.48, 470.60, 708.26 and 1,282.47 µg/mL, respectively. Moreover, the highest anti-proliferative effect on Jurkat cells was MLE/e with IC_{50} value of 553.52 ± 14.07 µg/mL, while the lowest one was MSE/w with IC_{50} value of 5,813.45 ± 111.25 µg/mL. MLE/w and MSE/e had anti-proliferative effect with IC_{50} values of 912.06 ± 16.86 and 2,385.95 ± 81.28 µg/mL, respectively. The highest anti-proliferative effect on normal lymphocytes was MLE/w with IC_{50} value of 1,140.52 ± 6.05 µg/mL which was followed by MLE/e and MSE/e with IC_{50} values of 1,356.17 ± 136.78 and 2,920.68 ± 155.38 µg/mL, respectively. The lowest anti-proliferative effect was MSE/w with IC_{50} value of 11,366.26 ± 266.28 µg/mL. The IC_{50} values of MLE/e and MLE/w were the most significant effects of all the extracts ($p < 0.05$). This study obviously indicated that the mintweed leaf extracts (MLE) possessed proliferative property on normal human

lymphocytes as opposed to the anti-proliferative property on human leukemia cell line. Apoptotic cell death on Jurkat cells was observed by morphological change and DNA fragmentation. All mintweed extracts, especially MLE/e, induced apoptotic cell death in Jurkat cells by morphological changes, which were cell shrinkage, chromatin condensation and nuclear fragmentation, after being treated with different concentrations of mintweed extracts for 24 h. MLE/e showed the highest activity for inducing the DNA fragmentation of Jurkat cells and DNA ladder formation with concentrations of 400 to 800 µg/mL. MLE/e killed Jurkat cells to a significant level by changing nuclear morphology with dose dependent manner. In addition, Western blot analysis showed that induction of apoptosis by mintweed extracts was accompanied by increase in the caspase-9, pro-apoptotic Bax and decrease in the Bcl-2 level. All the extracts, MLE/e is the most active extract that should be further investigated for targeting of the apoptosis signaling pathway and for a promising strategy for the development of a novel cancer chemotherapeutic agent.

APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

A.1 Folin-Ciocalteau method

- Gallic acid stock solution (1mg/mL)
  - Gallic acid 110 mg
  - 10% Ethanol 100 mL
- 2% Sodium carbonate solution
  - Sodium carbonate 2 g
  - DI water 100 mL
- Folin-Ciocalteau reagent (1: 1)
  - Folin solution 1 mL
  - Methanol 1 mL

A.2 Aluminium trichloride method

- 10% $AlCl_3.6H_2O$
  - $AlCl_3.6H_2O$ 1 g

  Adjust volume up to 10 mL with DI water
- 5% $NaNO_2$
  - $NaNO_2$ 0.5 g
  - DI water 10 mL

- 1 N NaOH
  - NaOH 4 g

  Adjust volume up to 100 mL with DI water

A.3 DPPH assay

- DPPH solution (0.04 mg/mL)
  - DPPH 0.004 g
  - DI water 10 mL

A.4 FRAP assay

- 0.1M Acetate buffer, pH 3.6
  - Sodium acetate trihydrate 0.82 g
  - Glacial acetic acid 1.6 mL

  Adjust pH 3.6 and made volume up to 100 mL with DI water
- 10 mM TPTZ
  - TPTZ 0.031 g
  - 1N HCl 400 µL

  Adjust volume up to 10 mL with DI water
- 20 mM Ferric chloride
  - $FeCl_3$ 0.0324 g

  Adjust volume up to 10 mL with DI water
- Working FRAP reagent
  - 0.1M Acetate buffer 10 mL
  - 10 mM TPTZ 1 mL
  - 20 mM Ferric chloride 1 mL

- **10 mM Ferrous sulfate stock solution**
  - $FeSO_4.7H_2O$ 0.027 g
  - DI water 10 mL

A.5 TBARS assay

- MDA Stock solution (1 mg/mL)
  - MDA 1 mg
  - Ethanol 1 mL
- Phosphatidyl chlorine (PC) Stock solution (10 mg/mL)
  - PC 0.02 g
  - Ethanol 2 mL
- 150 mM KCl Stock solution
  - KCl 1.12 g
  - DI water 100 mL
- 10 mM $FeCl_3$ Stock solution
  - $FeCl_3$ 0.0162 g
  - DI water 10 mL
- 10 mM Sodium-L-ascorbate Stock solution
  - Sodium-L-ascorbate 0.01 g
  - DI water 5 mL
- Stop solution I
  - Trichloroacetic acid (TCA) 20 g
  - 0.25 N HCl 2.08 mL
  - 0.01% BHT (Stock 1%) 100 µL

  Adjust volume up to 100 mL with DI water

- Stop solution II

  - Thiobarbituric acid (TBA) 0.4 g

  Adjust volume up to 100 mL with 75 mL of acetic acid and 25 mL of 1N NaOH

- Working stop solution (1: 1)

  - Stop solution I 50 mL
  - Stop solution II 50 mL

A.6 Spay reagent for thin layer chromatography

- Potassium hydroxide (KOH)

  - 5% ethanolic potassium hydroxide, spray and evaluate under UV 366 nm

- 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

- 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 100°C for 5-10 min then evaluated under visible light.

- 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 110°C for 5-10 min then evaluated under visible light.

- Iron (III) chloride reagent ($FeCl_3$)
  - 10% iron (III) chloride aqueous solution, spray and evaluate under visible light.
- DPPH reagent
  - 1 mg DPPH dissolve in 10 mL methanol, spray and evaluate under visible light.
- Kedde reagent
  - 5 mL of 3% ethanolic 3,5-dinitrobenzoic acid are mixed with 5 mL of 2 NaOH. The plate is sprayed with 10 mL of freshly prepared mixture, evaluated under visible light.

APPENDIX B

PREPARATION OF REAGENTS FOR CELL CULTURE

B.1 RPMI 1640 medium

- RPMI 1640 medium 1 pack
- Sodium bicarbonate 1.5 g
- Sodium pyruvate 0.11 g

Add DDI water to 800 mL, adjust pH to 7.2 and bring the volume to 1000 mL with DDI water.

B.2 Complete RPMI 1640 medium (200 mL)

- RPMI 1640 medium 176 mL
- Fetal Bovine Serum (FBS) 20 mL
- Penicillin Streptomycin (Pen Strep) 2 mL
- HEPES 2 mL

B.3 1X 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 to 1000 mL with DI water, filter sterile (store at 4°C).

B.4 Hoechst 33258 staining

- Fixing solution (4% *p*-formaldehyde)
  - 37% formaldehyde 2.2 mL
  - PBS 17.8 mL
- Hoechst staining solution
  - Hoechst 33258 (1 mg/mL) 40 µL
  - 1% TritonX-100 400 µL
  - DI water 3,560 µL

  (Store at -20°C)

B.5 DNA fragmentation

- Lysis buffer
  - 10 mM Tris-HCl, pH 8 0.157 g
  - 0.1 M NaCl 0.584 g
  - 1 mM EDTA (stock 0.5 M) 200 µL
  - 10% SDS 10 g

  Adjust pH to 8.0 with 1N HCl and made volume up to 50 mL with DI water (store at 4°C)
- Storage DNA maker buffer
  - 10 mM Tris-HCl 0.0157 g
  - 10 mM NaCl 0.0058 g
  - 1 mM EDTA (stock 0.5 M) 20 µL

  Adjust pH to 7.5 with 1N HCl and made volume up to 50 mL with DI water (store at 4°C)

- Reconstituted proteinase K
  - 50 mM Tris-HCl 0.3940 g
  - 10 mM $CaCl_2$ 0.0735 g

  Adjust pH to 8.0 with 1N HCl and made volume up to 50 mL with DI water (store at 4°C)
- TE buffer
  - 10 mM Tris-HCl 0.157 g
  - 0.5 M EDTA 0.2 mL

  Adjust pH to 8 with 1N HCl and made volume up to 100 mL with DI water (store at 4°C)
- 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 1000 mL (store at 4°C)

B.6 Western blot

- RIPA buffer
  - 50 mM Tris, pH 8 0.600 g
  - 150 M Nacl 0.877 g
  - 5 mM EDTA (stock 0.5 M) 1000 µL
  - 0.5% NP-40 500 µL

  Made volume up to 100 mL with DI water

- Lysis buffer
  - RIPA buffer 955 µL
  - 200 mM PMSF 5 µL
  - Protease inhibitor cocktail (25x) 40 µL
- 5x Sample loading buffer
  - 0.5 M Tris-Hcl, pH 6.8 5,000 µL
  - SDS 1 g
  - Glycerol 3,000 µL
  - 5% Bromophenol blue 500 µL
  - β-Merceptoethanol 500 µL

  Made volume up to 10 mL with DI water, aliquot 1 mL and store at -20°C.

  Make to 1x in sample mixture and boil for 5 min.
- 10x Running buffer
  - Tris base 29 g
  - Glycine 144 g
  - SDS 10 g

  Made volume up to 1000 mL with DI water
- 1x Running buffer
  - 10x Running buffer 100 mL
  - DI water 900 mL

  Do not adjust the pH, it should be around 8.1-8.4.

- 10x Transfer buffer
  - 25 mM Tris-base 29 g
  - 192 mM glysine 144 g

  Made volume up to 1000 mL with DI water
- 1x Transfer buffer
  - 10x Transfer buffer 100 mL
  - 20% methanol 200 mL
  - DI water 700 mL
- 10x TBS
  - 20 mM Tris-base 24.22 g
  - 150 mM NaCl 87.66 g

  Made volume up to 1000 mL with DI water
- 1x TBS
  - 10x TBS 100 mL
  - DI water 900 mL
- 0.1% Tween20 in TBS (TBST)
  - 10x TBS 100 mL
  - Tween-20 1 mL
  - DI water 899 mL
- Blocking solution (3%)
  - Bovine serum albumin (BSA) 3 g
  - TBST 100 mL

- 30% Acrylamide (29.2:0.8)
  - Acrylamide 29.2 g
  - Bis-Acrylamide 0.8 g

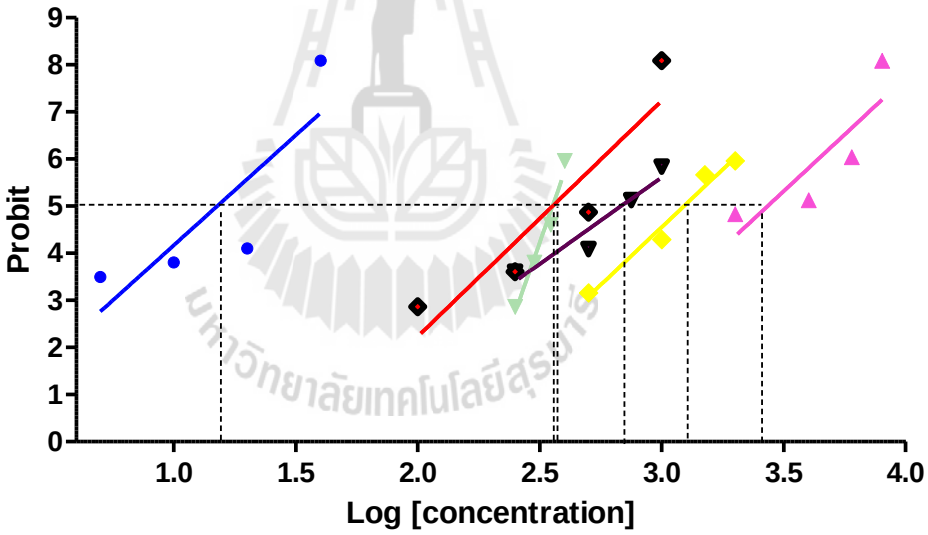
  Dissolve in 100 mL of DI water and filter through Whatman paper.

- Seperating gel (12%) (for 2 gel, 15 mL)
  - 1.5 M Tris-Hcl, pH 8.8 3,750 µL
  - 20% SDS 75 µL
  - Bis-Acrylamide 6,000 µL
  - 10% Ammonium persulphate 75 µL
  - TEMED 10 µL
  - DI water 5,090 µL
- Stacking gel (4%) (for 2 gel, 5 mL)
  - 0.5 M Tris-Hcl, pH 8.8 1,250 µL
  - 20% SDS 25 µL
  - Bis-Acrylamide 670 µL
  - 10% ammonium persulphate 25 µL
  - TEMED 5 µL
  - DI water 3,025 µL

APPENDIX C

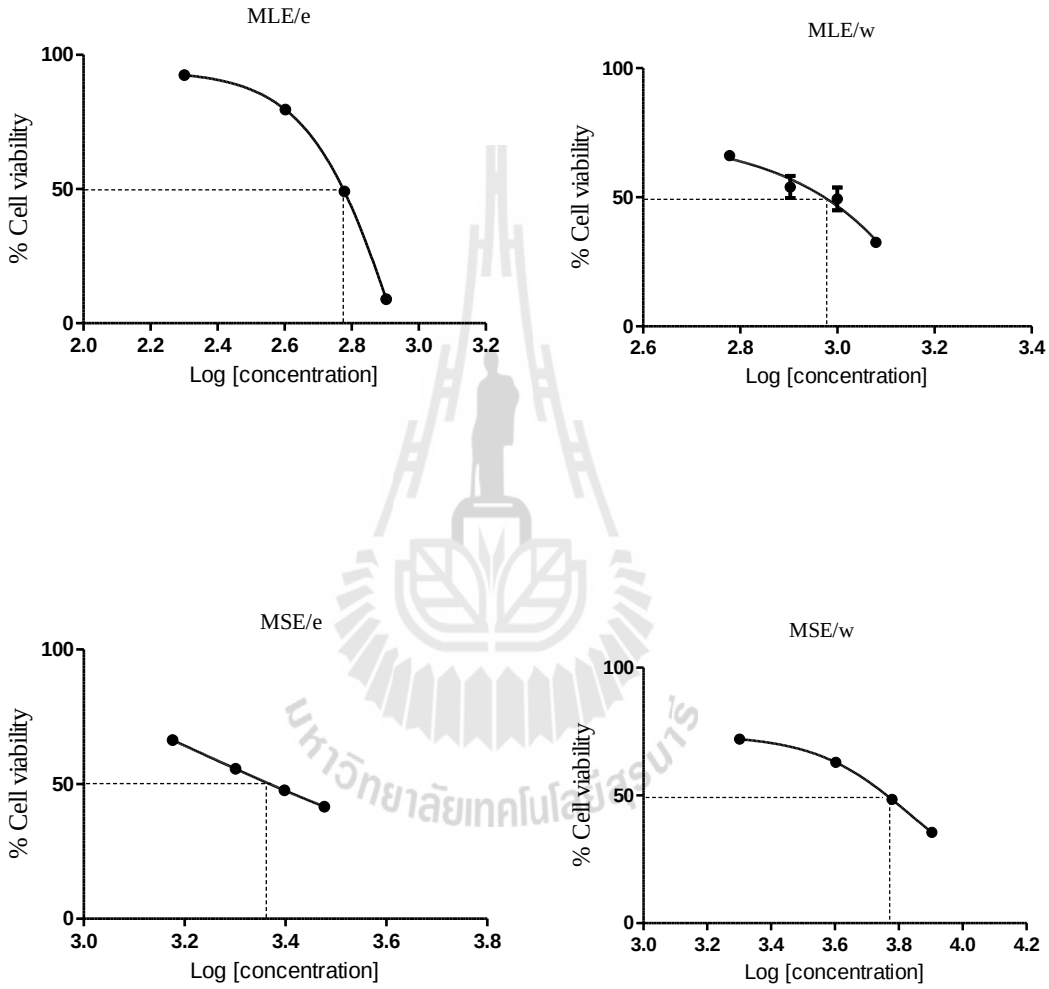
DOSE RESPONSE CURVE OF MINTWEED EXTRACTS

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



- Ascorbic acid, y= 4.6733x - 0.5032
- Catechin, y= 4.8157X - 11.536
- MLE/e, y= 14.696x - 32.507
- MLE/w, y= 4.8997x - 10.143
- MSE/e, y= 3.6789x - 5.4228
- MSE/w, y= 5.0059x - 7.7769

C2. The anti-proliferative effects of mintweed extracts on Jurkat cancer cells at 24 h incubation



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Poster presentations

1. Sumalee Musika and Korakod Indrapichate. (2008). Some phytochemical properties and antioxidant activities of mintweed (*Hyptis suaveolens*) extracts. The 2nd International Conference on Natural Products for Health and Beauty (NATPRO). 17th to 19th December. Naresuan University, Phayao, Thailand.
2. Sumalee Musika and Korakod Indrapichate. (2010). Phytochemical, antoxidant, antiproliferative properties and apoptotic induction in human acute leukemia, Jurkat T cells, by mintweed (*Hyptis suaveolens* (L.) Poit) leaf extract. The 2nd Asian Conference on Environmental Mutagens Harmonize Gene & Environment Asian Health Promotion (ACEM). 15th to 18th December. Dusit Thani Pattaya Hotel, Pattaya, Thailand.