การชักนำให้เกิดเอพอพโตซิสในเซลล์เจอร์กัตโดยตำรับยามะเร็งร้อยแปด

นางสาวปัทมา เทพภูษาวัฒนา

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INDUCTION OF APOPTOSIS IN JURKAT CELLS BY MARENG-ROIPAD FORMULATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmacology
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Thesis Title	Induction of apoptosis in Jurkat cells by Mareng-roipad formulation
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ปัทมา เทพภูษาวัฒนา : การชักนำให้เกิดเอพอพโตซิสในเซลล์เจอร์กัตโดยตำรับยามะเร็งร้อยแปด. (INDUCTION OF APOPTOSIS IN JURKAT CELLS BY MARENG-ROIPAD FORMULATION) อ. ที่ปรึกษา: ผศ. ดร. วัชรี ลิมปนสิทธิกุล, อ. ที่ปรึกษาร่วม: ศ. พญ. ธาดา สืบหลินวงศ์ 88 หน้า.

มะเร็งร้อยแปดเป็นตำรับยาแพทย์แผนไทยที่ใช้ในการรักษามะเร็งมานานกว่าร้อยปี การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ของตำรับยาต่อเซลล์มะเร็งเม็ดเลือดขาว (เซลล์เจอร์กัต) และต่อเซลล์เม็ดเลือดขาวปกติ โดยการใช้สารสกัดน้ำและสารสกัดแอลกอฮอล์เพื่อศึกษาถึงความเป็นพิษต่อเซลล์ ฤทธิ์การชักนำให้เซลล์ตายแบบเอพอพโตซิส และกลไกการออกฤทธิ์ในการชักนำให้เซลล์ตายแบบเอพอพโตซิส จากการใช้วิธีย้อมเซลล์ตายด้วยสี propidium iodide (PI) พบว่าสารสกัดทั้งสองชนิด (10-100 ไมโครกรัม/มิลลิลิตร) มีความเป็นพิษต่อเซลล์เจอร์กัตตามความเข้มข้นของสารสกัดและตามระยะเวลาที่เซลล์ได้รับสารสกัด โดยสารสกัดน้ำมีความแรงมากกว่าสารสกัดแอลกอฮอล์ ในการศึกษาฤทธิ์การชักนำให้เซลล์เจอร์กัตตายแบบเอพอพโตซิสของสารสกัดทั้งสอง (10-100 ไมโครกรัม / มิลลิลิตร) ทำโดยการย้อมเซลล์ด้วย annexin V-FITC และ PI และวัดผลโดยใช้ flow cytometer ผลการทดลองพบว่าสารสกัดทั้งสองชนิดชักนำให้เซลล์ตายแบบเอพอพโตซิสตามความเข้มข้นของสารสกัด โดยมีค่า IC_{50} ของสารสกัดน้ำและแอลกอฮอล์เป็น 15 และ 150 ไมโครกรัม/มิลลิลิตร ตามลำดับ ซึ่งฤทธิ์นี้ทดสอบยืนยันได้ด้วยวิธีวัดปริมาณดีเอนเอที่ลดลงกว่าเซลล์ปกติ (hypodiploid DNA) ในเซลล์ที่ตายแบบเอพอพโตซิส สารสกัดทั้งสองมีฤทธิ์ต่ำมากในการชักนำให้เซลล์เม็ดเลือดขาวจากอาสาสมัครปกติตายแบบเอพอพโตซิส ในการศึกษากลไกการชักนำให้เซลล์เจอร์กัตตายแบบเอพอพโตซิส ทำโดยการใช้สารยับยั้งการทำงานของเอนไซม์ caspases คือ z-VAD-fmk และสารขัดขวางการทำงานของ Fas ligand (FasL) คือ แอนติบอดีต่อ FasL พบว่า สารสกัดออกฤทธิ์ผ่านการสื่อสารระหว่าง Fas และ FasL ซึ่งเป็น death receptor และผ่านการกระตุ้นการทำงานของเอนไซม์ caspase ผลจากการศึกษาครั้งนี้แสดงให้เห็นว่าสารสกัดน้ำและสารสกัดแอลกอฮอล์ของตำรับยามะเร็งร้อยแปดทำให้เซลล์มะเร็งเม็ดเลือดขาวตายแบบเอพอพโตซิส โดยมีกลไกการออกฤทธิ์ผ่าน death receptor ตำรับยานี้น่าจะมีความสามารถเป็นยาแผนไทยในการรักษามะเร็งเม็ดเลือดขาวได้

สาขาวิชา เภสัชวิทยา
ปีการศึกษา 2550

ลายมือชื่อนิสิต.....ปัทมา เทพภูษาวัฒนา.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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PATTAMA TEPPUSAWATTANA: THESIS TITLE. (INDUCTION OF APOPTOSIS IN JURKAT CELLS BY MARENG-ROIPAD FORMULATON) THESIS ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., THESIS COADVISOR: PROF. TADA SUEBLINVONG, M.D., 88 pp.

Mareng-roipad formulation is a traditional medicine which is alternatively used for cancer treatment longer than a century. This study intended to evaluate anti-tumor activity of the formulation on human acute leukemic T cells, Jurkat cells, and on normal human peripheral blood mononuclear cells (PBMCs). The water and the ethanol extracts of the formulation were investigated for cytotoxicity, apoptotic induction and mechanisms of apoptotic induction. By propidium iodide (PI) staining assay, both water and ethanol extracts (10-1000 μg/ml) exhibited cytotoxicity in a dose and time-dependent manner with the higher potency of the water than the ethanol extract. The Jurkat cell apoptosis induced by the extracts (10-1000 μg/ml) was examined by annexinV-FITC staining monitored by fluorescence flow cytometer. Both extracts induced apoptosis of Jurkat cells in a dose-dependent manner with IC_{50} at 15 μg/ml of the water and 150 μg/ml of the ethanol extract. The apoptotic activity of both extract was confirmed by assessing hypodiploid DNA in apoptotic cells after PI staining. These extracts had much less apoptotic activities on PBMCs from healthy subjects. The mechanisms of apoptotic induction of the extracts were also investigated by using a general caspase inhibitor, z-VAD-fmk, and an anti-Fas ligand (FasL) antibody. It was evaluated that the extracts induced Jurkat cell apoptosis mainly by Fas-FasL signaling pathway and in part by caspase activation. The results in this study indicate that the water and the ethanol extracts of Mareng-roipad formulation can induce human leukemic cell apoptosis mainly by the death receptor pathway. This formulation may have potential to be a traditional medicine for treatment of leukemia.

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

AICD	activation-induced cell death
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
APAF 1	apoptosis protease activating factor 1
BAD	BCL2 antagonist of cell death
BAP31	BCl-2-associated protein-31
BAX	BCL2 associated x protein
BCL2	B-cell CLL/Lymphoma 2
BH	BCL2 homology domain
BH3	BCL2 homology 3 domain
BID	BH3 interacting domain death agonist
CARD	caspase recruitment domains
CO_2	carbon dioxide
DD	death domain
DED	death effector domain
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FADD	Fas associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
FLIP	FLICE-inhibitory protein
FLIPL	FLICE -inhibitory protein long form
FLIPS	FLICE -inhibitory protein short form
h	hour
HCl	hydrochloric acid
IAPs	inhibitors of apoptosis proteins
mg	milligram(s)
ml	milliliter(s)

M	molar (mole per liter)
ng	nanogram(s)
NaCl	sodium chloride
NF-κB	nuclear factor κB
PBS	phosphate buffer saline solution
PBMCs	peripheral blood mononuclear cells
PT	permeability transition
pH	the negative logarithm of hydrogen ion concentration
r.p.m	revolution per minute
S.E.M.	standard error of mean
Smac	second mitochondria-derived activator of caspases
TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
VDAC	voltage-dependent anion channel
°C	degree Celsius
μg	microgram(s)

CHAPTER I

INTRODUCTION

Background and Rationale

Cancer is still one of the leading causes of death in people all over the world and Thailand as well [1]. It is a highly heterogeneous disease, occurring in multiple tissue types and displaying great genetic diversities. Recent understanding reveals that the underlying etiology of cancer is a consequence of both dysregulated proliferation and apoptosis suppression [2]. Each of these defects provides an obvious opportunity for intervention. Many currently used chemotherapeutic agents were designed to perturb proliferation. For many years, the cytotoxic actions of these agents were ascribed solely to their ability to induce genotoxic damage. During the past decade, however, the evidence is gradually demonstrated that they also utilize the apoptotic pathway in induce cancer cell death. Although the mechanisms by which chemotherapeutic agents can kill tumors via apoptotic pathways is still controversial, the killing of tumors through the induction of apoptosis has been now recognized as a preferable strategy for the identification of anticancer drugs [3].

Apoptosis is a tightly regulated programmed cell death that permits the removal of damaged, senescent or unwanted cells in multicellular organism, without damage to the cellular environment. It also plays an important role in the development of various diseases including cancer [4]. Recent knowledge on apoptosis has provided the basis of many novel targets in apoptotic process for cancer therapy and prevention. Much effort has been directed toward the search for new compounds as well as the identification of herbs that influence apoptosis and its mechanism of action.

There have been worldwide efforts to discover new anticancer agents from plants. One of the approaches used for the selection of plants that may contain new biologically active compounds is the ethnomedical data approach, in which the selection of a plant is based on the prior information on the folk medicinal use of the plant. Thai traditional medicinal herbs have been used in the treatment of different

diseases in the country for centuries. There have been claims that some traditional herbs can successfully treat cancer when using in combined formulation. However, the detailed pharmacological activities and mechanisms of action of this formulation have never been elucidated. Mareng-roipad is an herbal formulation consisting of over 40 kinds of dried and powdered plants. This formulation is used as traditional medicine to treat cancer in and around Petchaburi province for more than a century without any scientific evidence.

When it comes to the successful eradication of cancer cells, ultimately, all roads lead to apoptosis-programmed cell death. In this study, the human leukemic T cell apoptosis induced by the water and the ethanol extracts from Mareng-roipad formulation were investigated. Their effects on apoptotic induction and mechanisms of action were performed.

Objective

1. To investigate the cytotoxicity and the apoptotic induction of the water and the ethanol extracts of Mareng-roipad formulation on human leukemic T cells.
2. To study the selectivity of the extracts on tumor cells by comparing to normal cells.
3. To elucidate mechanisms of apoptotic induction of the extracts on human leukemic T cells.

Hypothesis

The water and the ethanol extracts of Mareng-roipad formulation can induce human leukemic T cells apoptosis

Keywords

Apoptosis

Jurkat cells

Mareng-roipad

CHAPTER II

LITERATURE REVIEWS

Recent progress has broadened our understanding of cancer and its underlying etiology. Cancer is a disease attributed to excessive cell proliferation as well as defective in apoptosis. This leads to cellular expansion and accumulation of tissue mass. Many current chemotherapeutic agents are designed to exploit excessive proliferation by inducing cytotoxicity or cytostasis to halt tumor progression. Previous studies have been demonstrated that these agents kill tumor cell by two alternative ways including apoptosis or programmed cell death and necrosis.

Necrosis is a violent form of cell death caused by a range of noxious chemicals, drugs, biological agents, or physical damage. It is associated with the rupture of cell membrane, swelling of cell and its organelles, release of lysosomal enzymes, and random disruption of cell structure. Necrotic cells cause the formation of an inflammatory process that damages the cells and their surrounding tissues.

In contrast, apoptosis or programmed cell death is the consequence of a series of precisely regulated events that are frequently altered in tumor cells. It is a cleaner type of death, where the chromatin is condensed, the DNA becomes fragmented and vesicles, known as 'apoptotic bodies', are formed. These are rapidly phagocytized by the macrophages with the result that the cell disappears without any inflammatory phenomena.

The basic differences between necrotic and apoptotic processes of cell death underscore the reason why apoptosis, and not necrosis, represents the most desirable target mechanism for the induction of cell death in tumor cells. The detailed of apoptosis is described below.

Programmed cell death by apoptosis

Apoptotic cells can be recognized by stereotypical morphological changes: The cell shrinks, shows deformation and looses contact to its neighboring cells. Its chromatin condenses and marginates to the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, calls “apoptotic bodies” which contain cytosol, the condensed chromatin, and organelles (Fig. 1). The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing any inflammatory response. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles **[5]**. Apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disruptive of the cells. During necrosis, the cellular contents are released uncontrolled into the cell’s environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue **[6]**.

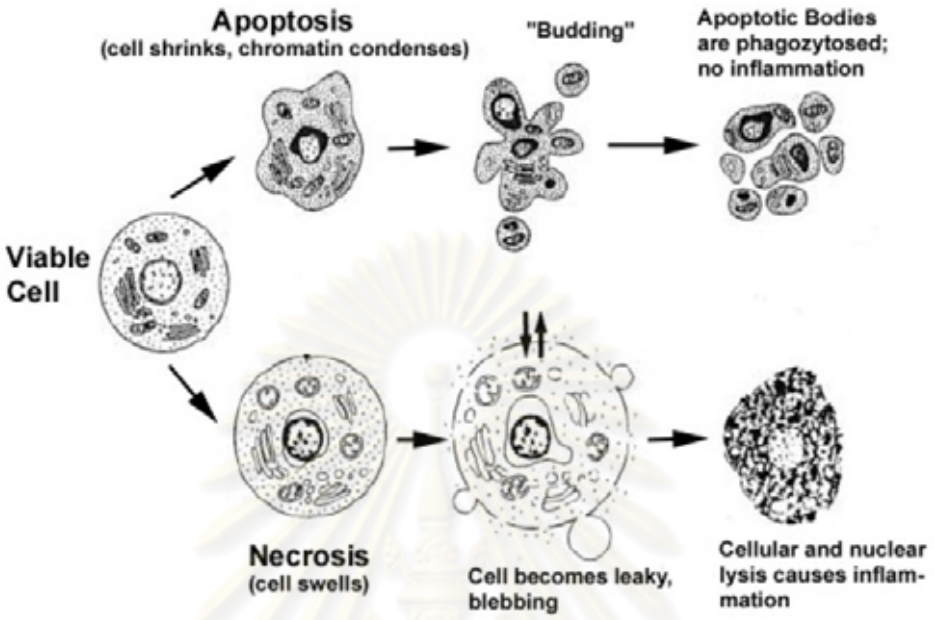


Figure 1: Hallmarks of the apoptotic and necrotic cell death process.

Molecular mechanisms of apoptosis signaling pathways

There are three major signaling pathways of apoptosis (Fig. 2): the death receptor pathway, the mitochrondial pathway, and a recently recognized endoplasmic reticulum (ER) stress pathway.

In the death receptor-signaling pathway, the signal is provided by the interaction between the ligand and death receptor, recruitment of adapter proteins, and activation of initiator and effector caspases. In the mitochondrial signaling pathway, a number of molecules are released from the mitochondria intermembrane space into the cytoplasm, where they interact with adapter proteins and activate initiator caspases, which are distinct from that of death receptor pathway activated initiator caspases. These initiator caspases then activate common effector caspases, resulting in apoptosis. In the ER-stress pathway,

apoptosis is mediated via activation of yet another initiator caspase (caspase-12) and then activation of common effector caspases as well as via its connection to the mitochondria [7].

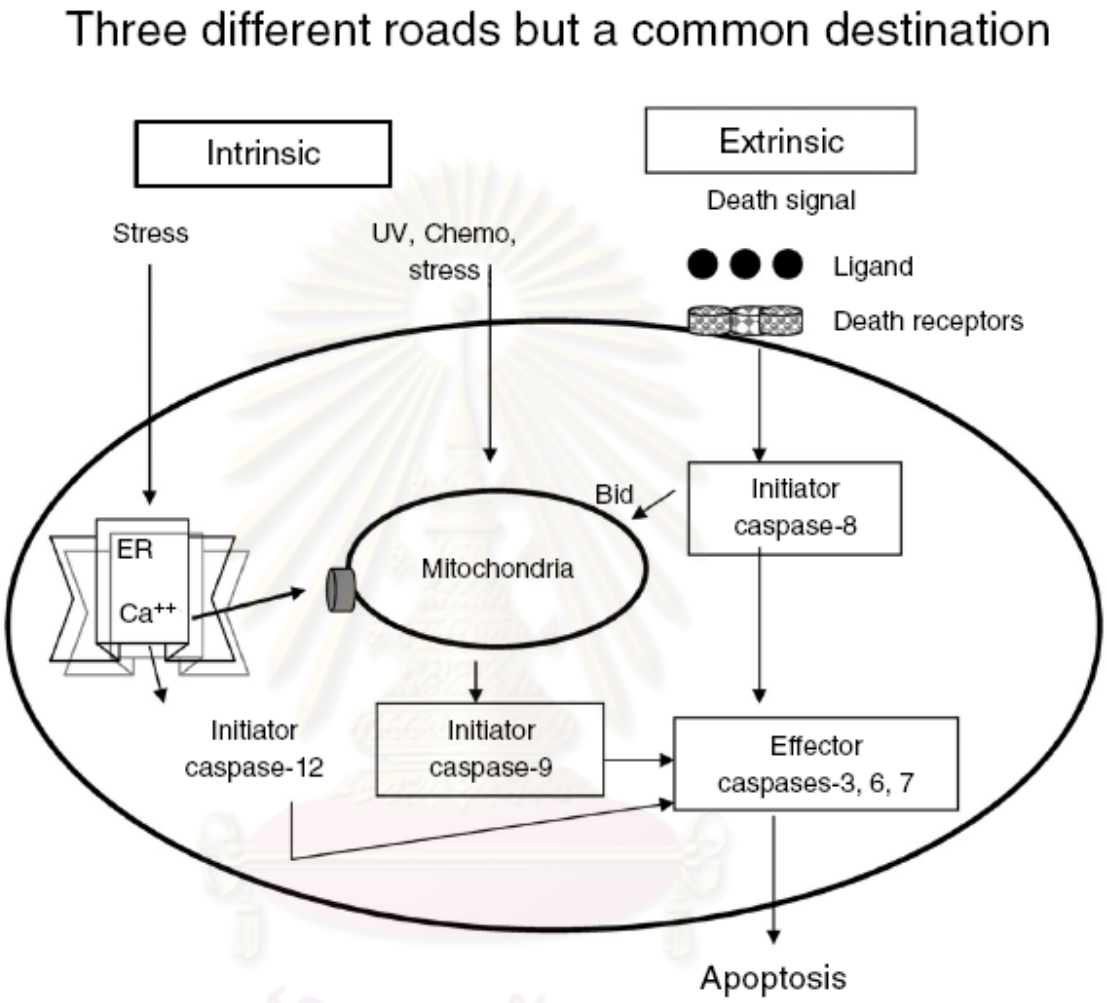


Figure 2: Three different pathways of apoptosis.

The death receptor pathway or the extrinsic apoptosis pathways

The death receptor pathway or the extrinsic pathway is mediated by the activation of death receptors on the cell surface that transmit apoptotic signals after ligation with their specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas or CD95, and the TNF-related apoptosis-

inducing ligand (TRAIL) receptors (DR-4 and DR-5) [8]. All members of the TNFR family consist of cysteine rich extracellular subdomains which allow them to specifically recognize their ligands. The molecular pathways by which TNFR1 and Fas effect apoptosis have been best characterized, with the role of TRAIL receiving attention more recently. Fundamentally, the death receptors share a similar means by which the caspase enzymatic cascade is activated (Fig. 3) and the Fas/Fas ligand (FasL) signaling pathway is discussed below.

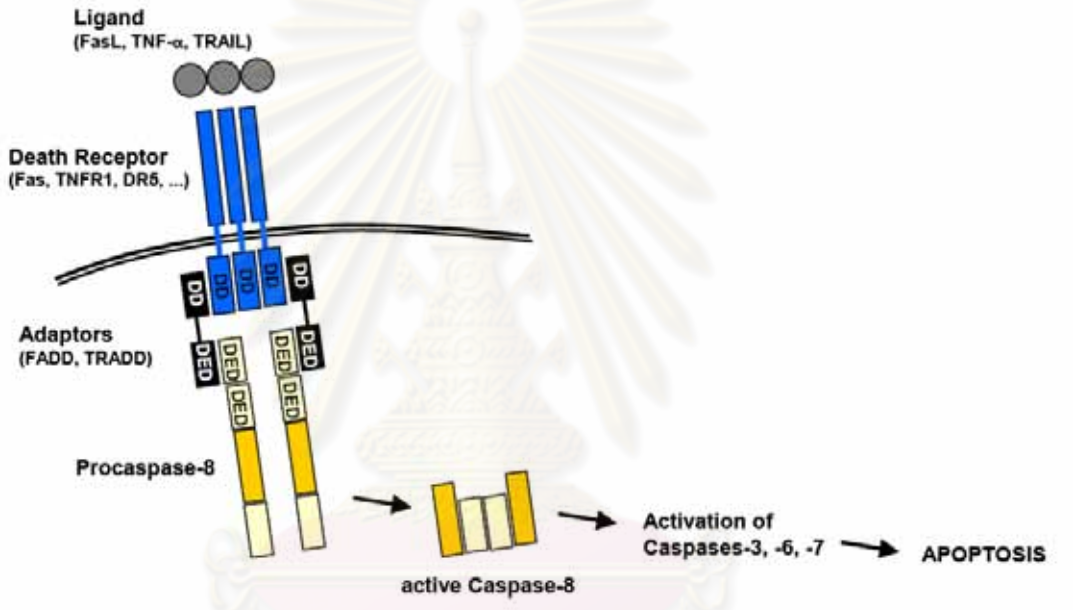


Figure 3: Receptor-mediated caspase activation at the DISC.

Fas/FasL pathway of apoptosis

Fas is a receptor that is widely expressed and constitutively present on lymphocytes; however, FasL displays a more restricted expression and is not present in resting lymphocytes. FasL is induced upon activation of lymphocytes and can be cleaved from the cell surface by metalloprotease. Therefore, FasL may be found in the soluble form

in vivo and can trigger apoptosis **[9]**. The steps of the Fas-mediated apoptosis-signaling pathway are shown in Fig. 4. Upon ligation with FasL or anti-Fas monoclonal antibodies, Fas undergoes trimerization. Because cytoplasmic domain does not have intrinsic enzymatic activity, it recruits an adapter protein, the Fas-associated death domain (FADD), which interacts with the homologous death domain (DD) of Fas by protein–protein interaction. FADD also contains a death effector domain (DED), and through homologous and protein–protein interaction, it recruits and binds to pro-caspase-8 (FLICE) to form a death-inducing signaling complex (DISC). Pro-caspase-8 undergoes dimerization and is activated without cleavage to generate active caspase-8. Active caspase-8 is released from the DISC into the cytoplasm, where it cleaves downstream effector pro-caspases to generate active effector caspases, which cleave a number of substrates, including transcription factors, enzymes (involved in DNA repair, cell cycle progression, and DNA cleavage), and structural proteins **[10]**,to elicit the characteristic morphological and biochemical features of apoptosis. Cells with Fas, harboring the capacity to induce such direct and mainly caspase-dependent apoptosis pathways were classified to belong to type I cells **[11]**. Fas is also called type I transmembrane receptor.

In type II cells, the signal coming from the activated receptor does not generate a caspase signaling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signaling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol **[12]**. Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with 7-fold symmetry that triggers the activation of the initiator procaspase-9 **[13]**. Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-6, and caspase-7, ultimately resulting in cell death **[14]**.

In type II cells, pro-caspase-8 levels are very low, and therefore, the caspase cascade is amplified via the mitochondrial pathway. Caspase-8 cleaves Bid, a Bcl-2 family member, to produce a truncated form of Bid, which then translocates from the cytoplasm to the mitochondria and exerts its pro-apoptotic effect by inducing conformational changes in Bax, resulting in the release of cytochrome c, activation of caspase-9, and finally, activation of effector caspases to induce apoptosis [15].

Apoptosis mediated by the Fas–FasL interaction is regulated by other DED-containing molecules, namely the FLICE-inhibitory protein (FLIP). This protein contains two DEDs. Cellular FLIP is present in two alternatively spliced isoforms, the long (FLIPL) and short (FLIPS) forms. FLIPL contains a COOH terminal domain beyond two DEDs and resembles caspase-8 and caspase-10. However, FLIPL is devoid of protease activity [16]. The DED of FLIP binds to Fas-FADD complexes and inhibits the recruitment and activation of pro-caspase-8, and therefore, FLIP acts as an anti-apoptotic molecule [16–19]. More recently, it has been reported that FLIP also promotes the activation of NF-κB and extracellular-regulated kinase signaling pathway by recruiting adapter proteins [20].

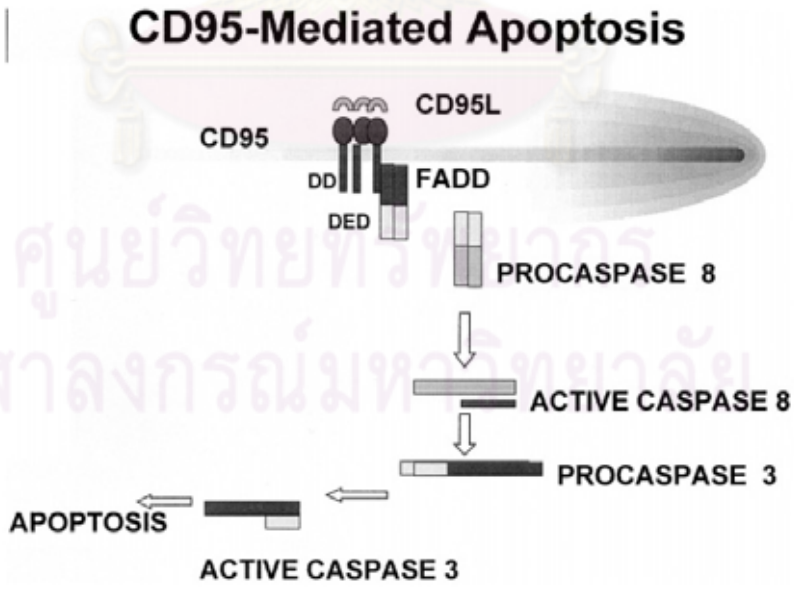


Figure 4: The Fas-FasL pathway of apoptosis

Intrinsic apoptosis pathways

The mitochondrial pathway of apoptosis

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs **[21-22]**. The mitochondrial pathway of apoptosis begins with the permeabilization of the mitochondrial outer membrane. It is suggested that permeabilization can be either permeability transition (PT) pore dependent or independent mechanism **[23]**. The PT pore is comprised of the matrix protein cyclophilin D, the inner mitochondrial membrane protein adenine nucleotide translocator (ANT), and the outer mitochondrial membrane protein voltage dependent anion channel (VDAC) **[24]**. The opening of the PT pore triggers the dissipation of the proton gradient created by electron transport, causing the uncoupling of oxidative phosphorylation. The opening of the PT pore also causes water to enter the mitochondrial matrix, which results in swelling of the intermembranal space and rupturing of the outer membrane causing the release of apoptogenic proteins **[23-24]**. Released proteins include cytochrome *c* **[25]**, apoptosis inducing factor (AIF) **[26]** and endonuclease G **[27-28]**. Once released from the mitochondria, cytochrome c binds to the adapter molecule apoptotic protease activating factor-1 (APAF-1) in the presence of ATP/dATP and recruits pro-caspase-9 to form apoptosome **[29]** (Fig. 5). This complex promotes the activation of caspase 9, which in turn activates effector caspases that collectively orchestrate the execution of apoptosis. AIF **[26]** and endonuclease G **[28]** both contribute to DNA fragmentation and subsequent chromosomal condensation, which are hallmark features of apoptosis. Other proteins released upon mitochondrial outer membrane permeabilization include Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP (inhibitor of apoptotic protein)-associated binding protein with low PI) and Omi/HtrA2 (high temperature requirement A2), which antagonize IAPs thereby promoting caspase activation **[30-31]**.

PT pore independent mitochondrial membrane permeabilization is regulated by Bcl-2 family members, which are characterized by Bcl-2 homology (BH) domains [23]. The Bcl-2 family can be subdivided into anti-apoptotic members such as Bcl-2 and Bcl-xL and pro-apoptotic species. Pro-apoptotic members are grouped into two categories based on the expression of BH domains (BH1-4). Multi-domain proteins comprise BH domains 1-3 and include Bax, Bak, and Bok. The other sub-group, the BH3 only proteins consists of Bad, Bik, Bid, Puma, Bim, Bmf and Noxa. The BH3 only proteins activate multi-domain pro-apoptotic species [32-33] and disrupt the function of anti-apoptotic Bcl-2 family members [33]. It is thought that multi-domain Bcl-2 family members form channels in the outer mitochondrial membrane through which apoptogenic proteins of the intermembranal space are released [34-36].

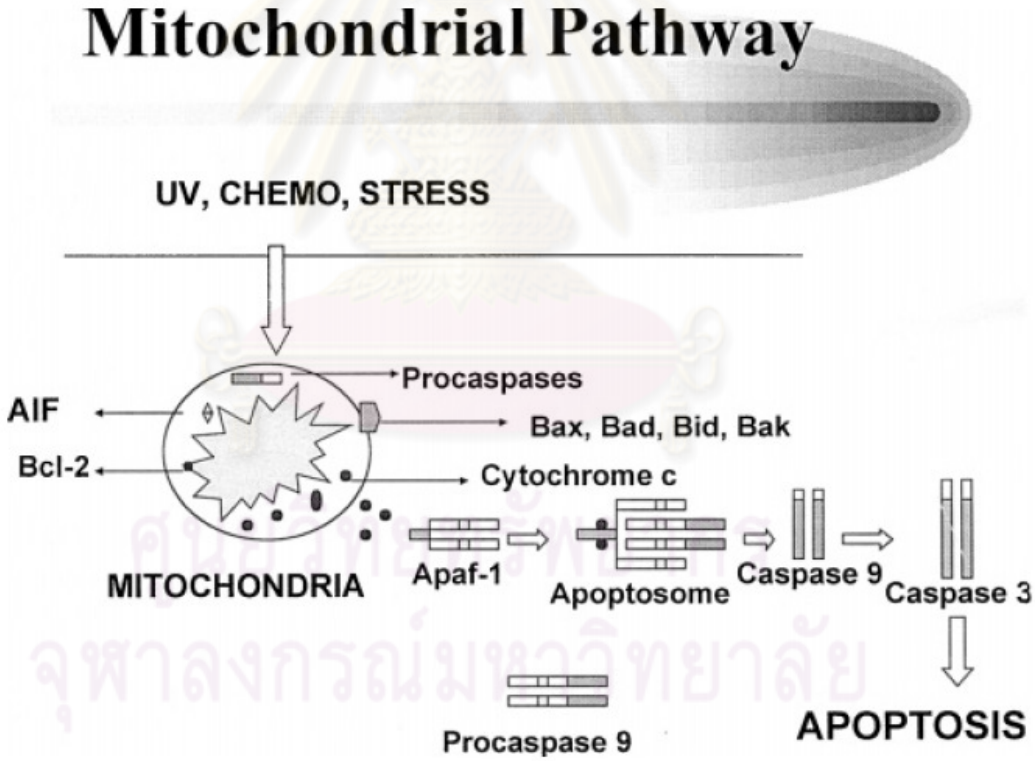


Figure 5: Mitochondria pathway.

Endoplasmic reticulum-stress pathway of apoptosis

Ca^{2+} storage and signaling as well as folding, modification, and sorting of newly synthesized proteins are among the main functions of the ER **[37]**. Disturbance of any of these functions can lead to ER stress, which in turn may induce apoptosis **[38-39]**. Both overload and depletion of the ER Ca^{2+} pool can result in changes in protein folding and in ER stress. Pathways of ER stress-induced signaling are shown in Fig. 6. Prolonged ER stress stimulates the activation of pro-caspase-12 localized in the ER membrane **[40]**. Once activated, caspase-12 acts on effector caspases to induce apoptosis. Therefore, these findings indicate that ER stress can be linked directly to caspase activation.

Several ER member proteins interact with Bcl-2 family members and influence apoptosis. Bcl-2-associated protein-31 (BAP31) contains a cytosolic domain, which interacts preferentially with pro-caspase-8, Bcl-2 and Bcl-xL **[41]**. Active caspase-8 can cleave BAP31, with an amino-terminal fragment remaining integrated in the ER and involved in the induction of apoptosis. This may provide a pathway linking the death receptor pathway with the ER-stress pathway of apoptosis.

There is now convincing evidence that the release of Ca^{2+} from the ER stores, followed by its translocation into the mitochondria, is an important signal for the activation of apoptosis. This finding is supported by the observation that Bcl-2 over expression could protect cells from thapsigargin-induced apoptosis. It was reported that Bcl-2 targeted to the ER is capable of blocking most although not all types of apoptosis **[42]**. Hacki *et al*. **[43]** demonstrated the existence of apoptotic crosstalk between the ER and mitochondria, which is controlled by Bcl-2 localized to the ER.

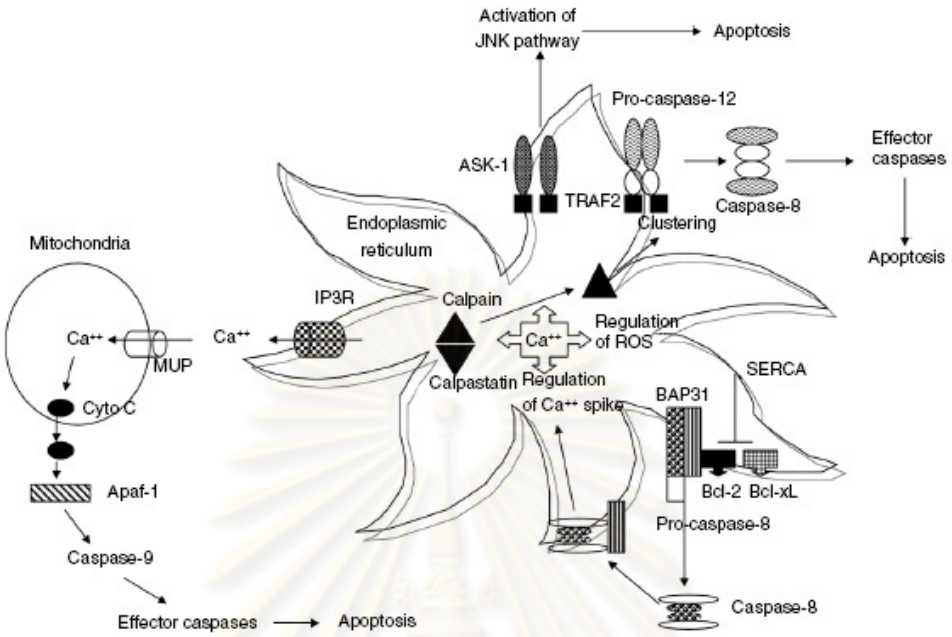


Figure 6: The endoplasmic reticulum (ER)-stress pathway.

Caspases are central initiators and executioners of apoptosis

The caspases are central importance in the apoptotic signaling networks which are activated in most cases of apoptotic cell death **[44]**. The term caspase is derived from cysteine-dependent aspartate-specific proteases: their catalytical activity depends on a critical cysteine-residue within a highly conserved active-site pentapeptide and the caspases specifically cleave their substrates after Asp residues. So far, 14 different caspase families have been described in mammals **[45-46]**. Caspase-1, -4 and -5 -11, and –12 are crucial regulators of the secretion of inflammatory cytokines such as IL-1ß, and IL-18 **[45]**, while caspase -3, -6, -7, -8, -9 and -10 are key enzymes in apoptotic cell death.

In the cell, caspases are synthesized as inactive zymogens, the so called procaspases, which at their N-terminus carry a prodomain followed by a large and a small subunit which sometimes are separated by a linker peptide. Upon maturation, the

procaspases are proteolytically processed, resulting in a small and a large subunit. The prodomain is also frequently but not necessarily removed during the activation process. A heterotetramer consisting of each two small and two large subunits then forms an active caspase. The proapoptotic caspases can be divided into the initiator caspases including procaspases-2, -8, -9 and –10, and the executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing death effector domains (DED) in the case of procaspases-8 and –10 or caspase recruitment domains (CARD) as in the case of procaspase-9 and procaspase-2. Via their prodomains, the initiator caspases are recruited to and activated at death inducing signaling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways).

In extrinsic apoptosis pathways (Fig. 4), e.g. procaspase-8 is recruited by its DEDs to the death inducing signaling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family **[47]**. When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by autoproteolysis **[45]**.

Intrinsic apoptosis pathways (Fig. 5) involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events at the so called apoptosome, a cytosolic death signaling protein complex that is formed upon release of cytochrome c from the mitochondria **[48]**. In this case it is the dimerization of procaspase-9 molecules at the Apaf-1 scaffold that is responsible for caspase-9 activation **[45]**. Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed **[49]**.

Inhibitors of apoptosis proteins

Expression levels of antiapoptotic proteins such as Bcl-2 and Bcl-X_L were reported to be up regulated by the transcription factor NF-κB, a central regulator of the innate and adaptive immune response as well as an antiapoptotic transcription factor **[50-51]**. Besides inducing the expression of pro-survival Bcl-2 members, NF-κB additionally transactivates a number of other antiapoptotic genes, such as the IAPs (inhibitors of apoptosis proteins).

IAPs are a family of antiapoptotic proteins whose prototype originally was described in baculovirus with many homologues found to be conserved across several species. So far, eight human IAP homologues have been identified, among others NAIP, c-IAP1, c-IAP2, XIAP and surviving (Fig. 7). All IAPs contain baculovirus IAP repeat (BIR) domains, 70 amino acid motifs, which are essential for the antiapoptotic properties of IAPs because it is the interaction between the BIR domains and caspases that is believed to confer most of the antiapoptotic activity of IAPs. Indeed, XIAP, c-IAP1 and c-IAP2 are thought to directly inhibit caspases-3, -7, and –9 **[52]**. In case of XIAP, it is the BIR3 domain that directly binds to the small subunit of caspase-9, whereas it is the BIR2 domain that interacts with the active-site substrate binding pocket of caspases-3 and –7 **[53-54]**.

In addition to the BIR domains, c-IAP1, c-IAP2, and XIAP contain a highly conserved RING domain at their C-terminal end which possesses E3 ubiquitin ligase activity. Via this RING domain, IAPs are able to catalyze their own ubiquitination, thereby targeting themselves for degradation by the proteasome **[55]**, but they also might target other proteins such as caspase-3 and –7 for ubiquitination and degradation **[56]**. Direct inhibition of caspase activity by c-IAPs is certainly a very important means of regulation when considered that signaling cascades mediated by proteolytic enzymes such as caspases is irreversible once activated and therefore must be precisely regulated in order to prevent locally and temporally inappropriate demise of cells. Importantly, Smac/Diablo, when released from the mitochondrial intermembrane space during mitochondrial apoptotic events, is able to counteract the inhibitory effect of IAPs on caspases since Smac/Diablo

can bind to e.g. XIAP in a manner that displaces caspases from XIAP and enables their activation. Thus, Smac/Diablo is a negative regulator of IAPs and in this way unfolds its apoptosis-enhancing property [57]. Essential for the ability of Smac/Diablo to bind to XIAP and to release caspases is a conserved tetrapeptide motif which is also present in HtrA2/Omi, another mitochondrial proapoptotic factor [58].

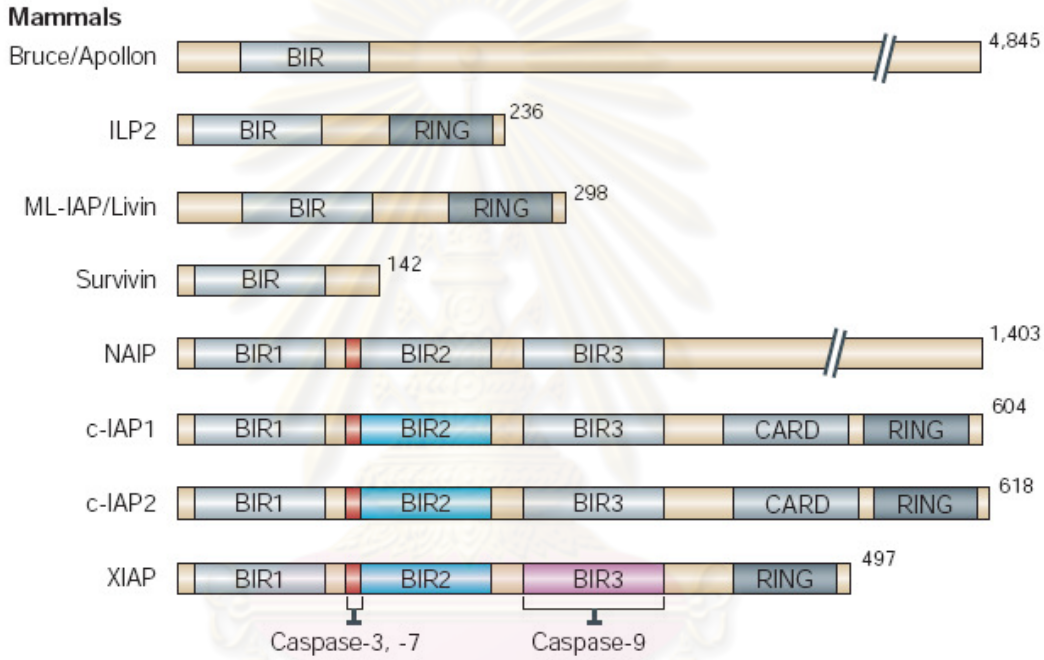


Figure 7: Proteins of the inhibitor of apoptosis (IAP) family

Apoptosis in cancer treatment

Many studies have demonstrated both in vivo and in vitro that a wide range of current anticancer drugs mediate cell death largely by way of apoptosis, as review in **[59]**. A mark increase in the number of apoptotic blasts was observed in a serial examination of peripheral blood mononuclear cells from acute leukemia patients undergoing induction therapy with various anticancer agents, including cytarabine, mitoxantrone, etoposide, paclitaxel, and topotecan **[60]**. Apoptotic induction was also demonstrated in solid tumors after treatment of mice with many cytotoxic drugs, including cytarabine, 5-fluorouracil, fludarabine, doxorubicin, cyclophosphamide, cisplatin, etoposide, dactinomycin, and camptothecin **[61]**. Most of these agents trigger tumor cell apoptosis by activating caspase enzymes. A number of studies have raised the possibility that anticancer drugs trigger apoptosis by inducing death receptor pathway.

The extrinsic death receptor mediated pathway has been proposed to plays an important role in chemotherapeutic drug-induced apoptosis in certain cell types **[62]**. Up regulation of the Fas and induction of its ligand FasL was observed after treatment of several tumor cell lines with chemotherapeutic drugs leading to apoptosis induced cell death **[63]**. However, the involvement of the death receptor pathway in anticancer induced apoptosis depends on several factors including the cell-type, the type of drug, drug concentrations and the duration of treatment **[64]**. A number of studies have failed to demonstrate the involvement of the Fas system in drug-induced apoptosis **[65]**. Collectively, these evidences suggest that many anticancer drugs can also trigger apoptosis in the absence of a functional Fas/FasL pathway.

Plants-derived anticancer agents

Drugs derived from plants make an enormous contribution to drug discovery. The examples of anticancer agents derived from plants that are currently used in clinical practice are;

- The vinca alkaloids, vinblastine and vincristine, are one of the most significant examples plant-derived anticancer drugs. These drugs are isolated from the periwinkle *Catharanthus roseus*, which inhibits microtubule assembly, inducing tubulin self-association into coiled spiral aggregates **[66]**. The introduction of vincristine was responsible for an increase in the cure rates for Hodgkin's disease and some forms of leukemia.
- Etoposide is an epipodophyllotoxin derivative, derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *Podophyllum emodi* **[67]**. It has significant activity against small-cell lung carcinoma **[68]** and has produced high cure rates in testicular cancer when use in combination with bleomycin. Etoposide is a topoisomerase II inhibitor, stabilizing enzyme–DNA cleavable complexes leading to DNA breaks **[69]**.
- The taxanes, paclitaxel and docetaxel, show impressive antitumor activity against breast, ovarian and other tumor types in the clinic. Paclitaxel originally isolated from the bark of the yew tree *Taxus brevifolia* stabilizes microtubules, leading to mitotic arrest **[70]**.
- The camptothecin derivatives, irinotecan and topotecan, have shown significant antitumor activity against colorectal and ovarian cancer respectively **[71]**. These compounds were initially obtained from the bark and wood of Nyssacea *Camptotheca accuminata* and act by inhibiting topoisomerase I **[72]**.

There are a large number of additional plant-derived agents that are currently under investigation. Flavopiridol is one of the most exciting agents currently in development, representing the first cyclin-dependent kinase inhibitor to enter the clinic **[73]**. It is a synthetic flavone derived from the plant alkaloid rohitukine, isolated from the leaves and stems of *Amoora rohituka* and *Dysoxylum binectariferum* **[73]**. The mechanism of action of flavopiridol involves interfering with the phosphorylation of cyclin-dependent kinases (cdk's), hampering their activation and blocking cell-cycle progression at growth phase 1 (G1) or G2 **[73]**. Another example, homoharringtonine is an alkaloid isolated from the Chinese tree *Cephalotaxus harringtonia* **[74]**, and has shown efficacy against various

leukemia [75]. The mechanism of action of homoharringtonine is the inhibition of protein synthesis, blocking cell-cycle progression [76]. 4-Ipomeanol is a pneumotoxic furan derivative isolated from the sweet potato *Ipomoeca batatas* [77] and has been under clinical evaluation as a lung-cancer-specific antineoplastic agent [78]. This compound is converted into DNA-binding metabolites upon metabolic activation by cytochrome P450 enzymes that are present in cells of the lung [77]. Finally, β-lapachone is a DNA topoisomerase I inhibitor that induces cell-cycle delay at G1 or S (synthesis) phase before inducing either apoptotic or necrotic cell death in a variety of human carcinoma cells, including ovary, colon, lung, prostate and breast [79]. Crude extracts from various plants were also evaluated for antitumor activity, such as *Nigella sativa* [80], *Dioscorea membranacea* [81], *Scutellaria barbata* [82], *Sutherlandia frutescens* [83], *Camellia sinensis* var. *assamica* [84], *Oroxylum indicum* [85] and *Ximenia Americana* [86].

Plants-derived compounds are also used in natural forms in traditional medicine for treatment of a variety of diseases all over the world as well as in Thailand. Some of the plant products have shown anti-tumor property against various human cancers and are used in the forms of herbal formulations, consist of many kinds of products in the preparations. Most of the herbal drugs are a mixture of a number of plant ingredients whose cumulative effect increases their efficacy in curing the diseases. Here are examples of herbal formulations which have been scientifically proved for anticancer activity;

- Triphala (TPL) is an herbal formulation consisting of the dried and powdered fruits of three plants, *Terminalia chebula, Emblica officinalis* and *Terminalia bellerica* in equal proportions. It is believed to promote health, immunity and longevity. This formulation is a frequently used Ayurvedic medicine to treat many diseases such as anemia, jaundice, constipation, asthma, fever and chronic ulcers. The potential antitumor activity of the water extract of TPL (250μg/ml, and 40 mg/kg) has been demonstrated, *in vitro* and *in vivo*, on human breast cancer cells MCF-7 and on the growth of mouse thymic lymphoma cells brcl-95 transplanted in Swiss mice. The extract had low toxicity on normal breast epithelia cell [87].

- Ge-Jee-Bok-Ryung-Hwan (GJBRH), a traditional herb formulation, is composed of eight medicinal plants. It has been used for women's diseases including cervical carcinoma in Korea, Japan and China. The water extract of GJBRH was investigated in HeLa human cervical carcinoma cells. The results showed that the extract at 2 mg/ml induced HeLa cell apoptosis in association with Bax translocation to mitochondria, Ca^{2+} signaling, and endoplasmic reticulum-stress **[88]**.
- Paljin-Hangahmdan (PH), an oriental herbal formulation from Korea, consists of 15 medicinal plants. The water extract of PH, at the concentrations of 200-800 μg/ml demonstrated cytotoxicity and induced apoptosis in human promyelocytic leukemia (HL-60) cells. It had less cytotoxicity in peripheral blood mononuclear cells isolated from a healthy subject **[89]**.
- PC-SPES is a eight-herb formulation sold directly to consumers in the USA; with efficacy in the treatment of prostate cancer. Ethanolic extracts of PC-SPES show significant cytostatic and cytotoxic activity against both prostrate cancer and non- prostate cancer cell lines. Decreased rates of cell proliferation, reduced clonogenicity, and cell cycle alterations have been observed. Cell cycle analysis has indicated that PC-SPES treatment results in the accumulation of cells in the G1 phase of division of prostate cancer cells. Animal studies have confirmed the anti-prostate activity of PC-SPES in vivo **[90-93]**.

Herbal formulations for cancer treatment are also available in Thailand. Mareng-roipad formulation is a herbal formulation consisting of over 40 kinds of dried and powdered plants, formulated from MoChon-Paungmalai at Pechaburi province. It has been used as traditional medicine to treat different types of tumor for more than a century without any scientific evidence. The anticancer activity of Mareng-roipad has not been elucidated. This study was, therefore, aimed to evaluate the anti-tumor effects of the formulation *in vitro* on human acute leukemic cells. The cytotoxicity and apoptotic induction of the extracts were investigated on Jurkat cells and human peripheral blood mononuclear cells. The mechanisms of action of the extracts were also studied. The results from this study may

point to the promise of Mareng-roipad formulation as a traditional anti-tumor medicine without any doubt.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Extracts of Mareng-roipad formulation

The Mareng-roipad formulation in the grounded powder form was gifted from Greenchat Natural Herbs (Thailand) Co., Ltd. The water and the ethanol extracts of the formulation were prepared by Assistant Professos Dr. Pathama Leewanich, Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University, Thailand. The water extract was prepared by decoction followed by lyophilization. The ethanol extract was prepared by soaking the powder in absolute ethanol for 72 h before evaporation. Both extracts were kept in closed containers at 4 °C until used. The identification of both extracts was performed by gas chromatography and mass spectrophotometry from the Institute of Health Research Chulalongkorn University.

1.2 Cell culture

- Human peripheral blood mononuclear cells (human PBMCs)

Human PBMCs were isolated from heparinized blood of healthy male blood donors with informed consent from the National Blood Bank, Thai Red Cross Society. The donors were aged between 20 - 35 years. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.5% L-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin; at 37 °C, 97% humidity, and 5% CO_2.

- Leukemic T cells (Jurkat cells)

The human acute leukemic cells (Jurkat) were purchased American Type Cell Culture (ATCC) (Rockville, MD). The cells were maintained in RPMI supplemented with 10% fetal calf serum, 0.5% L-glutamine, 100 mg/ml streptomycin, and 100 units/ml

penicillin; at 37 ^{o}C, 97% humidity, 5% CO_2. The cells were subcultured three times weekly.

1.3 Equipments and Instruments

The following instruments were used in this study; autoclave (Hirayama, Japan), autopipette (Gilson, France), Biohazard lamina-flow hood (Science, Gelman), flow cytometer (Becton Dickinson, USA), Freezer – 70^{o} C (Sanyo, Japan), hemocytometer (Boeco, Germany), Incubator (Olympus, Japan), light microscope (Olympus, Japan), 24 multi-well plates (Nunc, Denmark), pH meter SA 520 (Orian, USA), pipette (Falcon, USA), refrigerator 4^{o}C and - 20^{o}C (Sanyo, Japan)

1.4 Reagents

The reagents used in this study were; dimethyl sulfloxide (Sigma, USA), doxorubicin (Ebewe phama, Austria), etoposide (Ebewe pharma, Austria), fetal bovine serum (Hyclone, USA), Hanks' balanced salts solution powder (Gibco, Germany), Heparin (Leo, Denmark), HEPES (Hyclone,USA), Histopaque® -1077 (Sigma, Germany), L-glutamine (Gibco, Germany), penicillin/streptomycin (Hyclone, USA), RPMI1640 medium (Sigma, USA), 0.4 % trypan blue dye (Sigma, USA), annexin V-FIT C apoptosis detection kit (Santa Cruz Biotecnology, USA), caspase inhibitor z-VAD-FMK (Promega, USA), and anti-Fas ligand Antibodies (Biolegend, USA)

2. Methods

2.1 Preparation stock solutions of Mareng-roipad formulation extracts

The stock solution of the water extract was prepared by dissolving the extract in double distilled water at the concentration of 10 mg/ml. The stock solution of the ethanol

extract was done by preparing the extract at the concentration of 10 mg/ml in 2% DMSO. Both solutions were sterilized by passage through a 0.20 µm filter and stored at -20°C until used.

2.2 Preparation of human peripheral blood mononuclear cells

PBMCs were isolated from 10 ml heparinzed blood of each donor by the following procedures:

1. Pipette 5 ml of Histopaque-1077 at room temperature into each 15 ml polypropylenes centrifuge tube.

2. Mix the blood sample 1:1 with Hanks' balanced salts solution (HBSS) containing 2 µl/ml heparin at room temperature.

3. Slowly add 9 ml of the blood/ HBSS mixture onto the top of Histopaque-1077 in each tube. Be careful not to mix the two parts together, and cap the tubes tightly.

4. Centrifuge the tubes at 400 g for 30 minutes at room temperature.

5. Carefully remove the top layer from each tube without disturbing the interface.

6. Collect cells at the interface (buffy layer containing PBMCs) from each tube.

7. Immediately transfer the PBMCs to a new sterile polypropylene 15 ml centrifuge tube.

8. Wash the cells twice with 12.5 ml HBSS (+ 2 µl/ml heparin + 1% fetal bovine serum) by centrifugation at 250 g for 10 min at room temperature.

9. Discard the supernatant and resuspend the pellet in 5 ml the completed RPMI medium (RPMI 1640 medium with 10 % fetal bovine serum, 0.5% L- glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin).

10. Determine viable cells by staining with 0.4% trypan blue dye solution at the ratio 1:1, count numbers of the cells on hemocytometer and adjust them to the required density with the completed RPMI 1640 medium.

The PBMCs, with cell viability more than 90%, were used in this study at the density of $1x10^6$ cells/ml.

2.3 Determination of Cytotoxic activity of the extracts

The cytotoxic effect of the Mareng-roipad formulation extracts on Jurkat cell was determined by fluorescence flow cytometry after propidium iodide (PI) staining. PI can enter death cells and intercalates with DNA, causing red fluorescence of the death cells.

Both extracts, at the final concentrations of 10, 30, 100, 300 and 1000 µg/ml were used in this experiment. The assay was performed in duplication as followed (n=3):

1. Aliquot of 900 µl Jurkat cells suspension in the completed RPMI 1640 medium, at the density $3x10^5$ cell/ml into each well of 24-well plate.

2. Add 100 µl of each sample solution into each well.

3. Incubated the cell at 37^0C, 97% humidity, 5% CO_2 for 24 and 48 h.

4. Collect the cells in each well to a micro-centrifuge tube and centrifuged at 13,000 rpm 25^0C for 1 minute.

5. Removed the supernatant. Wash the cells twice with 500 µl cold PBS by centrifugation at 13,000 rpm, 25^0C for 1 minute.

6. Discard the supernatant, resuspend the cell pellet in 100 µl of assay buffer and transferred into a flow cytometer tube.

7. Add 1 µl of 0.05 µg/ml PI into each tube.

8. Incubate the tubes in the dark at room temperature for 15 minutes.

9. Subsequently add 400 µl of assay buffer and immediately analyze the cells by flow cytometer. For each sample, ten thousand cells were measures.

The completed RPMI 1640 medium and 1.5 µg/ml doxorubicin solution were used as the negative and the positive controls, respectively.

2.4 Determination of apoptotic induction activities of the extracts

2.4.1 Analysis of phosphatidyl serine exposure (Annexin V-FIT C apoptosis detection)

Loss of plasma membrane asymmetry is one of the earliest features of apoptotic cells. The membrane phospholipid, phosphatidylserine (PS), is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid binding protein that has a high affinity for PS, and binds to cells with exposed PS. Since externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining can identify an earlier stage of apoptosis.

The effect on the apoptotic induction of the Mareng-roipad formulation extracts on Jurkat cells and the human PBMCs were determined by Annexin V-FITC and PI binding assay with flow cytometer.

The water and the ethanol extracts, at the final concentrations of 10, 30, 100, 300 and 1000 µg/ml, were used for determining dose dependent properties of the extracts. The incubated times with the extracts for 6, 18, and 24 h were also performed for determining the time dependent effect of the extract. The assay was performed in duplication as followed (n=3-4):

1. Aliquot 900 µl Jurkat cells suspension in the completed RPMI 1640 medium, at the density $3x10^5$ cells/ml, into each well of 24-well plates.

2. Add 100 µl of each sample solution into each well.

3. Incubated the cell at 37^0C, 97% humidity, 5% CO_2 for 6, 18 and 24 h.

4. Collect the cells in each well to a micro-centrifuge tube and centrifuged at 13,000 rpm 25^0C for 1 minute.

5. Removed the supernatant. Wash the cells twice with 500 µl cold PBS by centrifugation at 13,000 rpm, 25^0C for 1 minute.

6. Discard the supernatant, resuspend the cell pellet in 100 µl of 1x assay buffer and transferred into a flow cytometer tube.

7. Add 1 µl of 0.05 µg/ml PI and 0.5 µl of annexin V-FITC in each tube.

8. Incubate the tube in the dark at room temperature for 15 minutes.

9. Subsequently add 400 µl of 1x assay buffer into each tube and immediately analyze 10,000 cells/sample by fluorescence flow cytometer.

The completed RPMI 1640 medium and 10 µg/ml etoposide solution were used as the negative and the positive controls, respectively.

10. Assess types of death cells as follow; annexin V-FITC$^-$/PI$^-$ cells as viable cells, annexin V-FITC$^+$ cells as apoptotic cells, PI$^+$ cells as nonapoptotic cells, and annexin V-FITC$^+$/PI$^+$ cells as late apoptotic or secondary necrotic cells.

2.4.2 Analysis of cellular DNA content (Propidium iodide staining assay)

A distinctive feature of apoptosis at the biochemical level is DNA fragmentation followed by the progressive loss of DNA from the apoptotis cells, due to activation of endonuclease. The decrease of cellular DNA can be determined by DNA-specific fluorochromes, such as propidium iodide, on ethanol fixed cells.

The apoptosis effect of the Mareng-roipad formulation extracts on Jurkat cell was determined by fluorescent flow cytometer after fixing in cold ethanol and staining with propidium iodide (PI).

Both extracts, at the final concentrations of 30, 100 and 300 µg/ml were used in this experiment. The assay was performed in duplication according to the following procedures (n=3-4):

1. Aliquot 900 µl Jurkat cells suspension in the completed RPMI 1640 medium, at the density 3x10^5 cells/ml, into each well of 24-well plates.

2. Add 100 µl of each sample solution into each well.

3. Incubated the cell at 37^{0}C, 97% humidity, 5% CO_2 for 24 and 48 h.

4. Collect the cells in each well to a micro-centrifuge tube and centrifuged at 13,000 rpm 25^{0}C for 1 minute.

5. Removed the supernatant. Wash the cells twice with 500 µl cold PBS by centrifugation at 13,000 rpm, 25^{0}C for 1 minute.

6. Discard the supernatant, fix the cells pellet at -20^{0}C for 15 minutes in 500 µl of cold ethanol. After that, cells were centrifuged at 13,000 rpm 25^{0}C for 1 minute.

7. Discard the supernatant, resuspend the cell pellet in 500 µl of 1x assay buffer, maintained at 25^{0}C for 15 minutes and transferred into a flow cytometer tube.

8. Add 5 µl of 0.05 µg/ml PI and 5 µl of 4 mg/ml RNase in each tube.

9. Incubate the tube in the dark at room temperature for 30 minutes.

10. Analyze 10,000 cells/sample by fluorescence flow cytometer.

The completed RPMI 1640 medium and 10 µg/ml etoposide solution were used as the negative and the positive controls, respectively.

2.5 Determination of mechanisms of apoptotic induction of the extracts

The Fas-Fas ligand dependent and the caspase-dependent mechanisms were evaluated in this study by using a caspase inhibitor and a FasL blocking agent. The assay was performed similar to 2.4.1.

2.5.1 Fas-Fas ligand dependent pathway

The water and the ethanol extracts, at their IC_{50} concentrations for apoptosis induction (15 and 150 µg/ml, respectively), were used in this experiment. The optimum concentration of anti-FasL antibody was screened (10-400 ng/ml) and the final concentration at 10 ng/ml was selected for using in the assay. Jurkat cells were pretreated with the antibody 1 h before adding the extracts. The assay was performed in duplication as followed (n=3):

1. Aliquot 900 µl Jurkat cells suspension in the completed RPMI 1640 medium, at the density 3x10^5 cells/ml, into each well of 24-well plates.

2. Add 10 µl of anti-FasL antibody into each well.

3. Incubated the cell at 37^{0}C, 97% humidity, 5% CO_2 for 1 h.

4. Add 100 µl of each sample solution into each well.

5. Incubated the cell at 37^{0}C, 97% humidity, 5% CO_2 for 18 h.

6. Performed the next procedures as in the step 4^{th}- 10^{th} of 2.4.1.

2.5.2 Caspase dependent pathway

The water and the ethanol extracts at the concentration of 15 and 150 μg/ml, respectively, were used in this experiment. Jurkat cells were pretreated with 50 μM z-VAD-fmk, a pan caspase inhibitor which inhibits caspase-3, -8 and -9 activity, 1 h before adding the extracts. The assay was performed in duplication as in 2.5.1 (n=3).

2.6 Statistical analysis

All data were presented as means and standard error of means (means $\pm$ S.E.M). Differences among means were analyzed by using one–way analysis of variance (ANOVA). All statistical analysis was performed according to the statistic program, SPSS. A value of $p < 0.05$ was considered to be significant.

CHAPTER IV

RESULTS

1. Cytotoxic activity of the extracts of Mareng-roipad formulation

To evaluate the cytotoxic activity of the water and the ethanol extracts of Mareng-roipad formulation, Jurkat cells were incubated with various concentrations (10-1,000 µg/ml) of the extracts. After 24 and 48 h, the cytotoxicity of the extracts was determined by the PI staining assay using fluorescent flow cytometer. The percentage of PI positive cells in each condition was interpreted as death cells. When compared to the negative control, both extracts significantly induced cytotoxicity in a concentration- and time-dependent manner, as illustrated in Fig. 9 and Table 1. Doxorubicin at the final concentration of 1.5 µg/ml was used as the positive control in this experiment. The potency of both extracts was similar at 24 h, but the potency of the water extract was higher than the ethanol extract at 48 h.

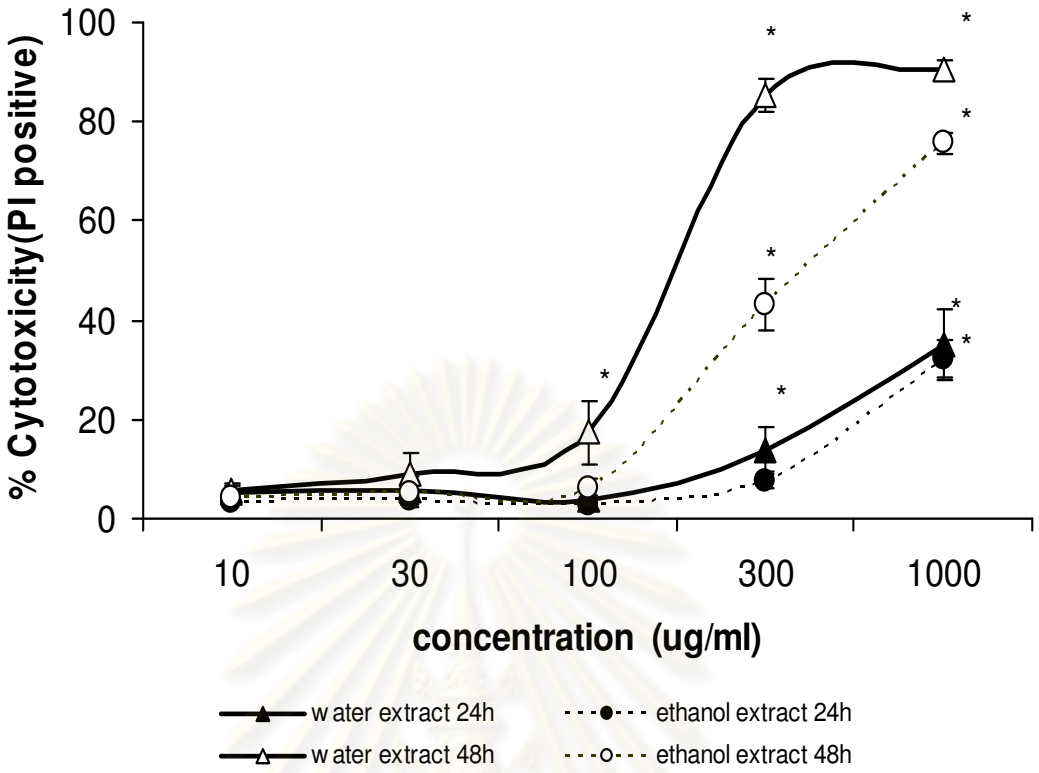


Figure 9: Cytotoxic effect of the extracts on Jurkat cells. Cells were treated with 10-1000 µg/ml extracts for 24 and 48 h. The death cells were determined by PI staining and monitored by flow cytometer. Percentage of PI positive cells was plotted against the concentrations of the extracts. Data were represented as mean ± S.E.M. of three independent experiments (n=3).

*significant difference when compared to the untreated control at $p<0.05$

Treatment	% PI POSITIVE			
	24 h		48 h	
	Water extract	Ethanol extract	Water extract	Ethanol extract
Negative control	3.12±1.11	3.48±0.69	3.9±0.81	3.9±0.98
Positive control	100±0	99.15±0.39	100±0	94.37±3.72
10µg/ml	5.08±3.05	3.17±0.45	5.55±2.98	4.38±1.63
30µg/ml	5.68±4.88	3.76±2.13	8.83±7.48	5.37±2.49
100µg/ml	3.93±1.37	2.83±1.46	17.45±11.2*	6.25±3.11
300µg/ml	13.95±7.49*	7.5±2.83	85.28±5.85*	43.03±9.01*
1000µg/ml	35.22±11.8*	32.03±7.1*	90.75±3.02*	75.7±3.79*

Table 1: Cytotoxic effect of the extracts on Jurkat cells. Cells were treated with 10-1000 µg/ml extracts for 24 and 48 h. The death cells were determined by PI staining and monitored by flow cytometer. Data were represented as mean ± S.E.M. of Three independent experiments (n=3).
*significant difference when compared to the untreated control at $p<0.05$

2. Apoptosis activity of Mareng-roipad extracts

2.1 On acute leukemic cells (Jurkat cells)

In order to characterize the nature of cell death caused by the extracts, Jurkat cells were treated with different concentrations (10-1,000 µg/ml) of the extracts for 18 h, and types of cell death were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The representative profiles of cell death were demonstrated in Fig. 10. Stained cells were interpreted as in the following; annexin V-FITC positive cells as apoptotic cells, PI positive cells as necrotic cells, double annexin V-FITC/PI positive cells as necrotic and late apoptotic cells, and double annexin V-FITC/PI negative cells as viable cells. It is clearly illustrated that both extract could induce cell death mainly by apoptosis, as shown in Fig. 11 and Table 2. They significantly induced Jurkat cell apoptosis in a concentration-dependent manner. The potency of the water extract was higher than the ethanol extract, with the IC_{50} concentrations at 15 and 150 µg/ml, respectively. Three concentrations around these IC_{50} concentrations were chosen for studying the time-dependency of the apoptosis induced by the extracts. There were 10, 30 and 100 µg/ml for the water extract, and 30, 100 and 300 µg/ml for the ethanol extract.

To determine whether the apoptotic activity of the extracts was time-dependent, Jurkat cells were incubated with the extracts at the previously mentioned concentrations for 6, 18 and 24 h. Apoptotic cells were identified by annexin V-FITC/PI staining assay using fluorescent flow cytometer. As shown in Fig. 12 and 13 the water extract induced Jurkat cells apoptosis in a concentration but not time-dependent fashion. However, its activity varied among the times used in the experiments. The activity at 18 h exposure was higher than at 6 and 24 h exposure. On the contrary, the apoptotic activity of the ethanol extract was time-dependent, especially at the highest concentration, 300 µg/ml, used in the experiment (Fig. 12 and 13).

To further confirm that both extracts induced apoptosis, Jurkat cells were treated with 10, 100 and 300 µg/ml of the extracts for 24 and 48 h. The cells were fixed with cold ethanol, stained with PI and analyzed for cellular DNA content by flow cytometer. Fig. 14

showed the representative DNA histograms of Jurkat cells treated with the extracts for 24 h from one experiment. The fraction of apoptotic cells was identified in the DNA histograms as a sub-G1 hypodiploid population. It was noticed that Jurkat cells treated with the extracts, at the concentration of 300 µg/ml, for 24 and 48 h had sub-diploid cell population, as shown in Fig. 15 and 16. This population was not observed at lower concentrations of both extracts (30 and 100 µg/ml). These results confirmed that the water and the ethanol extracts of Mareng-roipad formulation induced Jurkat cells apoptosis. However, this confirmation was noticed only at the highest concentration of the extracts. The percentage of apoptotic cells was not as high as the results from dual annexin V-FITC/PI staining assay.

2.2 On human PBMCs

In order to evaluate the pharmacological effect of both extracts on normal cells, human PMBCs from normal male subjects were treated with three concentrations of both extracts for 18 h. The percentage of apoptotic cells was analyzed by annexin V-FITC/PI staining assay using flow cytometer. As shown in Fig. 17, both extracts distinctly had no effect on normal cells. These results demonstrate that the extracts were much more harmful to cancer cells, at least to acute leukemic cells, than to normal cells. The comparative activity of both extracts on Jurkat cells and on human PBMCs was shown in Fig. 18.

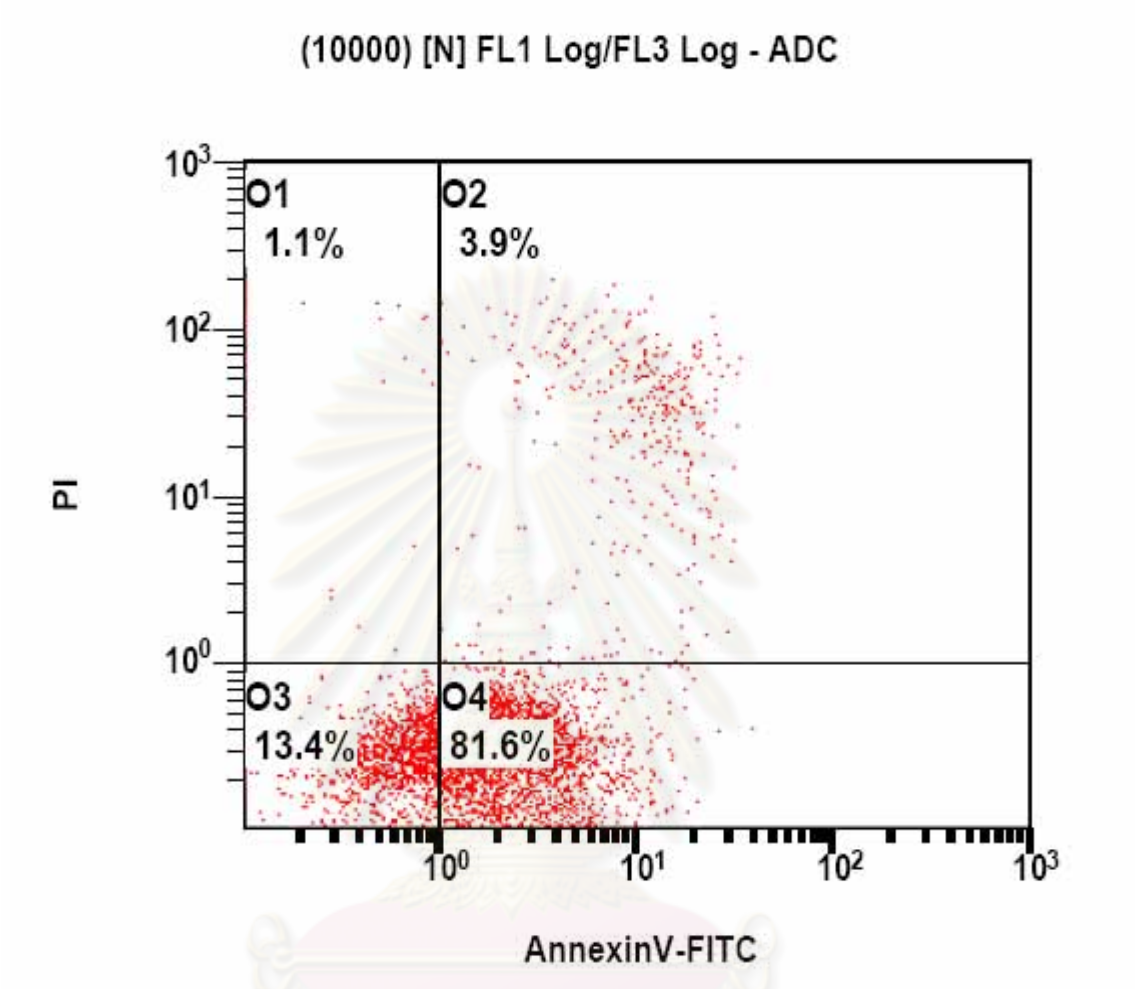


Figure 10: Representative dot plot and percentages of histogram analysis of etoposide-treated Jurkat cells by annexin V-FITC/PI staining. Cells in each quadrants are as followed; necrotic cells (PI positive) in the quadrant O1, necrotic and late apoptotic cells (double annexin V-FITC/PI positive) in the quadrant O2, viable cells (double annexin V-FITC/PI negative) in the quadrant O3, and apoptotic cells (annexin V-FITC positive) in the quadrant O4.

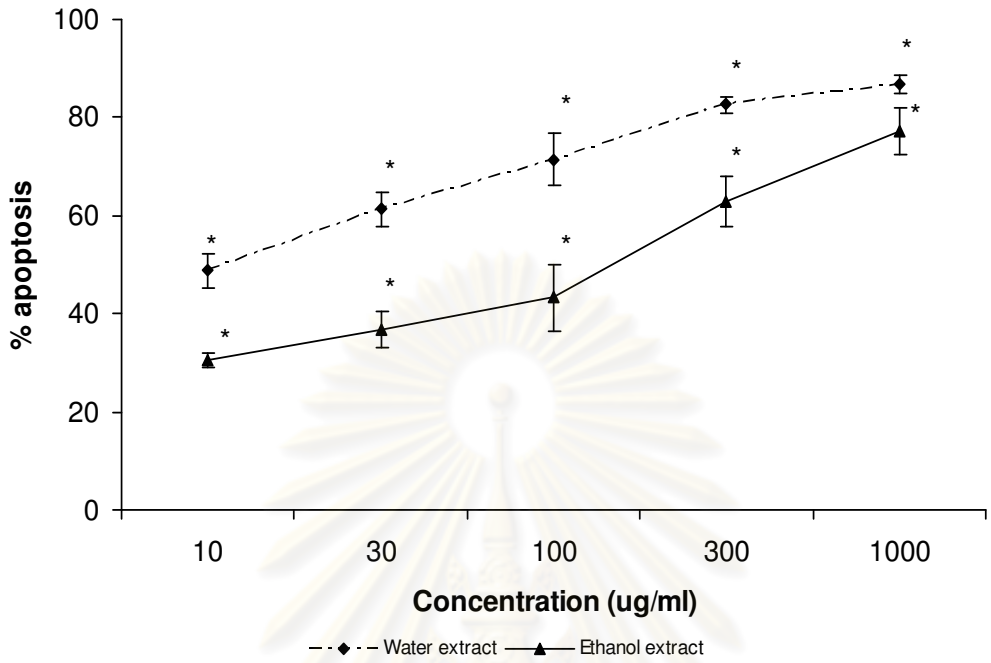


Figure 11: The Jurkat cell apoptosis induced by the extracts. Cells were treated with 10-1000 µg/ml extracts for 18 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Percentage of apoptotic cells (annexin V-FITC positive cells) was plotted against the concentrations of the extracts. Data were represented as mean ± S.E.M. of Three independent experiments (n=3).

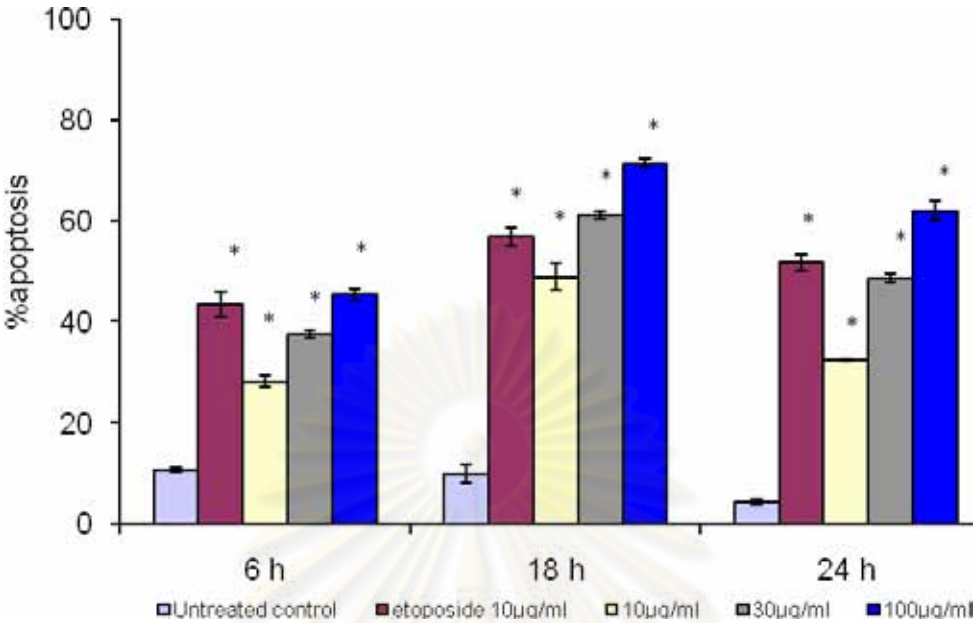
*significant difference when compared to the untreated control at $p<0.05$

Treatment	%Viable cells		% Death cells					
			Apoptosis		PI+		Double positive	
	Water extract	Ethanol extract	Water extract	Ethanol extract	Water extract	Ethanol extract	Water extract	Ethanol extract
Untreated control	87.07±0.46	87.63±1.29	9.97±0.86	8.57±2.00	1.87±0.68	2.5±0.90	1.13±0.34	1.3±0.45
Etoposide 10µg/ml	37.58±7.23	24.95±4.45	56.87±4.53	72.5±3.89	0.42±0.17	0.13±0.08	5.15±3.95	2.47±0.64
10µg/ml	50.67±3.45	68.87±1.37	48.83±3.50*	30.52±1.45*	0.03±0.02	0.13±0.05	0.45±0.13	0.68±0.12
30µg/ml	38.77±4.00	62.55±3.71	61.23±3.56*	36.70±3.76*	0.07±0.02	0.12±0.05	0.45±0.17	0.63±0.08
100µg/ml	24.83±6.88	55.83±6.53	71.40±5.40*	43.32±6.76*	1.23±0.61	0.13±0.10	2.53±1.09	0.68±0.24
300µg/ml	12.20±3.26	33.42±5.16	82.55±1.55*	62.85±5.29*	0.85±0.47	0.62±0.48	4.27±2.44	3.17±1.92
1000µg/ml	7.47±1.63	16.88±3.87	86.75±1.72*	77.27±4.70*	0.6±0.31	0.65±0.38	5.20±2.37	5.17±0.69

Table 2: The Jurkat cell apoptosis induced by the extracts. Cells were treated with 10-1000 µg/ml extracts for 18 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data were represented as mean ± S.E.M. of Three independent experiments (n=3).

*significant difference when compared to the untreated control at $p<0.05$

(a) water extract



(b) ethanol extract

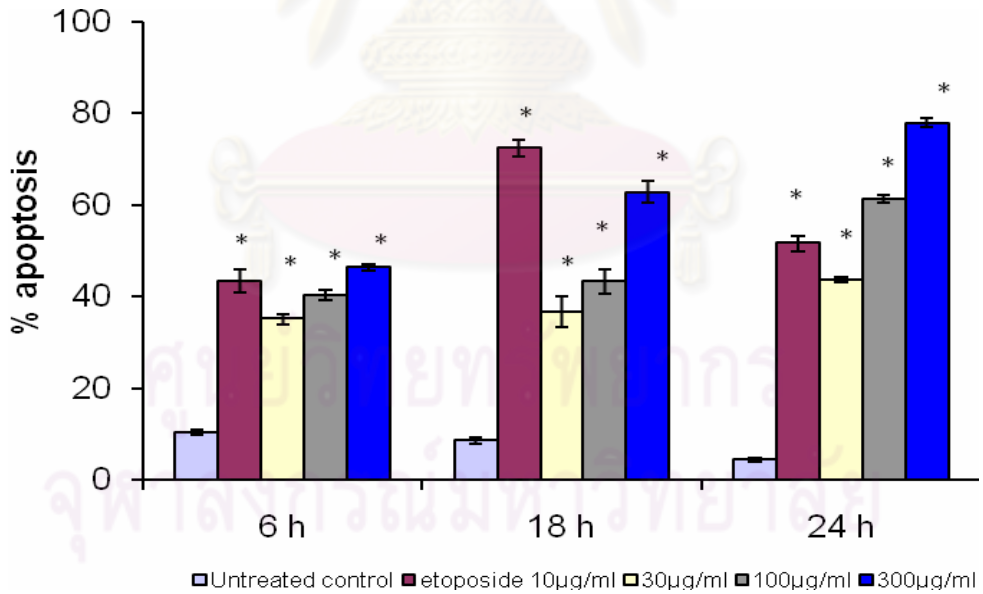
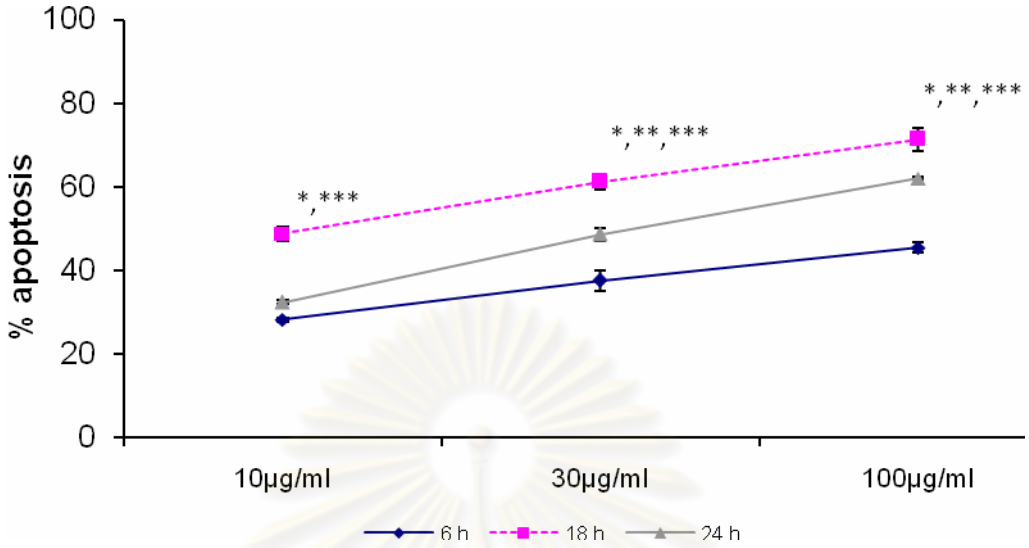


Figure 12: The time-dependency of the Jurkat cell apoptosis induced by the extracts. Cells were treated with 10-1000 µg/ml extracts for 6, 18 and 24 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data represented the percentages of the apoptotic cells (annexin V-FITC positive cells) as mean ± S.E.M. of four independent experiments (n=4).

*significant difference when compared to the untreated control at $p<0.05$

(a) water extract



(b) ethanol extract

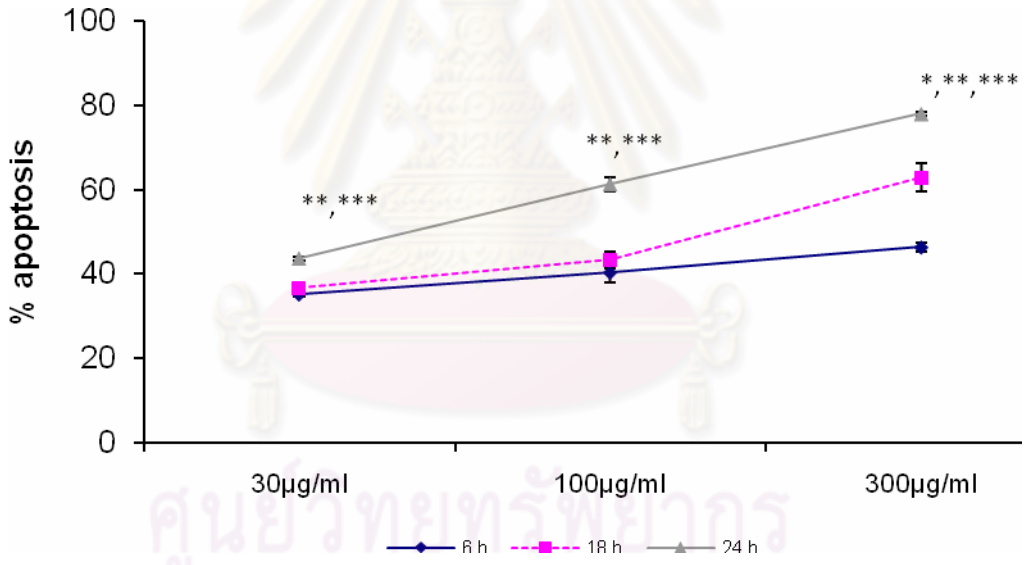


Figure 13: The time-dependency of the Jurkat cell apoptosis induced by the extracts. Cells were treated with 10-1000 µg/ml extracts for 6, 18 and 24 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Percentage of apoptotic cells (annexin V-FITC positive cells) was plotted against the concentrations of the extracts. Data represented the mean ± S.E.M. of four independent experiments (n=4).

* Significant difference when compared between 6 h and 18 h at $p<0.05$

** Significant difference when compared between 6 h and 24 h at $p<0.05$

*** Significant difference when compared between 18 h and 24 h at $p<0.05$

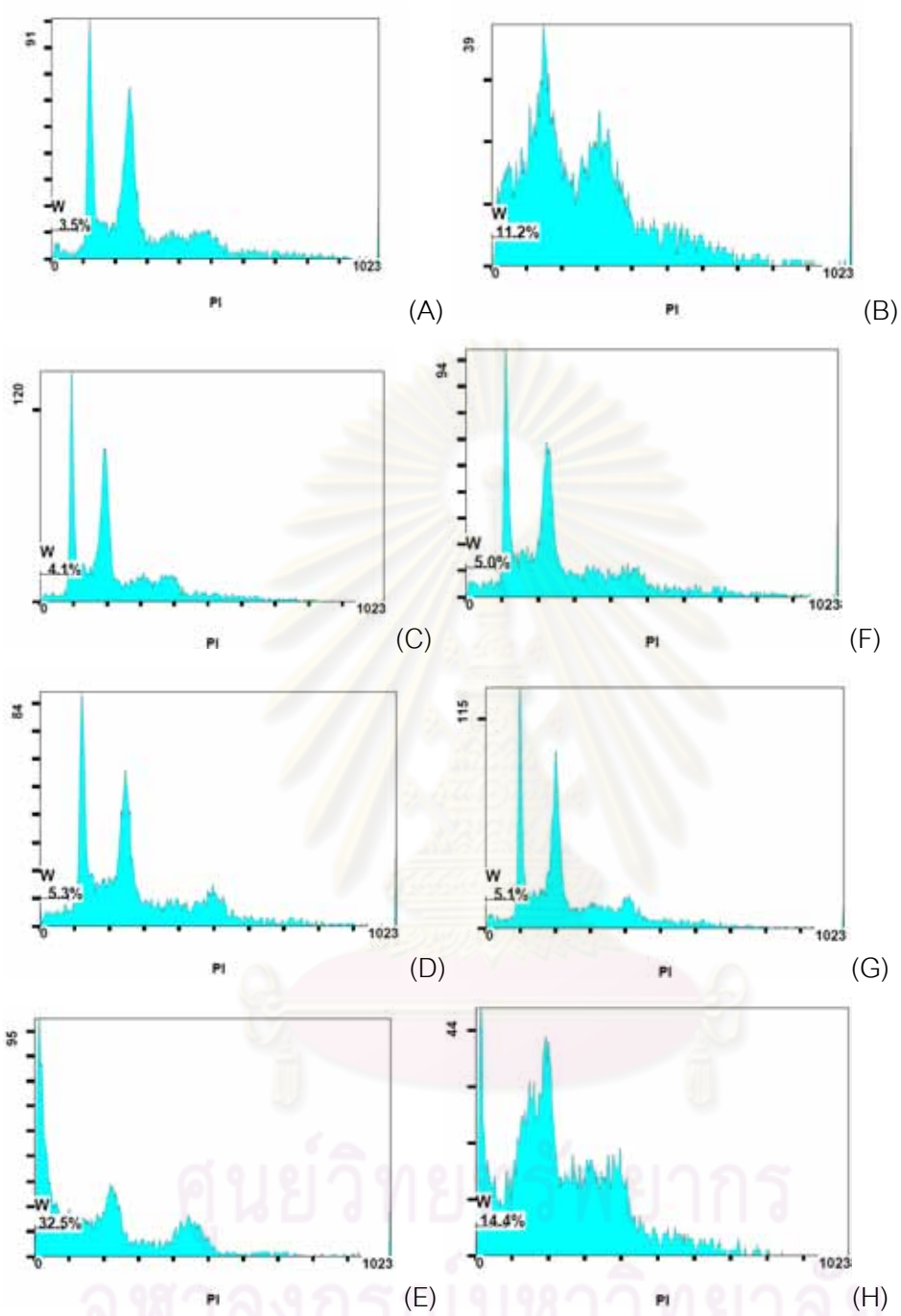


Figure 14: Representative DNA histogram of PI staining of Jurkat cells treated with the extracts for 24 h from one experiment. The fraction of apoptotic cells was determined in a DNA histogram as a sub-G1 hypodiploid population. (A) Untreated control; (B) 10 µg/ml Etoposide; (C, D, E) 30,100, 300 µg/ml water extract; and (F, G, H) 30,100, 300 µg/ml ethanol extract.

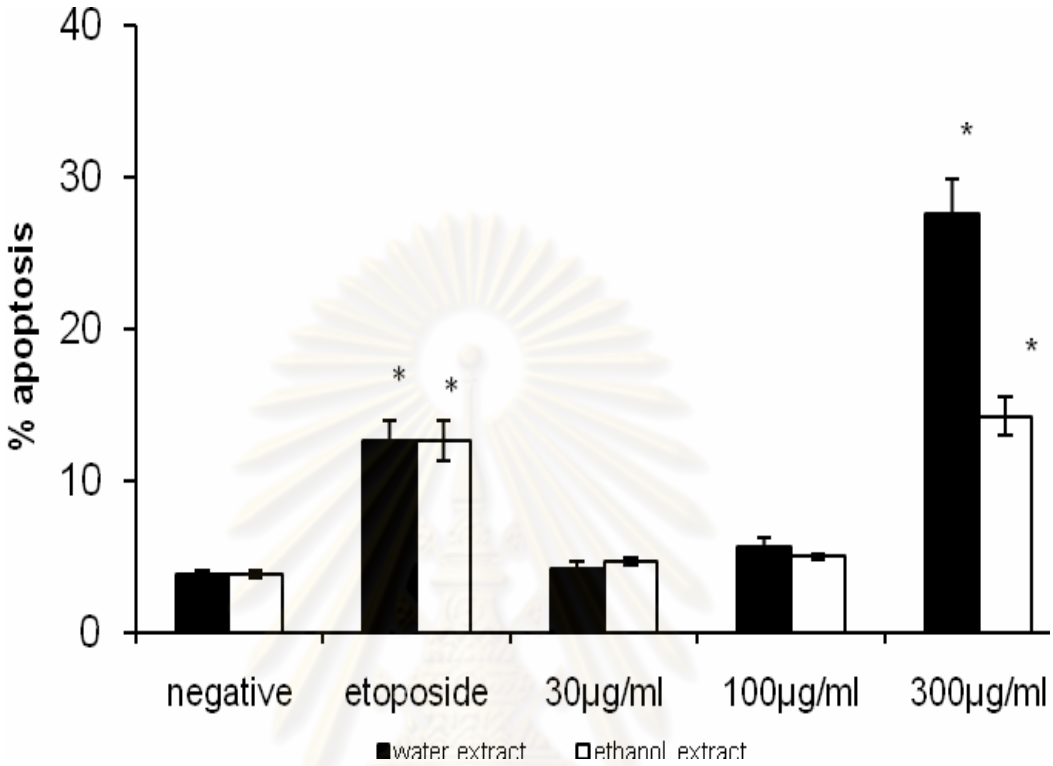


Figure 15: Analysis of DNA content in Jurkat cells after treatment with the extracts for 24 h. Cells were treated with 30, 100, and 300 µg/ml extracts for 24 h. The presence of hypodiploid cells was analyzed by flow cytometry after staining ethanol-fixed cells with PI. The percentage of apoptotic cells (hypodiploid cells) was plotted against the concentrations of the extracts. Data were represented as mean ± S.E.M. of four independent experiments (n=4).

*significant difference when compared to the negative control at $p<0.05$

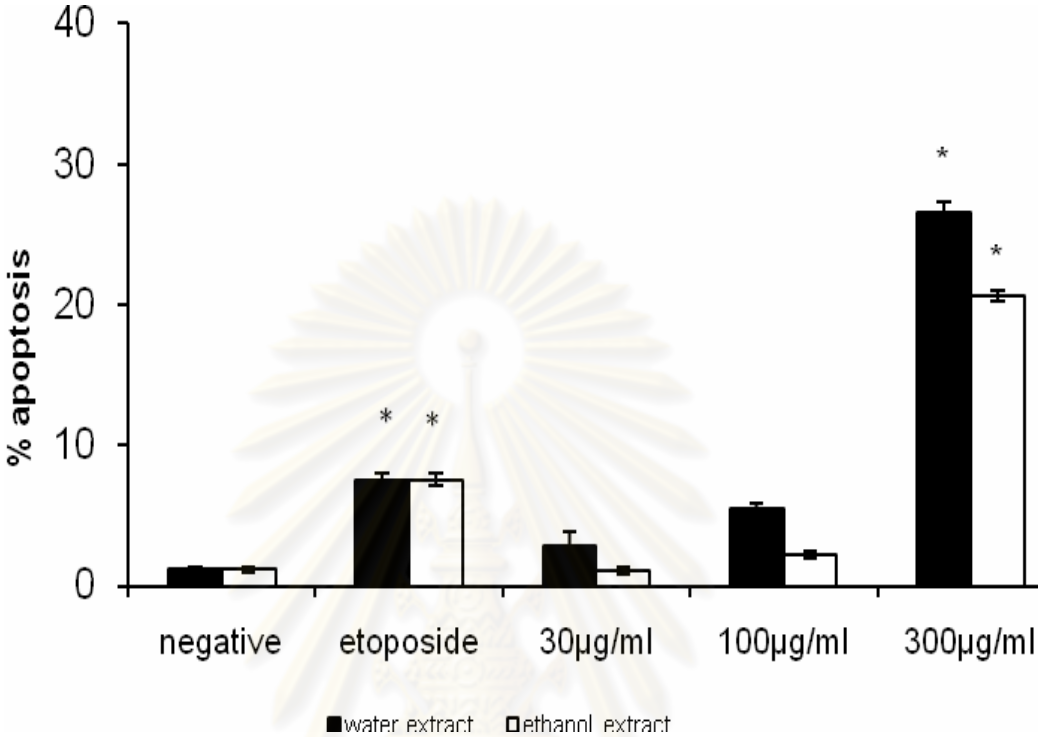


Figure 16: Analysis of DNA content in Jurkat cells after treatment with the extracts for 48 h. The assay was performed as in the Fig. 15, except changing the incubation time from 24 h to 48 h. Data were represented as mean ± S.E.M. of three independent experiments (n=3).

*significant difference when compared to the negative control at $p<0.05$

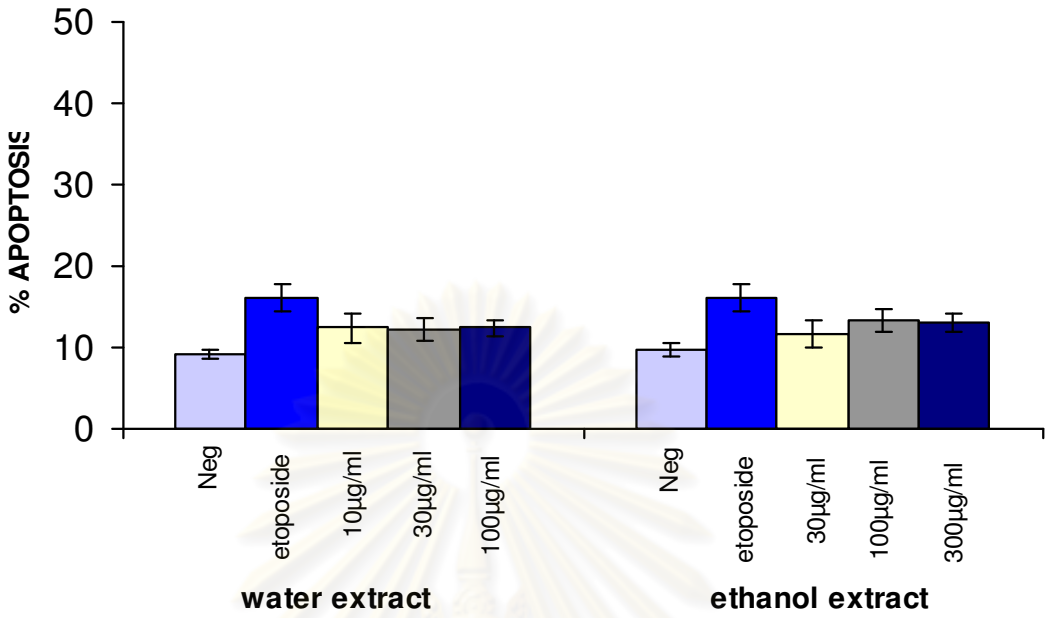
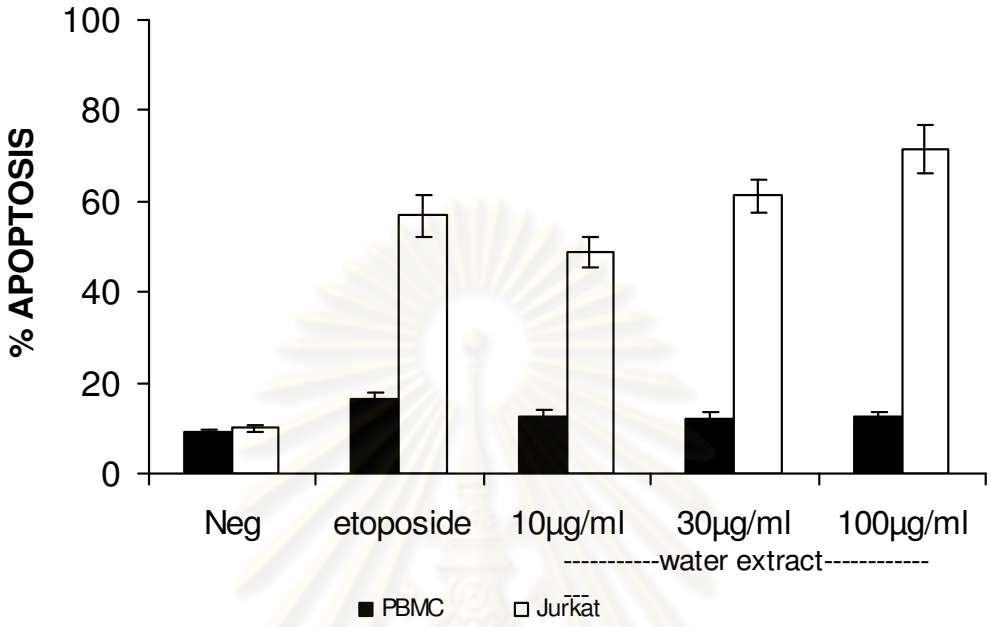


Figure 17: The apoptosis induced by the extract on normal cells. Human PBMCs were treated with three concentrations of the extracts for 18 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data represented the percentages of the apoptotic cells (annexin V-FITC positive cells) as mean ± S.E.M. of four independent experiments (n=4).

(a) water extract



(b) ethanol extract

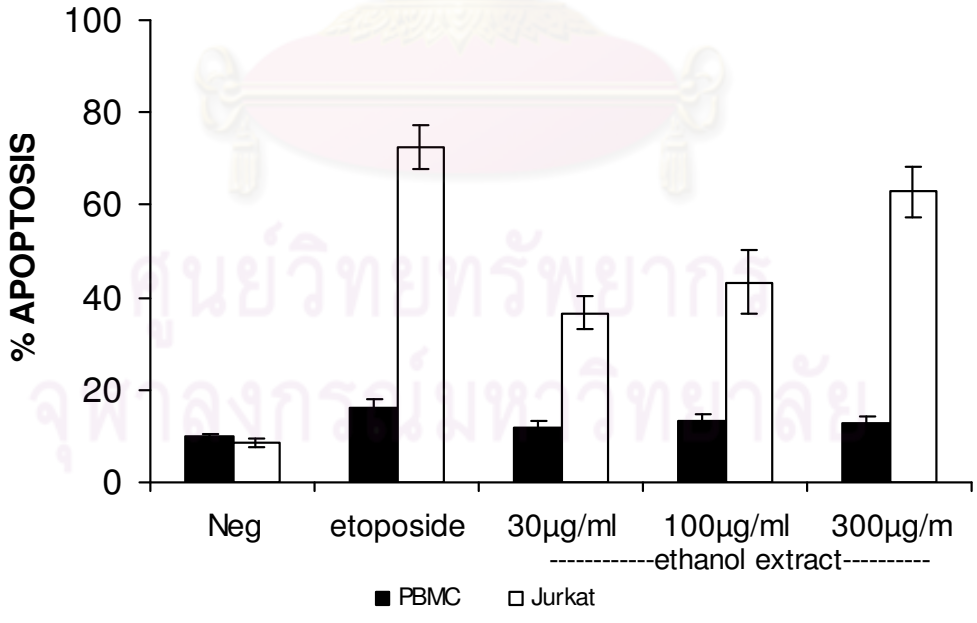


Figure 18: The comparison between human PBMC and Jurkat cell apoptosis induced by the extracts. Data represented as mean ± S.E.M. of four independent experiments (n=4)

3. Mechanisms of Mareng-roipad formulation induced apoptosis

To evaluate the molecular effector pathway of apoptosis induced by the extracts from Mareng-roipad formulation, the roles of Fas-FasL interaction and caspases were examined by blocking FasL with an anti-FasL antibody and inhibiting caspases with a caspase inhibitor.

To investigate the role of Fas-FasL interaction, Jurkat cells were pretreated with 10 ng/ml anti-human FasL antibody (NOK-1) for 1 h before being treated with the extracts for 18 h. The percentage of apoptotic cell was analyzed by annexin V-FITC/PI staining assay using flow cytometer. As shown in Fig.19, the apoptosis induced by both extracts was significantly inhibited, from 52.67% to 14.80% for the water extract and from 51.13% to 22.98% for the ethanol extract. These results suggest that the extracts from Mareng-roipad formulation induce Jurkat cell apoptosis mediated, in part, by the Fas/FasL signaling pathway.

To examine the involvement of caspases in the apoptosis induced by the extracts, Jurkat cells were pretreated with the pan-caspase inhibitor Z-VAD-fmk for 1h, followed by the extract treatment for 18 h. The caspase inhibitor significantly rescued the cells from the apoptosis induced by both extract, from 48.83% to 24.68% for the water extract and from 51.80% to 26.83% for the ethanol extract (Fig. 20). These studies suggest that the apoptosis triggered by the extracts was mediated by caspase activation.

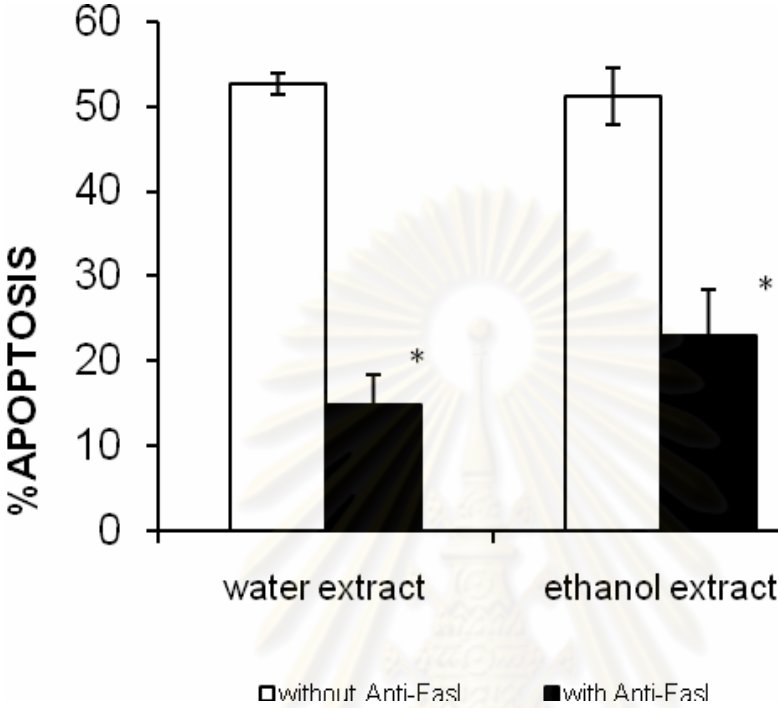


Figure 19: Effect of Fas-FasL signaling pathway on the Jurkat cells apoptosis induced by the extracts. Cells were 1 h pretreated with 10 ng/ml anti-FasL antibody before treatment with the extracts for 18 h. The apoptotic cells were detected with flow cytometer after staining with annexin V-FITC/PI. Data represented the percentage of the apoptotic cells (annexin V-FITC positive) as mean ± S.E.M. of three independent experiments (n=3).

* Significant difference when compared between the anti-FasL antibody-treated and the untreated conditions at $p<0.05$

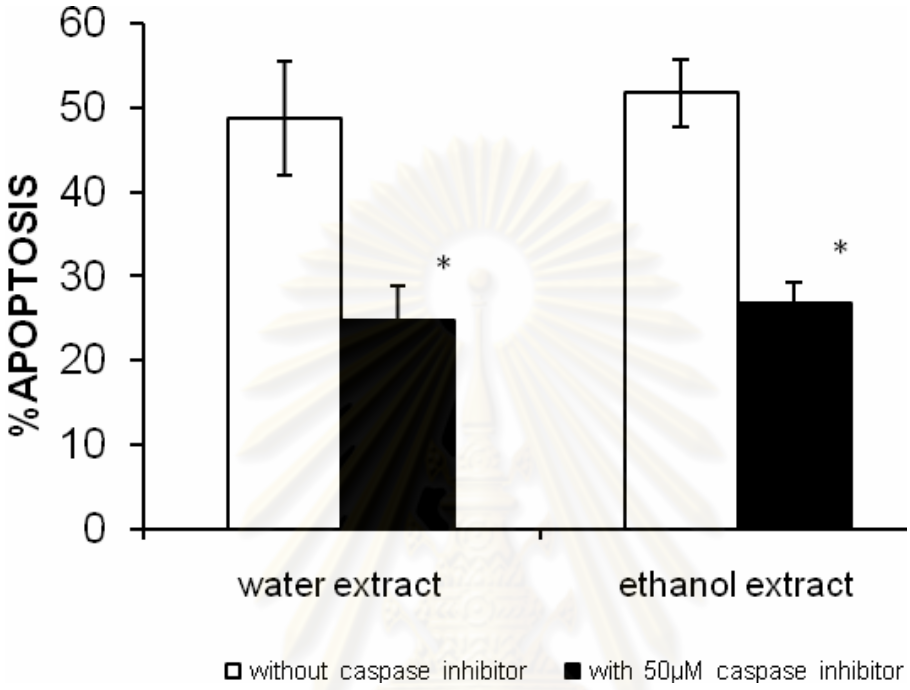


Figure 20: Effect of caspases on the Jurkat cells apoptosis induced by the extracts. Cells were 1 h pretreated with 50 μM z-VAD-fmk before treatment with the extracts for 18 h. The apoptotic cells were detected with flow cytometer after staining with annexin V-FITC/PI. Data represented the percentage of the apoptotic cells (annexin V-FITC positive) as mean ± S.E.M. of three independent experiments (n=3).

* significant difference when compared between the caspase inhibitor-treated and the untreated conditions at $p<0.05$

CHAPTER V

DISCUSSION AND CONCLUSION

The anti-tumor activity of Mareng-roipad formulation was evaluated for the first time in this study even though this formulation has been used as a traditional medicine for cancer treatment for more than a century. Many current anti-cancer agents primarily exert their antitumor effect by triggering apoptosis in cancer cells **[59]**. The cytotoxic effect of both water and ethanol extracts of Mareng-roipad formulation was also determined in term of apoptosis, which is the most desirable target mechanism for the induction of cell death in tumor cells. Both extracts of the formulation demonstrated cytotoxicity against human acute cells, Jurkat cells, in a concentration and time-dependent manner when determined by PI staining assay. PI enters into death cells only, and intercalates DNA. The PI-stained death cells can be detected by fluorescence flow cytometer, which is a very sensitive method for quantization amount of cells with different characteristics. The water extract demonstrated more potent cytotoxicity than the ethanol extract.

The nature of cell death induced by the extracts was investigated in this study by using annexin V-FITC/PI staining and monitored by fluorescence flow cytometer. This method can be used to identify apoptotic cells as followed; annexin V-FITC positive cells, necrotic cells as PI and double annexin V-FITC/PI positive cells, and viable cells as double negative cells. Annexin V was used to detect early apoptosis. It specifically binds to phosphatidylserine (PS) which is translocated from the inner to the outer leaflet of the plasma membrane at the early stage of apoptosis **[17]**. The extracts induced Jurkat cell apoptosis was determined by the loss of lipid asymmetry in apoptotic cells by measuring PS externalization by annexin-V staining. The Jurkat cell cytotoxicity induced by the extracts involved apoptosis because annexin V-FITC positive cells was significantly increased, in a concentration-dependent fashion. The water extract was highly more potent than the ethanol extract, as determined from their IC_{50} (15 μg/ml versus 150 μg/ml) at 18 h of incubation. It is very interesting to notice that the extracts

induced mainly Jurkat cell apoptosis with few amount of necrotic cells. If this effect happens in vivo, the death cells as apoptotic cells will not be harmful to their surrounding tissues. The time dependent assay with both extracts indicated that the ethanol extract increased apoptotic cells according to the time of incubation, 6, 18, and 24 h. However, the water extract induced Jurkat cell apoptosis with maximum effect at 18 h. The time-dependent effect of the extract was observed between 6 and 18 h of incubation, but not at 24 h. It was possible that the apoptotic cells turned into particles of apoptotic bodies at 24 h and these particles were not gated during monitoring by flow cytometer. The effect of the extracts on cell death by apoptosis was confirmed by assessing hypodiploid DNA in apoptotic cell after PI staining. Both extracts did not induce apoptosis on normal human PBMCs. It is suggests that both water and ethanol extracts of Mareng-roidpad formulation preferentially induce apoptosis in tumor cells, at least in human acute leukemic cells, *in vitro*.

The mechanisms responsible for apoptotsis by the extracts were also explored in this study. A number of previous studies have raised the possibility that anticancer drugs trigger apoptosis by inducing death receptor pathway and activating caspase enzymes **[46]**. The mechanisms of action of the extracts were evaluated by inhibiting caspase activities with zVAD-fmk, a broad peptide inhibitor of caspases, and blocking Fas-FasL signaling pathway with NOK-1, an anti-FasL antibody. Flow cytometric staining of annexin V-FITC positive cells revealed that the anti-FasL antibody and z-VAD-fmk significantly attenuated apoptosis induced by both extracts. However, both inhibitors could not completely inhibit the effects of the extracts, even at their higher concentrations which were also tested in the study (data not shown). It is suggests that active compounds in Mareng-roipad formulation may induce apoptosis in tumor cells in part by caspase-dependent and Fas-FasL signaling pathway. Others apoptotic pathways that may involve in the anti-tumor effect of the formulation should be investigated.

In summary, this study revealed for the first time that Mareng-roipad formulation exhibit anti-tumor activity. The water and the ethanol extracts of the formulation induced preferentially tumor cell death, at least on leukemic T cell, mainly by apoptosis which is the safest way of cell death. They induced tumor cell apoptotic by Fas-FasL signaling

pathway and by activating caspases. The caspase-independent, which is need to be investigated in the future, may also be a apart of the apoptotic activity of the extracts. The results from this study suggest that Mareng-roipad has a possibilty of an anticancer formulation in traditional medicine. It may be a candidate for developing lower toxicity anti-leukemic agents. Further studies on individual active components in the formulation are required to be undertaken.

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APPENDICES

APPENDIX A

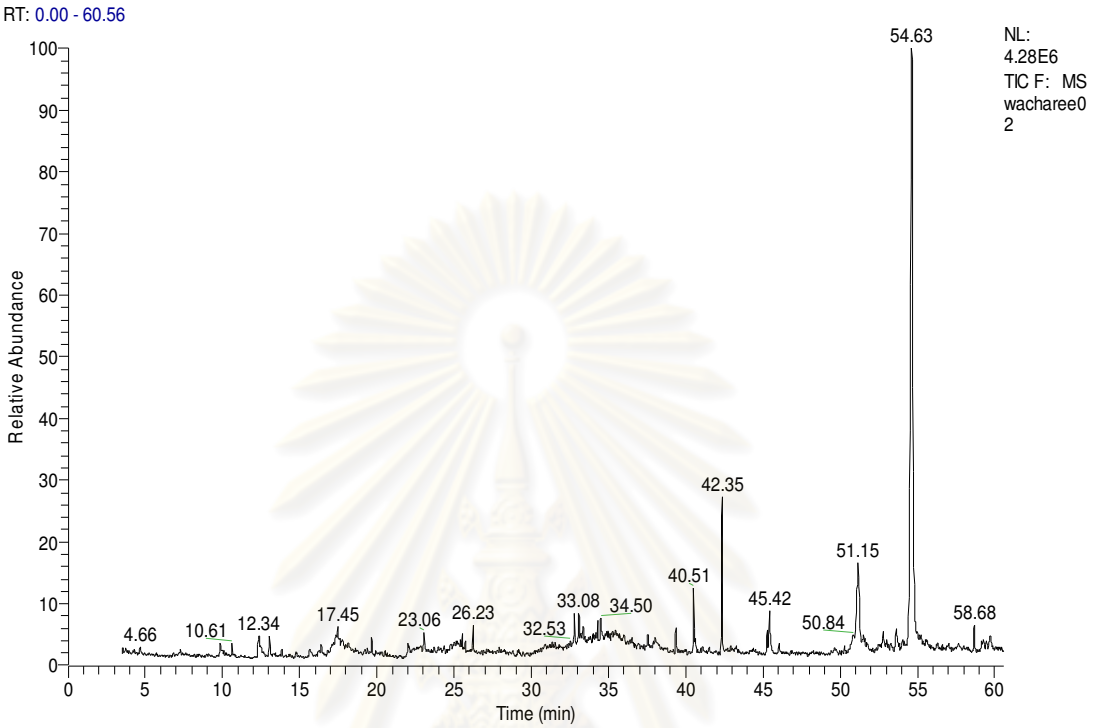
Identification of extracts

Identification of the water and the ethanol extracts of Mareng-roipad formulation was done by gas chromatography and mass spectrophotometry.

Instruments and Method

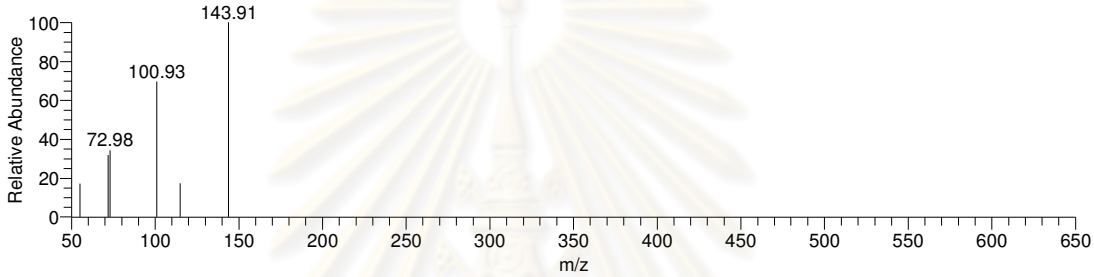
- Finnigan Trace GC ultra
- Finnigan DSQ Quadrupole detector
- BPX5 fused silica column (30 m x 0.25 mm, 0.25 um film thickness)
- The injector temperature was 180°C
- Sample 1 μl (20 mg/ml in methanol), was injected by splitter (1:10) into capillary column.
- Helium was used as carrier gas (flow rate 1 ml/min).
- MS was performed by EI positive mode at 70 eV ionization voltages.
- The constituents of the oil were identified by matching their mass spectra and retention indices with NIST02 MS library and the percentage composition was computed from GC peak areas.
- For ethanol extract: the oven temperature was start at 100°C, ramp with the rate of 3.3°C/ min to 240°C hold for 5 min, then ramp with the rate of 10°C/ min to 300°C hold for 10 min.
- For water extract: the oven temperature was hold at 60°C for 1 min, then ramp with the rate of 3.3°C/ min to 240°C hold for 5 min.

1. Identification of water extract by gas chromatography and mass specrophotometry

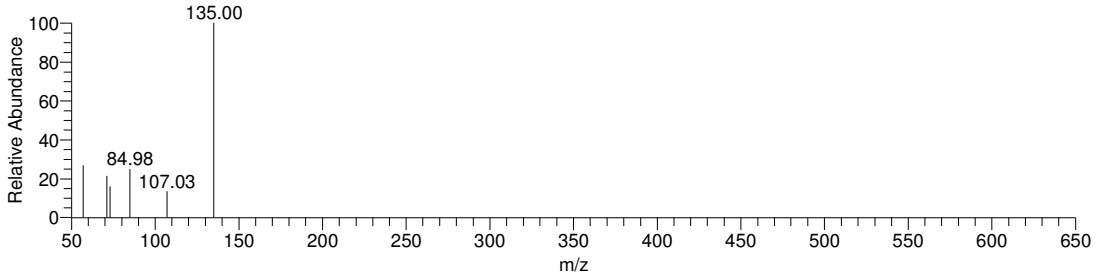


RT	Chemical Composition	Area %
12.34		1.45
13.06		0.91
26.23		1.20
32.79		1.40
33.08		1.75
34.50		1.13
39.37		1.09
40.51	Palmitic acid	2.79
42.35	Cyclic octaatomic sulfur	5.77
45.28		0.89
45.42	Oleic Acid	2.50
51.15	Phenol, 2-(1-methyl-2-buthenyl)-4-methoxy-	10.17

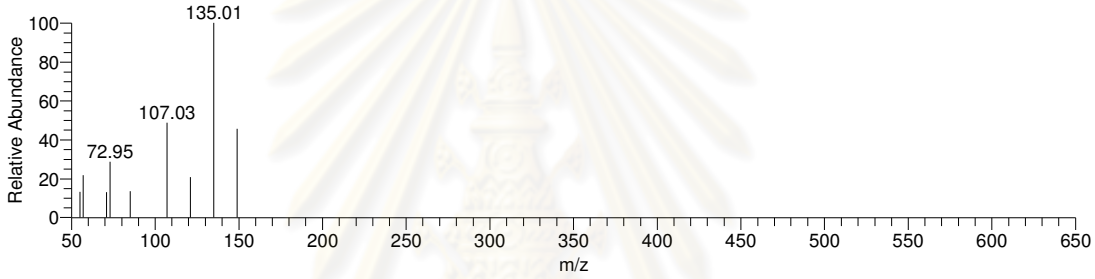
51.51		1.01
52.78		1.27
53.63		1.60
54.63	Piperine	60.58
55.24		1.14
58.68		1.31
59.15		1.09
59.70		0.94



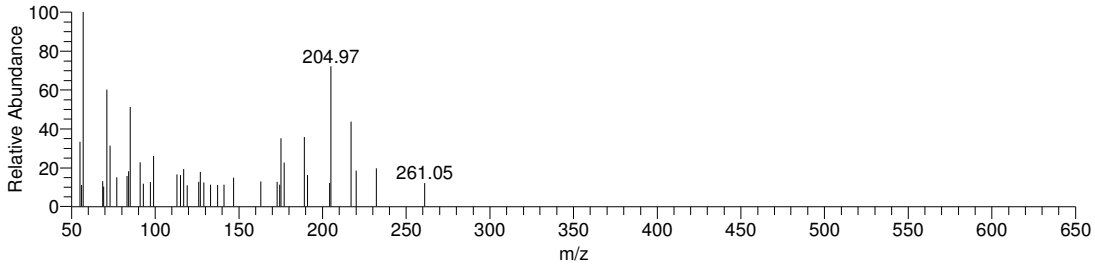
RT	Name	SI	Library	Formula
12.34	2-Butanol, 2-nitroso-, acetate (ester)	404	mainlib	$C_6H_{11}NO_3$
12.34	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	719	mainlib	$C_6H_8O_4$
12.34	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	726	replib	$C_6H_8O_4$



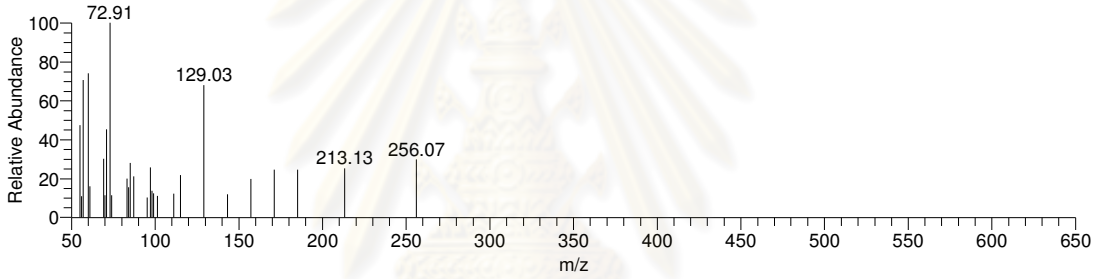
RT	Name	SI	Library	Formula
32.79	1-n-Hexyladamantane	432	mainlib	$C_{16}H_{28}$
32.79	Hydroxylamine, O-decyl-	443	mainlib	$C_{10}H_{23}NO$
32.79	Eicosane	462	replib	$C_{20}H_{42}$



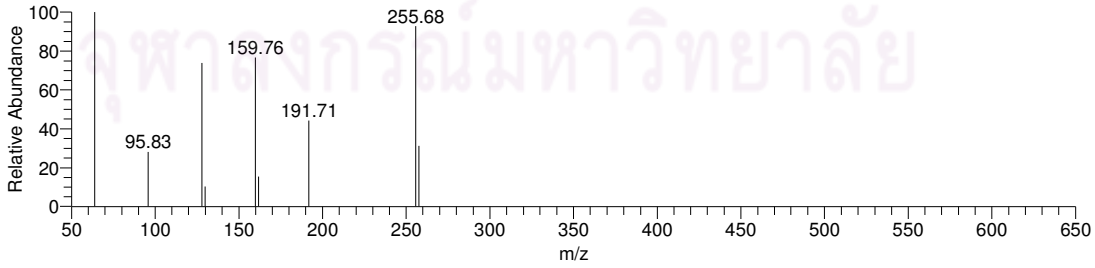
RT	Name	SI	Library	Formula
33.08	Picein	287	mainlib	$C_{14}H_{18}O_7$
33.08	Propanoic acid, 2,2-dimethyl-, 2-(1,1-dimethylethyl)-6-methylphenyl ester	333	mainlib	$C_{16}H_{24}O_2$
33.08	13,14-Benzo-1,4,8,11-tetrathiacyclopentadecane	405	mainlib	$C_{15}H_{22}S_4$



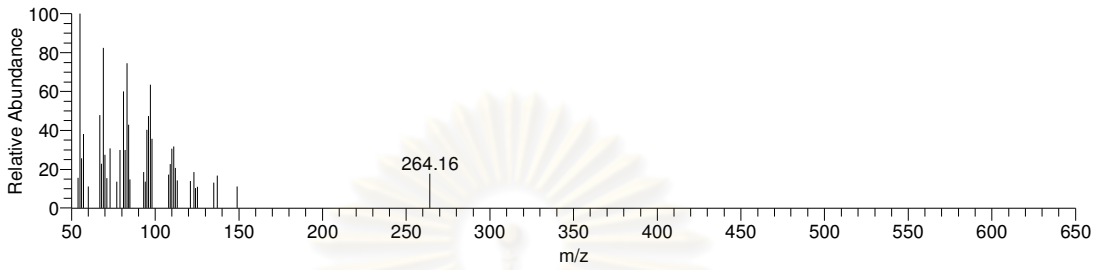
RT	Name	SI	Library	Formula
39.37	2,6-Bis(1,1-dimethylethyl)-4-methyl-4-isopropylcyclohexa-2,5-dien-1-one	317	mainlib	$C_{18}H_{3}0_{O}$
39.37	Pentadecane	412	replib	$C_{15}H_{32}$
39.37	Eicosane	418	replib	$C_{20}H_{42}$



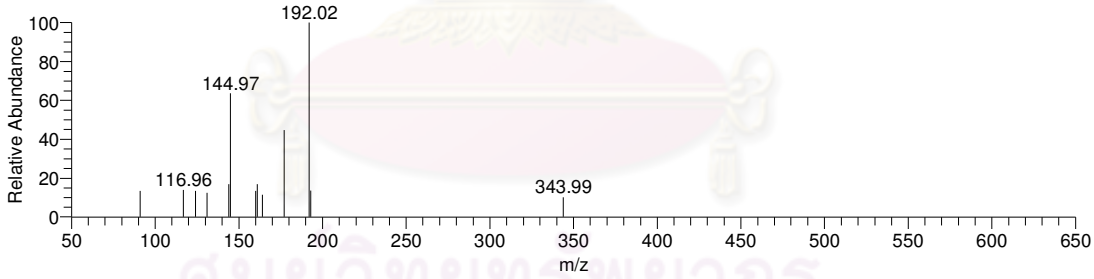
RT	Name	SI	Library	Formula
40.51	Tridecanoic acid	685	replib	$C_{13}H_{26}O_{2}$
40.51	Tetradecanoic acid	704	replib	$C_{14}H_{28}O_{2}$
40.51	n-Hexadecanoic acid	752	replib	$C_{16}H_{32}O_{2}$



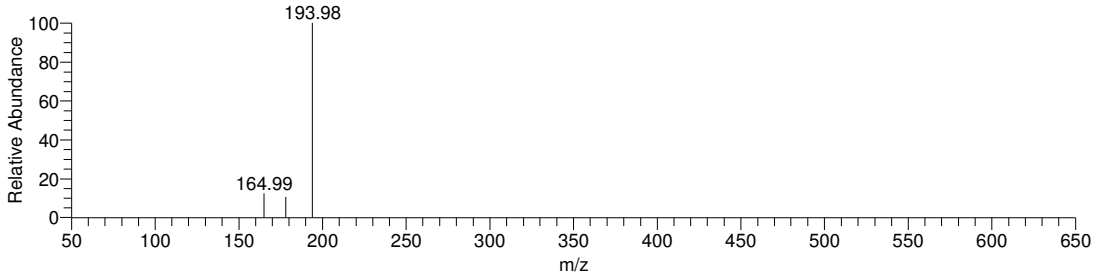
RT	Name	SI	Library	Formula
42.35	Sulfur	526	mainlib	S_6
42.35	Cyclic octaatomic sulfur	803	replib	S_8
42.35	Cyclic octaatomic sulfur	875	mainlib	S_8



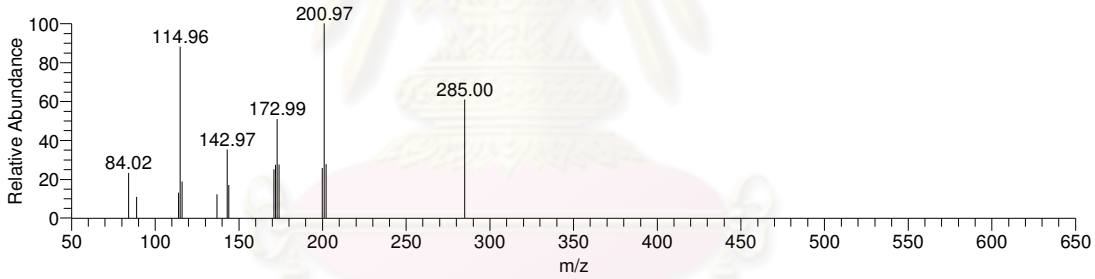
RT	Name	SI	Library	Formula
45.42	1-Hexyl-2-nitrocyclohexane	282	mainlib	$C_{12}H_{23}NO_2$
45.42	1-Hexyl-1-nitrocyclohexane	286	mainlib	$C_{12}H_{23}NO_2$
45.42	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	463	mainlib	$C_{20}H_{40}$



RT	Name	SI	Library	Formula
51.15	2H-1,4-Ethanoquinolin-3(4H)-one	376	replib	$C_{11}H_{11}NO$
51.15	7-Hydroxy-8-methoxy-2-methyl-1-(4'-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline	418	mainlib	$C_{19}H_{23}NO_3$
51.15	[2]Benzopyrano[4,3-b][1]benzopyran-7-ol, 5,6a,7,12a-tetrahydro-2,3,10-trimethoxy-, [6aS-(6aà,7à,12aá)]-	438	mainlib	$C_{19}H_{20}O_6$

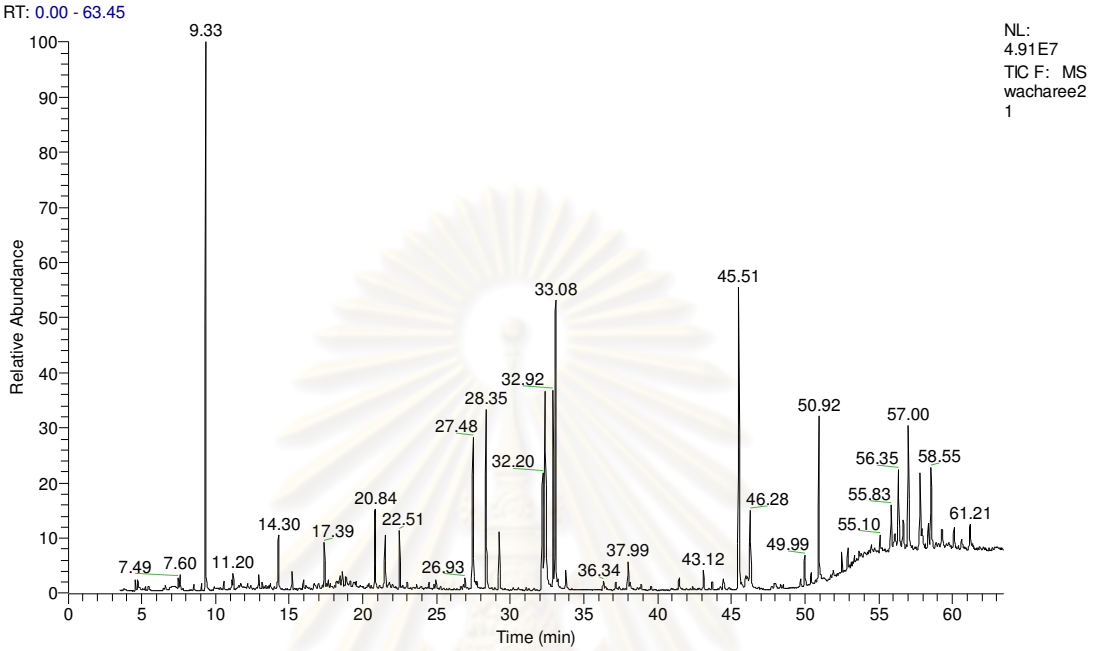


RT	Name	SI	Library	Formula
53.63	2-Naphthalenecarboxylic acid, 1-[[(2-methylphenyl)imino]phenylmethoxy]-, methyl ester	446	mainlib	$C_{26}H_{21}NO_3$
53.63	1H-Pyrrolo[2,3-b]pyridine, 2-phenyl-	495	mainlib	$C_{13}H_{10}N_2$
53.63	Benzoic acid, 3-methyl-2-[[(2-methylphenyl)imino]phenylmethoxy]-, methyl ester	515	mainlib	$C_{23}H_{21}NO_3$



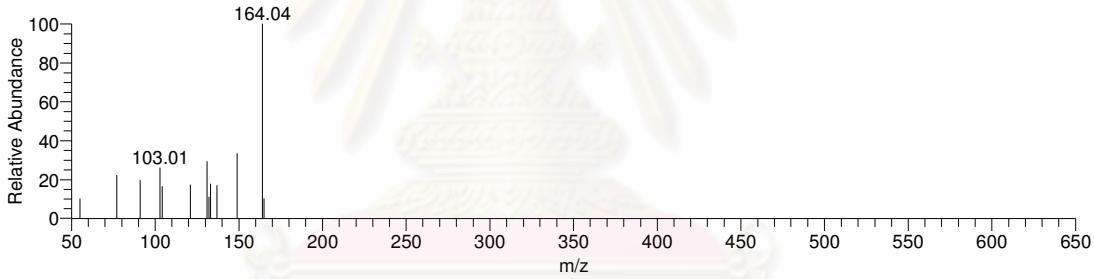
RT	Name	SI	Library	Formula
54.63	Piperine	800	replib	$C_{17}H_{19}NO_3$
54.63	Piperine	882	replib	$C_{17}H_{19}NO_3$
54.63	Piperine	907	replib	$C_{17}H_{19}NO_3$

2. Identification of ethanol extract by gas chromatography and mass specrophotometry

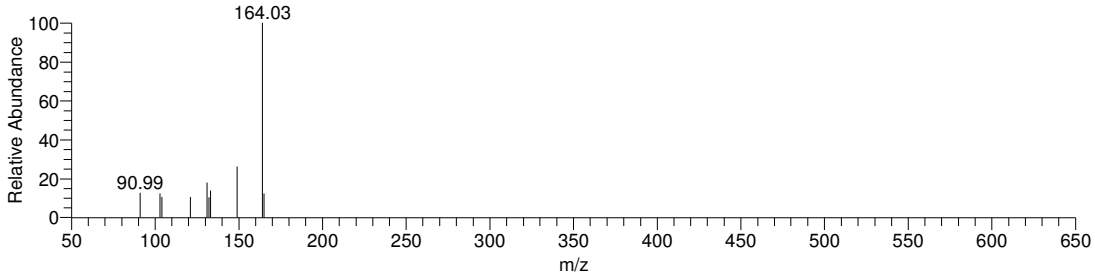


RT	Chemical Composition	Area %
9.33	Eugenol	12.46
14.30	Eugenol acetate	1.71
17.39	Apiol	1.08
20.84		2.52
21.53	Myristic acid	1.62
22.51	Myristic acid, ethyl ester	1.49
27.48	Palmitic acid	6.27
28.35	Palmitic acid, ethyl ester	4.97
29.25	Cyclic octaatomic sulfur	1.81
32.20	Linoleic acid	4.93
32.37	Oleic Acid	10.43
32.92	9,12-Octadecadienoic acid, ethyl ester	5.80
33.08	Ethyl Oleate	9.45
37.99	2,6-Diphenyl-1,7-heptadiene	0.77

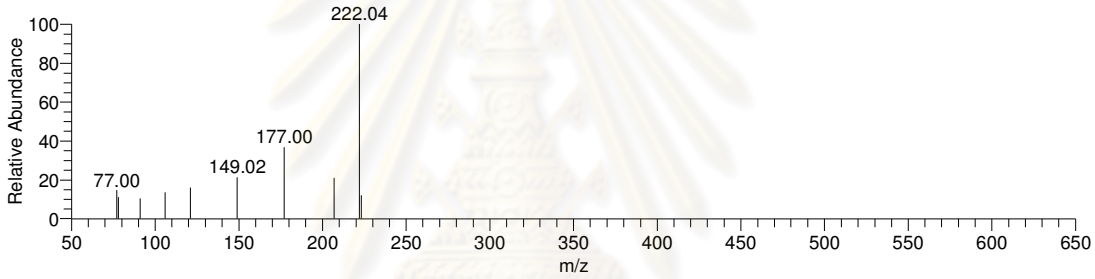
43.12		0.65
45.51	*para*-Anisoin	12.12
RT	**Chemical Composition**	**Area %**
45.99		0.68
46.28	Dihydroeugenol	3.54
49.99		0.80
50.92	Piperine	4.59
55.83		0.70
56.35	*alpha*-Amyrin	2.59
57.00	Lupeol	3.72
57.81	*beta*-Amyrin	2.43
58.55	Lupeol acetate	2.86



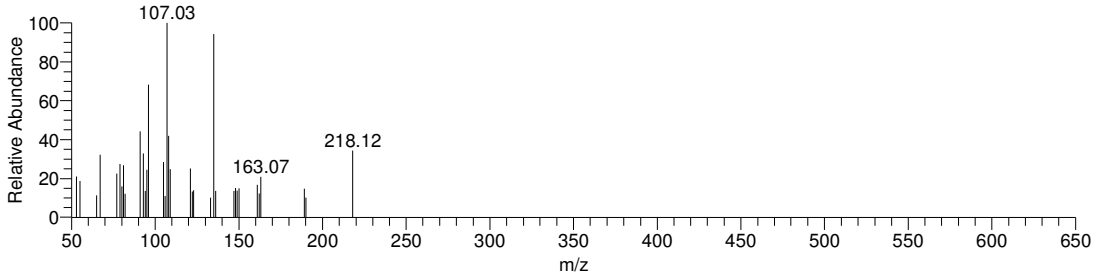
RT	Name	SI	Library	Formula
9.33	Phenol, 2-methoxy-4-(1-propenyl)-	892	replib	$C_{10}H_{12}O_2$
9.33	Eugenol	913	replib	$C_{10}H_{12}O_2$
9.33	Eugenol	940	replib	$C_{10}H_{12}O_2$



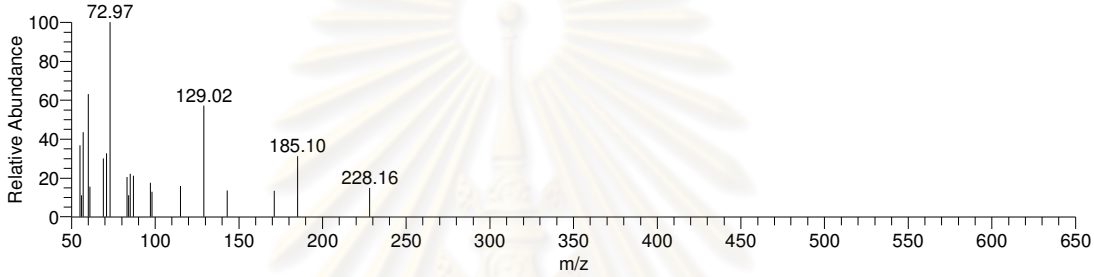
RT	Name	SI	Library	Formula
14.30	Phenol, 2-methoxy-4-(1-propenyl)-	831	replib	$C_{10}H_{12}O_2$
14.30	Eugenol	833	replib	$C_{10}H_{12}O_2$
14.30	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	900	replib	$C_{12}H_{14}O_3$



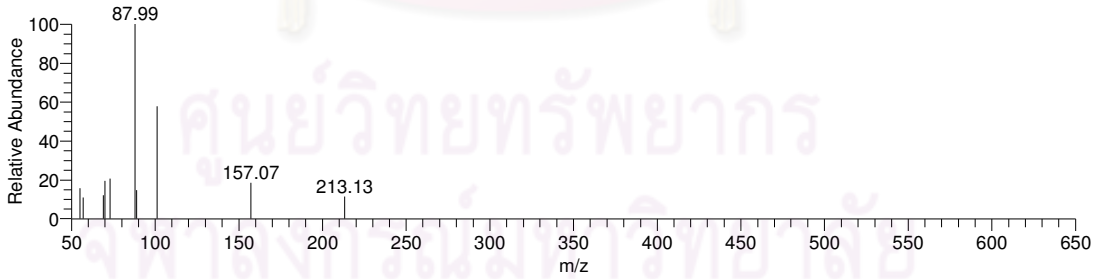
RT	Name	SI	Library	Formula
17.39	(1'S,4a'S,8a'R)-7-[(1,4,4a,6,6,8a-Hexahydro-2,5,5,8a-tetramethyl-6-oxo-1-naphthalenyl)methoxy]-6,8-dimethoxy-2H-1-benzopyran-2-one	514	mainlib	$C_{26}H_{30}O_6$
17.39	Apiol	766	replib	$C_{12}H_{14}O_4$
17.39	Apiol	878	replib	$C_{12}H_{14}O_4$



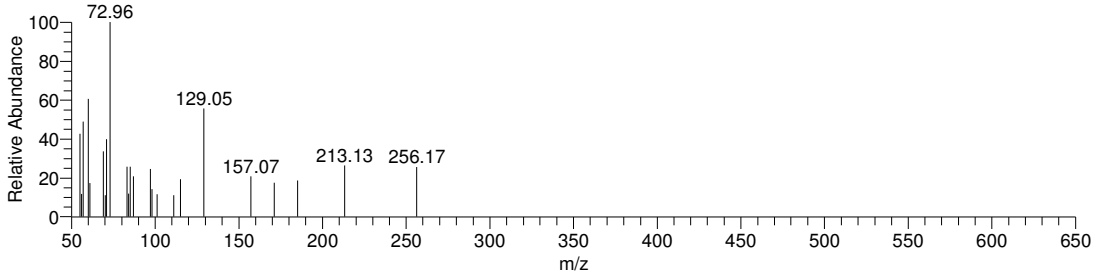
RT	Name	SI	Library	Formula
20.84	2R-Acetoxymethyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1t-cyclohexanol	381	mainlib	$C_{17}H_{30}O_3$
20.84	1H-3a,7-Methanoazulene, 2,3,6,7,8,8a-hexahydro-1,4,9,9-tetramethyl-, (1à,3aà,7à,8aá)-	618	replib	$C_{15}H_{24}$
20.84	2,6,10-Cycloundecatrien-1-one, 2,6,9,9-tetramethyl-, (E,E,E)-	859	mainlib	$C_{15}H_{22}O$



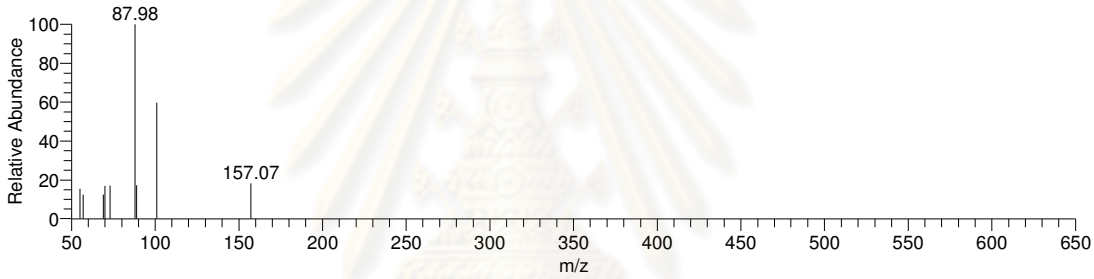
RT	Name	SI	Library	Formula
21.53	Undecanoic acid	729	replib	$C_{11}H_{22}O_2$
21.53	Tetradecanoic acid	876	replib	$C_{14}H_{28}O_2$
21.53	Tetradecanoic acid	880	replib	$C_{14}H_{28}O_2$



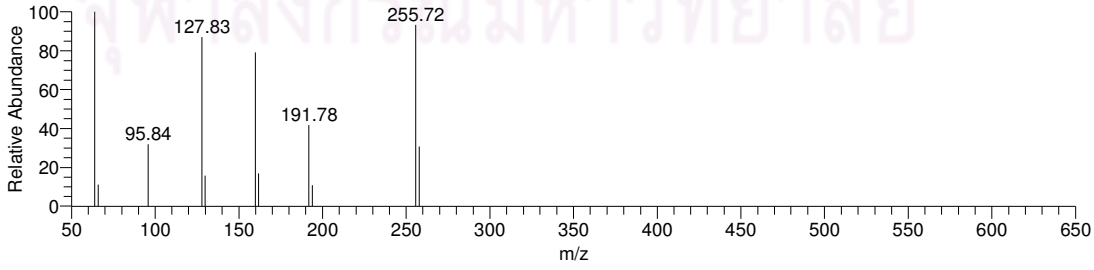
RT	Name	SI	Library	Formula
22.51	Dodecanoic acid, ethyl ester	747	replib	$C_{14}H_{28}O_2$
22.51	Tetradecanoic acid, ethyl ester	836	replib	$C_{16}H_{32}O_2$
22.51	Tetradecanoic acid, ethyl ester	883	mainlib	$C_{16}H_{32}O_2$



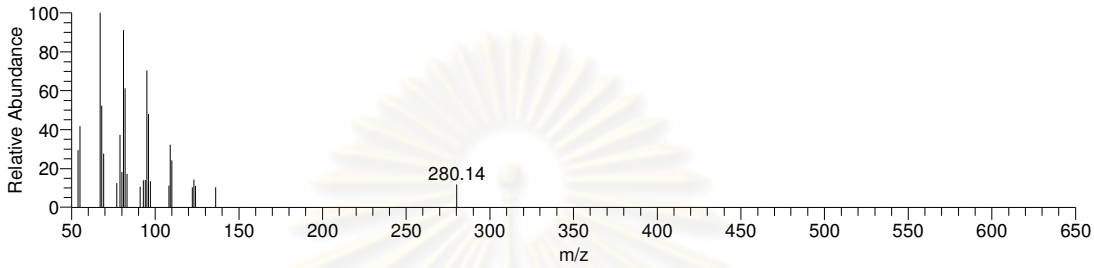
RT	Name	SI	Library	Formula
27.48	Tridecanoic acid	767	replib	$C_{13}H_{26}O_2$
27.48	Tetradecanoic acid	773	replib	$C_{14}H_{28}O_2$
27.48	n-Hexadecanoic acid	884	replib	$C_{16}H_{32}O_2$



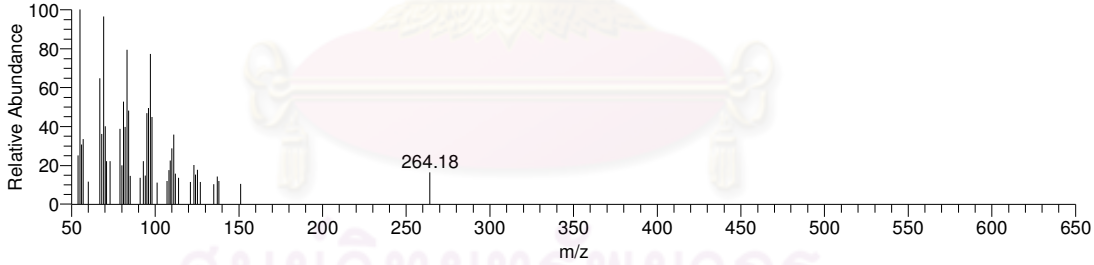
RT	Name	SI	Library	Formula
28.35	Dodecanoic acid, ethyl ester	742	replib	$C_{14}H_{28}O_2$
28.35	Hexadecanoic acid, ethyl ester	874	replib	$C_{18}H_{36}O_2$
28.35	Hexadecanoic acid, ethyl ester	908	mainlib	$C_{18}H_{36}O_2$



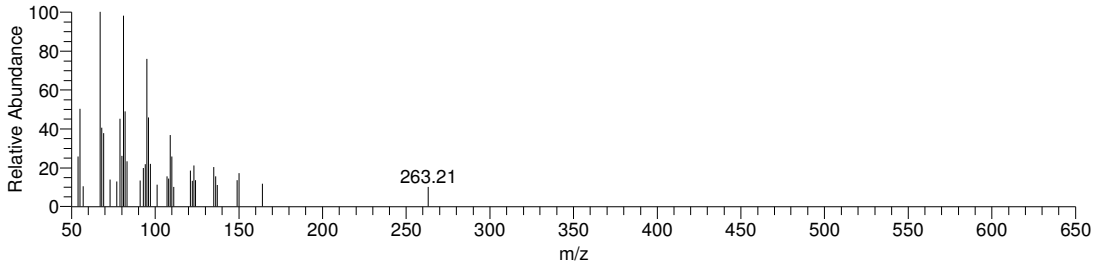
RT	Name	SI	Library	Formula
29.25	Sulfur	564	mainlib	S_6
29.25	Cyclic octaatomic sulfur	807	replib	S_8
29.25	Cyclic octaatomic sulfur	910	mainlib	S_8



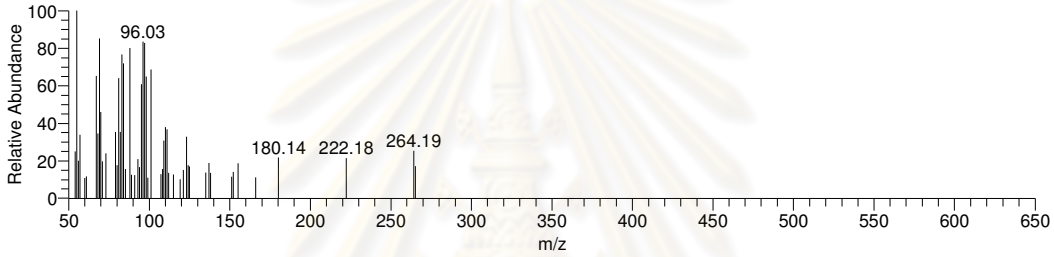
RT	Name	SI	Library	Formula
32.20	9,12-Octadecadienoic acid, methyl ester, (E,E)-	817	replib	$C_{19}H_{34}O_2$
32.20	11,14-Eicosadienoic acid, methyl ester	818	mainlib	$C_{21}H_{38}O_2$
32.20	9,12-Octadecadienoic acid (Z,Z)-	825	replib	$C_{18}H_{32}O_2$



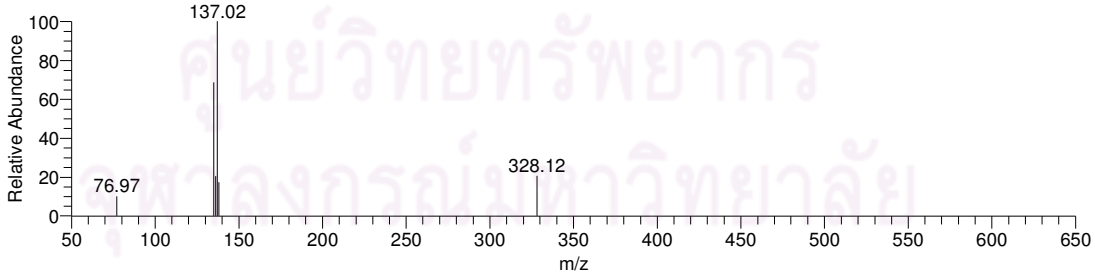
RT	Name	SI	Library	Formula
32.37	1-Hexyl-2-nitrocyclohexane	311	mainlib	$C_{12}H_{23}NO_2$
32.37	1-Hexyl-1-nitrocyclohexane	324	mainlib	$C_{12}H_{23}NO_2$
32.37	Oleic Acid	888	replib	$C_{18}H_{34}O_2$



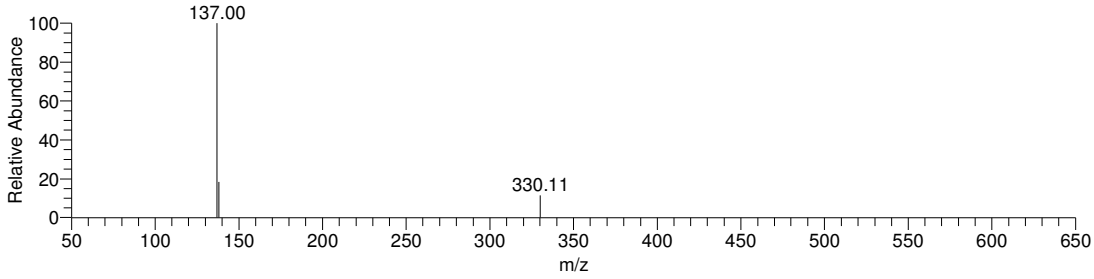
RT	Name	SI	Library	Formula
32.92	Linoleic acid ethyl ester	787	replib	$C_{20}H_{36}O_2$
32.92	Linoleic acid ethyl ester	832	replib	$C_{20}H_{36}O_2$
32.92	9,12-Octadecadienoic acid, ethyl ester	903	mainlib	$C_{20}H_{36}O_2$



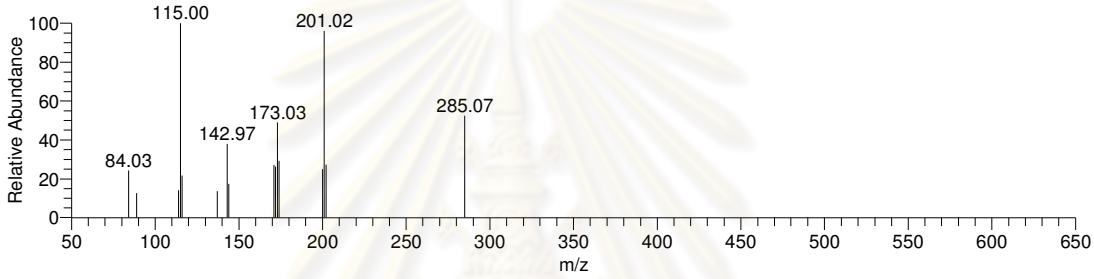
RT	Name	SI	Library	Formula
33.08	Ethyl Oleate	873	replib	$C_{20}H_{38}O_2$
33.08	(E)-9-Octadecenoic acid ethyl ester	894	mainlib	$C_{20}H_{38}O_2$
33.08	Ethyl Oleate	904	replib	$C_{20}H_{38}O_2$



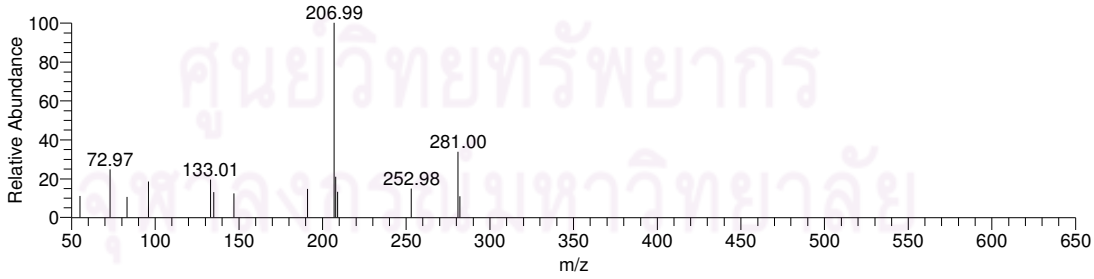
RT	Name	SI	Library	Formula
45.51	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-	507	mainlib	$C_{10}H_{12}O_3$
45.51	Ethanone, 2-hydroxy-1,2-bis(4-methoxyphenyl)-	605	mainlib	$C_{16}H_{16}O_4$
45.51	Ethanone, 2-hydroxy-1,2-bis(4-methoxyphenyl)-	643	replib	$C_{16}H_{16}O_4$



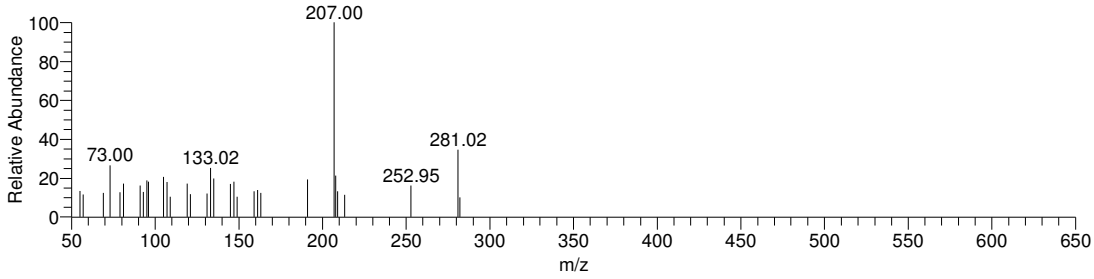
RT	Name	SI	Library	Formula
46.28	(-)-Nortrachelogenin	650	mainlib	$C_{20}H_{22}O_7$
46.28	Homovanillyl alcohol	665	replib	$C_9H_{12}O_3$
46.28	Phenol, 2-methoxy-4-propyl-	666	mainlib	$C_{10}H_{14}O_2$



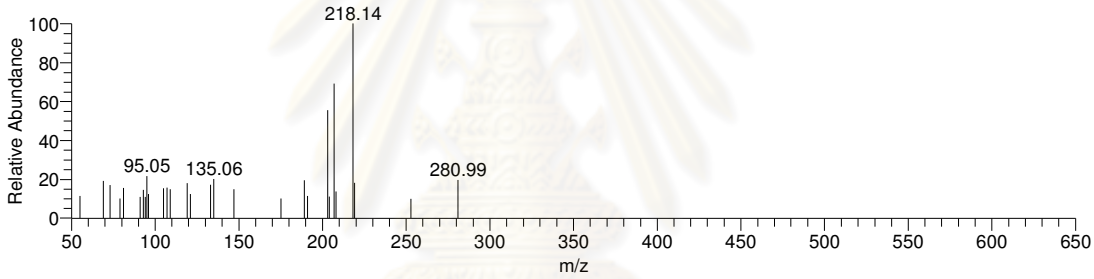
RT	Name	SI	Library	Formula
50.92	Piperine	792	replib	$C_{17}H_{19}NO_3$
50.92	Piperine	883	replib	$C_{17}H_{19}NO_3$
50.92	Piperine	907	replib	$C_{17}H_{19}NO_3$



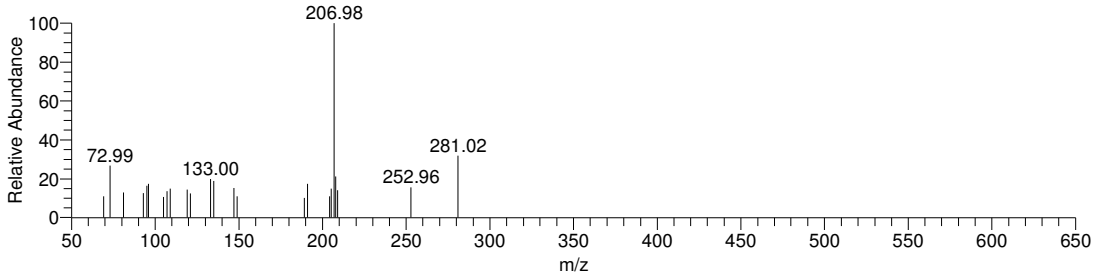
RT	Name	SI	Library	Formula
55.10	Cyclotrisiloxane, hexamethyl-	318	replib	$C_6H_{18}O_3Si_3$
55.10	1,1,1,3,5,5,5-Heptamethyltrisiloxane	370	mainlib	$C_7H_{22}O_2Si_3$
55.10	Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane	396	mainlib	$C_{17}H_{30}OSi$



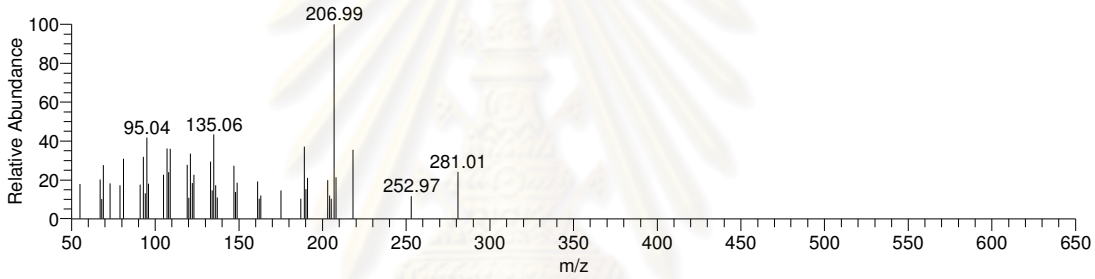
RT	Name	SI	Library	Formula
55.83	1,1,1,3,5,5,5-Heptamethyltrisiloxane	320	mainlib	$C_7H_{22}O_2Si_3$
55.83	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	383	mainlib	$C_{15}H_{24}O_2Si$
55.83	ç-Sitosterol	554	replib	$C_{29}H_{50}O$



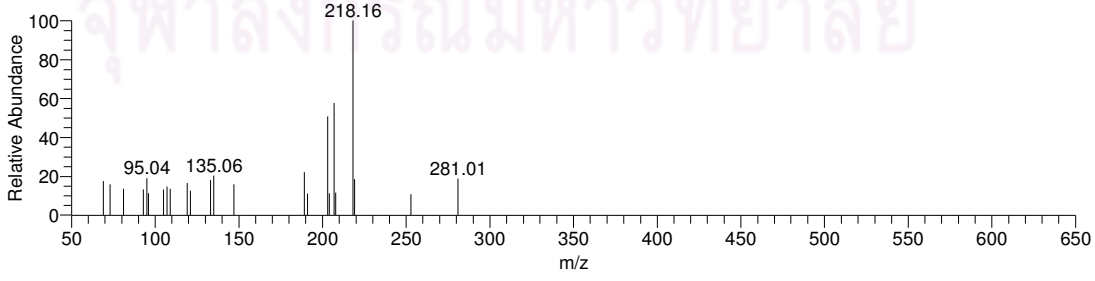
RT	Name	SI	Library	Formula
56.35	2H-Cyclopropa[a]naphthalen-2-one, 1,1a,4,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, (1aà,7à,7aà,7bà)-	599	mainlib	$C_{15}H_{22}O$
56.35	Olean-12-ene	655	mainlib	$C_{30}H_{50}$
56.35	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	655	mainlib	$C_{31}H_{48}O_3$



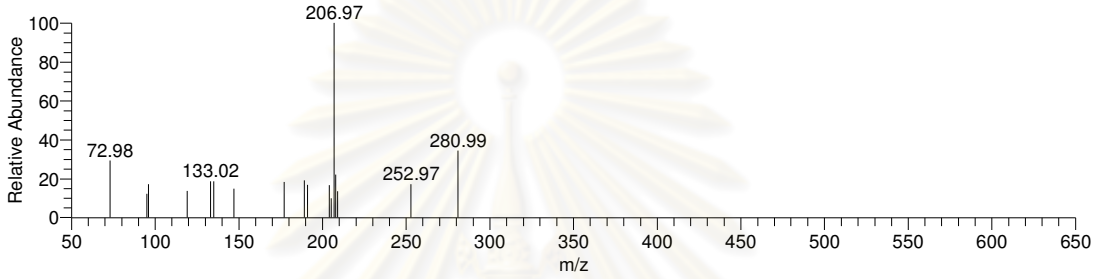
RT	Name	SI	Library	Formula
56.67	Cyclotrisiloxane, hexamethyl-	305	replib	$C_6H_{18}O_3Si_3$
56.67	1,1,1,3,5,5,5-Heptamethyltrisiloxane	349	mainlib	$C_7H_{22}O_2Si_3$
56.67	Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane	366	mainlib	$C_{17}H_{30}OSi$



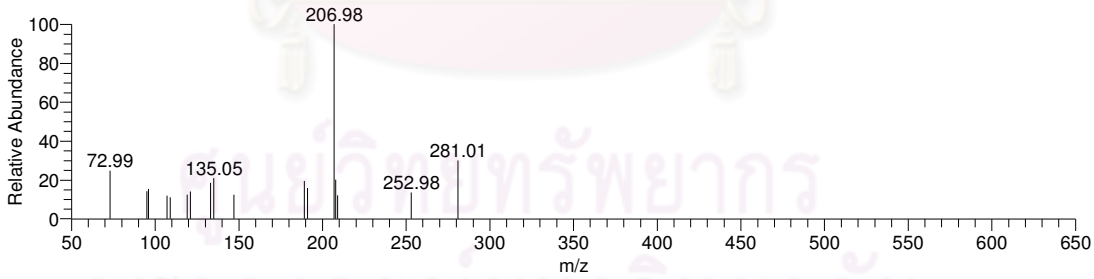
RT	Name	SI	Library	Formula
57.00	Hop-22(29)-en-3á-ol	380	mainlib	$C_{30}H_{50}O$
57.00	5,6-Azulenedimethanol, 1,2,3,3a,8,8a-hexahydro-2,2,8-trimethyl-, (3aà,8á,8aà)-	561	mainlib	$C_{15}H_{24}O_2$
57.00	Lupeol	803	mainlib	$C_{30}H_{50}O$



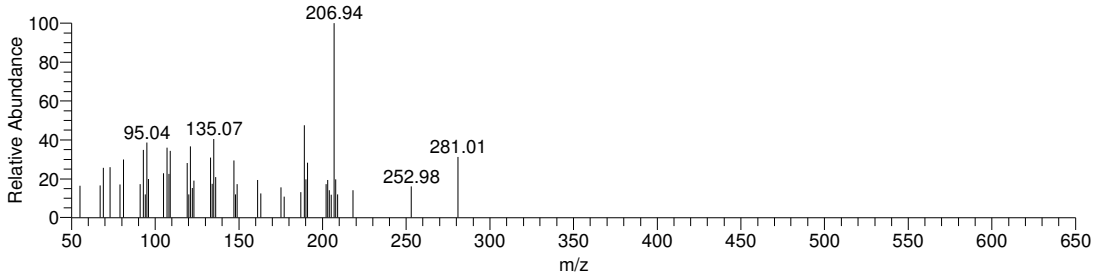
RT	Name	SI	Library	Formula
57.81	2H-Cyclopropa[a]naphthalen-2-one, 1,1a,4,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, (1aà,7à,7aà,7bà)-	600	mainlib	$C_{15}H_{22}O$
57.81	Olean-12-ene	655	mainlib	$C_{30}H_{50}$
57.81	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	674	mainlib	$C_{31}H_{48}O_3$



RT	Name	SI	Library	Formula
57.96	5-Methyl-2-trimethylsilyloxy-acetophenone	183	mainlib	$C_{12}H_{18}O_2Si$
57.96	4-Methyl-2-trimethylsilyloxy-acetophenone	187	mainlib	$C_{12}H_{18}O_2Si$
57.96	1,1,1,3,5,5,5-Heptamethyltrisiloxane	363	mainlib	$C_7H_{22}O_2Si_3$



RT	Name	SI	Library	Formula
58.38	5-Methyl-2-trimethylsilyloxy-acetophenone	201	mainlib	$C_{12}H_{18}O_2Si$
58.38	4-Methyl-2-trimethylsilyloxy-acetophenone	204	mainlib	$C_{12}H_{18}O_2Si$
58.38	1,1,1,3,5,5,5-Heptamethyltrisiloxane	375	mainlib	$C_7H_{22}O_2Si_3$



RT	Name	SI	Library	Formula
58.55	2,6,10-Dodecatrienoic acid, 3,7,11-trimethyl-9-(phenylsulfonyl)-, methyl ester, (E,E)-	207	mainlib	$C_{22}H_{30}O_4S$
58.55	Glaucyl alcohol	517	mainlib	$C_{15}H_{24}O$
58.55	Urs-12-ene	607	mainlib	$C_{30}H_{50}$

APPENDIX B

Buffers and Reagents

1. RPMI 1640 stock solution 1 liter

RPMI powder	10.4	g
$NaHCO_3$	1.5	g
Glucose	4.5	g
HEPES	10	ml
Penicillin/Streptomycin	10	ml
ddH_2O	900	ml

Adjust pH to 7.2 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by filtering through a 0.45 membrane filter

2. Complete RPMI 1640 medium 20 ml

RPMI stock	18	ml
Fetal Bovine Serum	2	ml

3. HBSS stock solution 1 liter

HBSS powder	9.25	g
$NaHCO_3$	0.35	g
ddH_2O	900	ml

Adjust pH to 7.2 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by filtering through a 0.45 membrane filter

4. 2µl/ml Heparin in HBSS

HBSS stock	22.5	ml
Heparin	45	µl

5. 10x Phosphate Buffered Saline (PBS) 1 liter

NCl	80	g
KCl	2	g
Na_2HPO_4	9.136	g
NH_2PO_4	2	g
ddH_2O	900	ml

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and sterilized by autoclaving

6. 1x Phosphate Buffered Saline (PBS) 1 liter

10xPBS	100	ml
ddH_2O	900	ml

Sterilized by autoclaving

7. 10x Assay Buffered 1 liter

HEPES	100	ml
$CaCl_2$	280	ml
NaCl	250	ml
ddH_2O	370	ml

8. 1x Assay Buffered 1 liter

10x Assay Buffered	100	ml
ddH_2O	900	ml

APPENDIX C

Results

Treatment	% Viable cells	% Death cells		
		Annexin-V^+ (Apoptosis)	PI^+ (Necrosis)	Double positive
Untreated control	88.81±0.91	10.71±0.90	0.14±0.04	0.33±0.05
10µg/ml Etoposide	55.14±4.89	43.39±4.97	0.15±0.08	0.69±0.14
10µg/ml water extract	70.14±2.59	28.34±2.52	0.39±0.14	0.59±0.08
30µg/ml water extract	61.60±1.50	37.59±1.48	0.16±0.06	0.64±0.13
100µg/ml water extract	53.66±2.00	45.44±1.94	0.20±0.10	0.68±0.19

Table 3: The Jurkat cell apoptosis induced by the water extract. Cells were treated with 10-100 µg/ml extract for 6 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data were represented as mean ± S.E.M. of four independent experiments (n=4).

Treatment	%Viable cells	% Death cells		
		Annexin-V^+ (Apoptosis)	PI^+ (Necrosis)	Double positive
Untreated control	88.81±0.91	10.71±0.90	0.14±0.04	0.33±0.05
10µg/ml Etoposide	55.14±4.89	43.39±4.97	0.15±0.08	0.69±0.14
30µg/ml ethanol extract	61.84±2.61	35.18±2.40	1.04±0.85	1.95±1.39
100µg/ml ethanol extract	58.78±2.40	40.39±2.45	0.25±0.13	0.61±0.12
300µg/ml ethanol extract	53.05±1.49	46.43±1.48	0.05±0.03	0.46±0.18

Table 4: The Jurkat cell apoptosis induced by the ethanol extract. Cells were treated with 10-100 µg/ml extract for 6 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data were represented as mean ± S.E.M. of four independent experiments (n=4).

Treatment	%Viable cells	% Death cells		
		Annexin-V^{+} (Apoptosis)	PI^{+} (Necrosis)	Double positive
Untreated control	92.83±0.97	4.38±0.99	2.02±0.66	0.75±0.03
10µg/ml Etoposide	21.31±5.39	51.72±3.32	7.05±3.11	19.78±4.58
10µg/ml water extract	66.13±5.77	32.45±3.59	0.18±2.77	1.23±6.28
30µg/ml water extract	46.05±4.19	48.63±1.95	0.85±0.62	4.43±1.89
100µg/ml water extract	27.80±0.87	62.07±0.50	2.87±0.09	7.25±0.53

Table 5: The Jurkat cell apoptosis induced by the water extract. Cells were treated with 10-100 µg/ml extract for 24h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data were represented as mean ± S.E.M. of three independent experiments (n=3).

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Treatment	%Viable cells	% Death cells		
		Annexin-V^+ (Apoptosis)	PI^+ (Necrosis)	Double positive
Untreated control	92.83±0.97	4.38±0.99	2.02±0.61	0.75±0.03
10µg/ml Etoposide	21.32±5.39	51.72±3.32	7.05±3.11	19.78±4.58
30µg/ml ethanol extract	49.12±3.42	43.71±1.95	1.18±0.51	6.50±1.20
100µg/ml ethanol extract	34.32±1.69	61.32±1.63	0.32±0.12	4.08±0.42
300µg/ml ethanol extract	17.48±1.24	77.97±0.87	0.85±0.25	3.72±1.71

Table 6: The Jurkat cell apoptosis induced by the ethanol extract. Cells were treated with 10-100 µg/ml extract for 24 h. The death cells were determined by annexin V-FITC/PI staining and monitored by flow cytometer. Data were represented as mean ± S.E.M. of three independent experiments (n=3).

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