ฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งของพรอพอลิสผึ้งพันธุ์ *Apis mellifera* จากจังหวัดน่าน ประเทศไทย

นางสาวดวงพร ธีระศรีปรีชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIPROLIFERATIVE ACTIVITY AGAINST CANCER CELLS OF *Apis mellifera* PROPOLIS FROM NAN PROVINCE, THAILAND

Miss Dungporn Teerasripreecha

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology
Faculty of Science
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By	Miss Dungporn Teerasripreecha
Field of Study	Zoology
Thesis Advisor	Associate Professor Chanpen Chanchao, Ph.D.
Thesis Co-advisor	Assistant Professor Preecha Phuwapraisirisan, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…………………………………… Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr. rer. nat.)

THESIS COMMITTEE

……………………………………………….. Chairman
(Associate Professor Kumthorn Thirakhupt, Ph.D.)

……………………………………………….. Thesis Advisor
(Associate Professor Chanpen Chanchao, Ph.D.)

……………………………………………….. Thesis Co-advisor
(Assistant Professor Preecha Phuwapraisirisan, Ph.D.)

……………………………………………….. Examiner
(Associate Professor Wichai Cherdshewasart, Ph.D.)

……………………………………………….. External Examiner
(Orawan Duangphakdee, Ph.D.)

ดวงพร ธีระศรีปรีชา : ฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งของพรอพอลิสผึ้งพันธุ์ *Apis mellifera* จากจังหวัดน่าน ประเทศไทย . (ANTIPROLIFERATIVE ACTIVITY AGAINST CANCER CELLS OF *Apis mellifera* PROPOLIS FROM NAN PROVINCE, THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. จันทร์เพ็ญ จันทร์เจ้า , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ปรีชา ภูวไพรศิริศาล, 120 หน้า.

พรอพอลิสเป็นผลิตภัณฑ์ชนิดหนึ่งของผึ้ง มีฤทธิ์ทางชีวภาพมากมาย เช่น ฤทธิ์ต้านแบคทีเรีย ฤทธิ์ต้านรา ฤทธิ์ต้านการอักเสบ และ ฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็ง เป็นต้น งานวิจัยนี้ศึกษาพรอพอลิสของผึ้งพันธุ์ *Apis mellifera* โดยทำการเก็บพรอพอลิสของผึ้งดังกล่าวจากจังหวัดน่าน ประเทศไทย ทำการสกัดขั้นต้นโดยใช้ 96% (ปริมาตร/ปริมาตร) เอทานอลและน้ำ นำสารสกัดหยาบมาทำการทดสอบความสามารถในการต้านการเพิ่มจำนวนของเซลล์มะเร็ง 5 ชนิดคือเซลล์มะเร็งเต้านม (บีที 474) เซลล์มะเร็งปอด (ชาโก) เซลล์มะเร็งตับ (เฮพจี 2) เซลล์มะเร็งกระเพาะอาหาร (คาโต 3) และเซลล์มะเร็งลำไส้ (เอสดับเบิลยู 620) นอกจากนี้มีการใช้เซลล์ไฟโบรบลาสต์ (เอชเอส) เป็นเซลล์ควบคุมปกติ โดยวิธีตรวจสอบที่ใช้สารเอมทีที พบว่าสารสกัดอย่างหยาบของพรอพอลิสด้วยเอทานอล (ซีอีอี) มีฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งต่อเซลล์ที่เลือกศึกษาได้ดี หลังจากนั้นทำการสกัดซีอีอีโดยทำการสกัดแยกส่วนโดยใช้เฮกเซน เมทิลลีนคลอไรด์ และเมทานอล เพื่อแยกสารประกอบตามความมีขั้วของสาร นำแต่ละส่วนที่แยกได้มาทำการทดสอบความสามารถในการต้านการเพิ่มจำนวนของเซลล์มะเร็ง ผลการทดลองแสดงให้เห็นว่าสารสกัดอย่างหยาบของพรอพอลิสด้วยเฮกเซน (ซีเอชอี) มีฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งที่ดีที่สุด จากนั้นจึงนำซีเอชอีมาทำให้บริสุทธิ์มากขึ้นโดยใช้โครมาโทกราฟีแบบต่างๆ นำทุกแฟรกชันที่ได้มาทดสอบกับเซลล์มะเร็งที่เลือกศึกษาข้างต้นโดยการตรวจสอบด้วยสารเอมทีทีอีกครั้ง จ ากการทดสอบพบว่าแฟรกชันที่ 3 (สารประกอบที่ 1) และแฟรกชันที่ 5 (สารประกอบที่ 2) มีฤทธิ์การต้านการเพิ่มจำนวนของเซลล์มะเร็งที่ดี ค่าของความเข้มข้นที่สามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งที่ 50 เปอร์เซนต์ (ไอซี 50) ของสารประกอบที่ 1 ที่มีต่อเอสดับเบิลยู 620, เฮพจี 2, บีที 474, คาโต 3, ชาโกและเอชเอส 27 มีค่าเท่ากับ 10.76, 21.52, 13.95, 10.93, 29.30 และ 21.35 ไมโครกรัมต่อมิลลิลิตร ค่าของไอซี 50 ของสารประกอบที่ 2 ที่มีต่อเอสดับเบิลยู 620, เฮพจี 2, บีที 474, คาโต 3, ชาโกและเอชเอส 27 มีค่าเท่ากับ < 3.125, 5.97, 4.03, 5.78, 4.41 และ 5.97 ไมโครกรัมต่อมิลลิลิตรตามลำดับ หลังทำการวิเคราะห์โครงสร้างทางเคมีโดยใช้นิวเคลียร์แมกเนติกรีโซแนนซ์และแมสสเปกโตรสโคปี พบว่าสารประกอบที่ 1 เป็นสารในกลุ่มคาร์ดานอลและสารประกอบที่ 2 เป็นสารในกลุ่มคาร์ดอล

ภาควิชา.....ชีววิทยา.....ลายมือชื่อนิสิต..........

สาขาวิชา.....สัตววิทยา.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..........

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DUNGPORN TEERASRIPREECHA: ANTIPROLIFERATIVE ACTIVITY AGAINST CANCER CELLS OF *Apis mellifera* PROPOLIS FROM NAN PROVINCE, THAILAND. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 120 pp.

Propolis is one of honeybee products. It provides a lot of bioactivities such as antibacterial, antifungal, anti-inflammatory, anti-tumor, etc. In this research, propolis of *Apis mellifera* was focused. It was collected from Nan province, Thailand. Propolis was initially extracted by 96% (v/v) EtOH and water. The antiproliferative activity of crude extract was performed against 5 cancer cell lines which were human duetol carcinoma (BT474), human lung undifferentiated (Chago), human liver hepatoblastoma (Hep-G_2), human gastric carcinoma (KATO-III), and human colon adenocarcinoma (SW620). Also, fibroblast cells (Hs27) were used as control. It was found that, by MTT assay, crude EtOH extract of propolis (CEE) had the good antiproliferative activity against those selected cells. Later, CEE was partitioned by hexane, CH_2Cl_2, and MeOH in order to separate compounds based on their polarities. Each part was tested against those selected cells. The result indicated that crude hexane extract of propolis (CHE) had the best antiproliferative activity against cancer cells. After that, CHE was further purified by various chromatographies. All obtained fractions were tested against those selected cells by MTT assay again. It was presented that fractions 3 (compound 1) and 5 (compound 2) had the good antiproliferative activity. The value of inhibition concentration at 50% (IC_{50}) of compound 1 for SW620, Hep-G_2, BT474, KATO-III, Chago, and Hs27 were 10.76, 21.52, 13.95, 10.93, 29.30, and 21.35 μg/ml, respectively. The IC_{50} values of compound 2 for SW620, Hep-G_2, KATO-III, Chago, BT474, and Hs27 were <3.125, 5.97, 4.03, 5.78, 4.41, and 5.97 μg/ml, respectively. After being analyzed by Nuclear Magnetic Resonance and Mass Spectroscopy, it was shown that compound 1 was in a cardanol group and compound 2 was in a cardol group.

Department: Biology	Student's Signature ....
Field of Study: Zoology	Advisor's Signature ....
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LIST OF ABBREVIATIONS

bp	Base pair
^{13}C NMR	Carbon-13 nuclear magnetic resonance
δ	Chemical shift
CDE	Crude dichloromethane extract of propolis
CEE	Crude ethanol extract of propolis
CEA	Crude ethyl acetate extract of propolis
CHE	Crude hexane extract of propolis
CME	Crude methanol extract of propolis
CBE	Crude n-butanol extract of propolis
COE	Crude oil extract of propolis
CPE	Crude petroleum ether extract of propolis
CWE	Crude water extract of propolis
COSY	Correlated spectroscopy
IC_{50}	Concentration required for 50% inhibition *in vitro*
°C	Degree Celsius
DNA	Deoxyribonucleic acid
ESIMS	Electrospray ionization mass spectrometry
EDTA	Ethylene diamine tetra-acetic acid
g	Gram
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear multiple quantum correlation
h	Hour
MeOH	Methanol

CH_2Cl_2	Methylene chloride or dichloromethane
µl	Microlitre
µg	Microgram
mg	Milligram
mM	Millimolar
ml	Millilitre
NMR	Nuclear magnetic resonance
1D NMR	One dimensional nuclear magnetic resonance
ppm	Part per million
%	Percentage
TMS	Tetramethylsilane
TLC	Thin layer chromatography
TBE	Tris-boric-ethylene diamine tetraacetic acid
2D NMR	Two dimensional nuclear magnetic resonance
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

Human body is composed of many cells and cell types. In general, normal cells divide and die eventually but not cancer cells. Their growth is out of control due to the DNA disorder. Invading cancer cells to other tissue of one organ is called tumor. A type of cancer is named after a target organ such as lung cancer, brain cancer, breast cancer, cervical cancer, skin cancer, hepatic cancer, etc.

Cancer is a leading cause of death worldwide. Although cancer treatments have been continuously developed, the number of new cancer patients is still highly increased every year. Cancer occurs by either internal factor (inherited mutations, hormones, and immune condition) and environment or external factor (tobacco, diet, radiation, infectious organisms, and alcohol.

In 2008, more than 10 million people worldwide were sick with cancer. Among these, only 5-10% of them are caused by genetics factor while the rest of 90-95% of them are caused by environmental factors. In 2020, the number of world cancer patients is expected to increase to be 7.5 billion.

At present, there are many ways to treat cancer such as surgery, radiotherapy, and chemotherapy but these treatments provide some side effects. For example, they were toxic to bone marrow cells and cells of the cardiovascular, respiratory, nervous, muscular, and reproductive systems. In addition, they are toxic to skin which can further cause hair loss, tinnitus, deafness, and immune suppression. Therefore, we are

interested in finding a new source for an anti-cancer agent which may be developed to be a drug in the future.

In this research, it was focused on propolis, one of honeybee products. As known, propolis is a sticky resin with black color. The word of "propolis" is derived from Greek which "pro" means the defense and "polis" refers to the city. Therefore, propolis means the defense of the city (Burdock, 1998). It was collected from leaf buds and tree barks. After propolis was collected, it was mixed with bee secretion and bee wax (Banskota, 2002). It will, then, be used to seal a crack within a hive and enclose a cadaver of enemy killed by worker bees (Kujumgiev *et al*., 1999). Considering food nutrition, propolis contains 50% (w/w) resin, 30% (w/w) fat, 10% (w/w) oil, 5% (w/w) pollen, and 5% (w/w) other compounds (Celli *et al*., 2004). Nowadays, propolis has been used as a nutritional supplement in foods and drinks for human health (Popova, *et al*., 2011). Moreover, propolis has been applied in medicinal products and cosmetics (Mohammadzadeh *et al*., 2007) because it has several biological activities such as antibacterial (Basim *et al*., 2006), antiviral (Erukhimovitch *et al*., 2006), anti-inflammatory (Paulino *et al*., 2003), and antifungal activities (Quiroga *et al*., 2006).

Since propolis is derived from plants, obviously main chemical compounds found in propolis are similar to those found in the plants. They belong to flavonoid group (flavones and apiginin) and phenolic group (caffeic acid phenethyl ester or CAPE) (Kartircioglu and Mercan, 2006). These chemical compounds were reported to provide many biological activities as mentioned above.

In this research, propolis from Nan province was tested for the antiproliferative activity on cancer cell lines [human duetol carcinoma (BT474), human lung undifferentiated (Chago), human liver hepatoblastoma (Hep-G_2), human gastric carcinoma (KATO-III), and human colon adenocarcinoma (SW620)] and normal cells [fibroblast cells (Hs27)] by MTT assay. The percentage of cell viability and IC_{50} value were estimated. The formular structure of active chemical compounds was investigated by Nuclear Magnatic Resonance (NMR) and Mass spectroscopy (MS).

CHAPTER II

LITERATURE REVIEW

2.1 Biology of *Apis mellifera*

Apis mellifera is native to the continents of Europe and Africa. It is well managed in a farming industry since it can provide high quantity of honey. In addition, it is not aggressive and does not swarm easily (Wongsiri, 1989). The characteristic of colony of *Apis mellifera* is shown in Figure 2.1.

In general, there are two types of bee nesting which are cavity-nesting and open nesting. *A. mellifera* is one of cavity-nesting bees and so is *A. cerana* (Oldroyd and Wongsiri, 2006). The mean of how to create a bee hive is in the manner to protect them from predators. For the development in beekeeping industry, a hive box of *A. mellifera* has been developed into many types and shapes such as WBC hive box, national hive box, modified dadant box, Smith hive box, and Langstroth hive box (Mace, 1976). Commonly, a hive box in an apiary is rectangular. Its roof is made from wood and flat. It is large enough to cover a brood chamber in order to protect bees from the rain and the snow. An entrance for a bee is always at the bottom of a hive box. The very important thing in a hive box is a comb that is built from wax secreting from worker's wax glands (D'ettorre *et al*., 2006; Raffiudin *et al*., 2007). As known, a wax gland is well developed in young bees. In a hive of *A. mellifera,* it consists of multiple combs which are useful for controlling temperature inside the hive. Within a hive, many combs are parallel to each other and are rearranged in the

vertical direction. A comb is the place for bees to accumulate food which is pollen, honey, and royal jelly. Also, it is the place for a queen to lay eggs (Mace, 1976). Considering the shape of bee cells in each comb, it is hexagonal which is believed to be well designed since each cell can be connected to each other without leaving a space. In each comb, there are two sides of cells connected to each other at the bottom end. Considering a cell, the edge will be thicker than the other part since adult bees live outside the cells and do many activities. Thus, the edge of bee cells needs to be strong (Dadant, 1927).

Within a colony, there is only one queen that is long-lived and reproductive. There are many males or drones those are short-lived and fertile and many workers those are non-reproductive females. Bees in these three castes are much different in morphology and behavior. For example, a queen has about 160-180 ovarioles per ovary while a worker has about 6 ovarioles per ovary. In addition, a queen and a drone lack pollen baskets but not workers (Oldroyd and Wongsiri, 2006). Especially in workers, they perform unique duties dependent on their ages (Nakaoka *et al*., 2008)

A. mellifera have widely been used as efficient pollinators for economic crops such as strawberries (Williams, 2000; Yoshiyama and Kimura, 2011). In addition, it was used as an animal model for the study of alcoholism. It could present the similar effect of alcohol in honeybees and in humans (Bozic *et al*., 2006). The honeybee is also a good model for studying olfactory cues such as pheromones, floral scents, and a major role in behavior (Iwama and Shibuya, 1998). Normally, a queen controls all bees within a hive by using different pheromones, including the control of the worker's ovary development. However, the ovary of workers can recover its function.

In the absence of the queen, some workers' ovaries will be functional and can and lay unfertilized eggs which finally will develop into drones (Human *et al*., 2007). The taxonomy of *A. melllifera* can be presented below:

Taxonomy identification of *A. mellifera* (Wongsiri, 1989)

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Superfamily Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *mellifera*

Figure 2.1 A colony of *Apis mellifera* (www.aopdb04.doae.go.th/).

2.1.1 Eusocial insect

Social insects have been evolved due to three main factors of food, protection, and propagation. Bees are one of eusocial insects those normally live together in a large family within a hive. No single honeybee is able to live alone for a long time by lacking of the relationship to other honeybees within the hive. Normally, one hive is composed of one family (colony). Within a hive, there are 3 castes of honeybees which are a queen, workers, and drones (Figure 2.2).

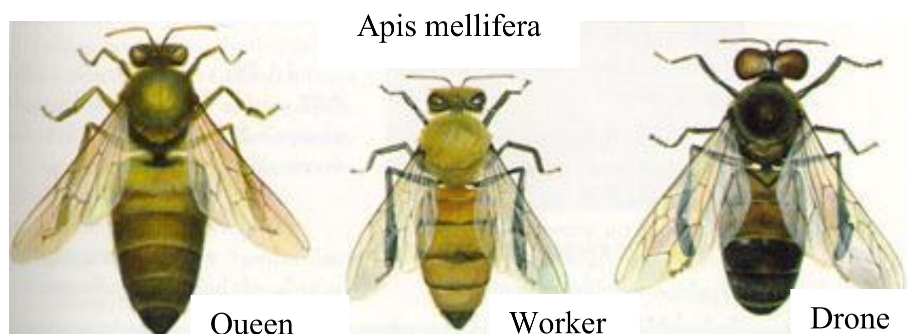


Figure 2.2 Three castes of *Apis mellifera*. (http://cdn.learners.in.th/).

Three castes of honeybees can be explained in details as below:

1. A queen: It is developed from a fertilized egg so it is diploid (2n). It has 16 pairs of chromosomes or 32 chromosomes. The meiosis of queen can cause the exchange of genetic information by crossing over (Oldroyd and Wongsiri, 2006). Normally, throughout the life time of one queen, it will mate to multiple males once but it can save all sperms in its spermatheca.

Considering its morphology, the wing length is much shorter than the body length. A queen is the largest in size within a hive since it has fed royal jelly by workers for the entire life. Usually, it moves slowly but, when in need, it moves fairly quickly. A queen is the only female in a hive that is fertile. It can lay about 2,000 eggs per day (Rueppell *et al*., 2007). Normally, a queen can spawn throughout a year and has a life span of 2-3 years.

2. Workers: They are developed from fertilized eggs so they are the same diploid as a queen. A worker is much smaller than a queen and drones in size. It is fed with royal jelly for only three days. Although it has only about 6 ovarioles per ovary, it does not mean that it cannot lay eggs. In the absence of a queen, some workers are able to activate their ovaries and lay eggs instead of a disappeared queen (Nakaoka *et al*., 2008). Uniquely, a worker has wax glands to produce wax, Nassanoff's glands to synthesize alarm pheromone, pollen baskets to collect pollen while foraging, and nurse glands to synthesize royal jelly, etc. Within a hive, workers perform all tasks. An assigned task depends on age of workers (Nakaoka *et al*., 2008). When it is young (0-2 days old) or is called a nurse bee, it does the cleaning cells. Later, it will feed bee larvae, cap broods, and take care of a queen (2-11 days old). When it is grown up (11-20 days old) to be a guard bee, it can build, repair the nest, and defend an enemy protruding the hive. Finally, when it is old (20 days old or until death) as a forager, it will go out of the hive to forage food (Seeley, 1982; Pratt, 1998).

3. Drones: They are developed from unfertilized eggs so they are haploid. Its chromosome numbers are 16. Although it is haploid, it can produce normal sperms because of the unnatural division during the first meiotic division. A haploid spermatocyte will duplicate its own chromosomes so one chromosome consists of two sister chromatids. When it divides, all chromosomes within one set can be completely transmitted to one daughter cell. In the second meiotic division, it occurs as usual (Oldroyd and Wongsiri, 2006). Their only one duty is to breed to a queen from outside. A drone's body size is wider and larger than a worker's body size. Unlike a queen and workers, drones lack of a sting (Oldroyd and Wongsiri, 2006).

Furthermore, they do not have pollen baskets and wax glands. It has large compound eyes and antennae which are specially developed to increase the vision and smell abilities (Menzel *et al*., 1991). Moreover, in order to enhance the ability to breed to a queen, the crowd of drones (about 3-7 days old) from many hives will fly to the Drone Congregation Area (DCA) where is far away from their own hives (Figure 2.3). The crowd of drones can be all together at one place due to the pheromone releasing by a queen. A multiple mating will be performed in order to increase the genetic variation of queen's daughters (Rua *et al*., 2009).

Figure 2.3 Fertilization in *Apis mellifera*. The breeding behavior between a queen and drones at the Drone Congregation Area (DCA) is shown (A). To increase the genetic variation of a queen's daughters within a hive, a queen will mate to many drones during the time (http://www.rakbankerd.com/). After fertilization, a reproductive organ of the drone will be affixed to the abdomen or vagina of a queen (B) (http://t1.gstatic.com/).

2.1.2 Propolis

Honeybees are beneficial to humans as they are important pollinators for crops. Furthermore, their products (honey, wax, royal jelly, pollen, venom, and propolis) are economic. Propolis is sticky resin that bees collect it from buds and barks of trees (Marcucci, 1995). It is used to seal a crack within a hive as in Figure 2.4 (Papachristoforou *et al*., 2011). Furthermore, it is used to enclose a corpse of enemy that is killed by workers in order that the contaminating microorganisms cannot be spread within a hive (Kujumgiev *et al*., 1999). Propolis consists of resin and balsam (50%), wax (30%), oil (10%), pollen (5%), and others (5%) (Celli *et al*., 2004). Moreover, it consists of vitamin B and flavonoid which performs the antibacterial activity (Castaldo *et al*., 2002).

In this research, propolis was focused because it was reported to perform many interesting bioactivities as mentioned in 2.2.

Figure 2.4 Guard bee uses propolis to seal a hole in its beehive.

2.2 Bioactivity of propolis

2.2.1 The antibacterial activity

In 2005, Silici and Kutluca reported the antimicrobial activity of propolis from three different subspecies of *A. mellifera* (*A. mellifera caucasica, A. mellifera carnica,* and *A. mellifera anatolica*). Crude ethanol extract of propolis (CEE) from those was tested on *Staphylococcus aureus* (ATCC 25923), *Escherichai coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). The result was shown that CEE from *A. mellifera caucasica* was sensitive to *S. aureus* (IC_{90} of 117 μg/ml) > *E. coli* (IC_{90} of 1,875 μg/ml), and > *P. aeruginosa* (IC_{90} of 3,750 μg/ml), respectively. In contrast, CEE from *A. mellifera carnica* and *A. mellifera anatolica* had the weak antibacterial activity.

In 2007, Mohammadzadeh *et al*. reported the antibacterial activity of CEE from Tehran-Khojir region (near the North of Iran). The CEE was tested on both Gram-positive bacteria which are *S. aureus* (ATCC 6538p), *S. epidermidis* (ATCC 12228), and *B. subtilis* (ATCC 6633) and Gram-negative bacteria which are *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 9027) by determining the minimum inhibition concentration (MIC) using microdilution method. It was shown that CEE could inhibit the growth of Gram-positive bacteria better than the growth of Gram-negative bacteria. The MIC of 125 μg/ml was obtained from both *S. aureus* and *S. epidermidis* while the MIC of 500 μg/ml was obtained from Gram-negative bacteria.

2.2.2 The antifungal activity

In 2006, Quiroga *et al*. reported that CEE (96%) from *A. mellifera* could inhibit the growth of fungi. After being purified, pinocembrin and galangin were the main active chemical components. Both pinocembrin and galangin were treated with *Aspergillus niger, Phomopsis* spp., *Fusarium* sp., *Trichoderma* spp., *Penicilium notatum*, and *Saccharomyces carlsbergensis.* The obtained result (MIC) was compared to ketoconazole and clortrimazole. Unfortunately, the MIC of pinocembrin and galangin was higher than the MIC of ketoconazole and clortrimazole. For example, considering the growth inhibition against *Saccharomyces carlsbergensis,* the MIC of galangin was 18 ± 0.02 µg/ml while the MIC of pinocembrin was 20 ± 0.01 µg/ml. In contrast, the MIC of clortrimazole and ketoconazole against *Saccharomyces carlsbergensis* were 1 ± 0.01 and 3 ± 0.01 µg/ml, respectively.

In 2011, Yang *et al*. reported the antifungal activity of propolis harvested in Hebei province, China. Propolis was extracted by bioassay-guided isolation and tested on *Penicillium italicum* that could cause a disease in fruits. Ethyl acetate, *n*-butanol, water, and petroleum ether were used as solvents. It was shown that crude ethyl acetate extract of propolis or CEA (200 mg/l) could completely inhibit the growth of *P. italicum*. In addition, crude petroleum ether (CPE) and *n*-butanol (CBE) extracts of propolis could provide the antifungal activity at 35% and 25%, respectively. In contrast, crude water extract of propolis (CWE) had the weak antifungal activity against *P. italicum*.

2.2.3 The antiparasitic activity

Hegazi *et al*. (2007) reported that propolis from Siwa oasis in Egypt had the antiparasitic activity against *Fasciola gigantic*, the parasite that could cause fasiolosis. Fasciolosis could be found in cattle. The symptom for this disease was that sick cattle would be anemia and often vomited. The CEE was tested against *F. gigantic* at concentration of 10, 20, and 30 µg/ml. In addition, the inhibition result was compared to Triclabendazone at the concentration of 10 and 20 µg/ml. The morphology of treated *F. gigantic* was observed by scanning electron microscopy (SEM). It was shown that the extract at the concentration of 10 and 20 µg/ml had the similar antiparasitic activity to triclabendazone. The oral and ventral suckers of parasite were destroyed by the swelling and cracking. That made the parasite not be able to hold the host's colon and die eventually.

Dantas *et al*. (2006) reported that CEE of Bulgarian propolis had the antiparasitic activity against *Trypanosoma cruzi* in Swiss mice. The Swiss mice were infected by 10^4 trypomastigotes. Then, they were treated by CEE at the doses ranged from 25 to 100 mg/kg body weight for 14 days. Swiss mice in a control group were treated by 5% EtOH. The body weight was measured every 2 days and the Pizzi–Brener method was selected to check the level of parasitemia. It was shown that CEE at 50 mg/kg body weight could decrease the parasitemia significantly, comparing to the control group.

2.2.4 Free radical scavenging activity

Ahn *et al*. (2007) reported the free radical scavenging activity of propolis from different areas (Heilongjiang, Neimongol, Hebei, Shandong, Shanxi, Gansu, Henan, Hubei, Sichuan, Hunan, Yunnan, and Hainan) in China. Crude ethanol extract of those samples was extracted (CEE). The free radical scavenging activity of CEE was compared to Butylated hydroxytoluene (BHT) which was an antioxidant used to prevent rancidity of fat and lipid oxidation of oil in food. It was shown that, by DPPH assay, CEE from Neimogol, Hebei, and Hubei had the strongest free radical scavenging activity (over 70%). This data was coincided to the amount of total polyphenol and flavonoid from those samples. The result was presented that propolis from Neimongol (284 ± 5.9 and 159 ± 2.1 mg/g of CEE), Hebei (302 ± 4.3 and 150 ± 2.4 mg/g of CEE), and Hubei (277 ± 5.5 and 138 ± 15.3 mg/g of CEE) had the highest amount of total polyphenol and flavonoid, too.

Kumazawa *et al*. (2004) also reported the free radical scavenging activity of propolis collected from different countries by DPPH assay. The CEE from Argentina, Australia, Brazil, Bulgaria, Chile, China (Hebei, Hubei, and Zhejiang), Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, the United States, and Uzbekistan were tested for the free radical scavenging activity. Also, the obtained activity was compared to the activity obtained by BHT. It was shown that the free radical scavenging activity of CEE from Australia, China, Hungary, and New Zealand was over 60% while CEE from South Africa had the weak free radical scavenging activity. Unfortunately, CEE from Thailand had the very weak free radical scavenging activity.

Furthermore, Choi *et al*. (2006) reported the free radical scavenging activity of CEE from many regions in Korea (Yangpyeong, Boryung, Cheorwon, and Yeosu). The obtained activity was compared to that of CEE from Brazil. The CEE concentration was varied to be 10, 20, 50, and 100 μg/ml. It was shown that CEE (50 μg/ml) from Cheorwon and Yeosu had the highest free radical scavenging activity at about 90%. In contrast, CEE from Brazil at the same concentration had the lowest free radical scavenging activity at about 70%.

In addition, Choi *et al*. (2006) reported the amount of total flavonoids and polyphenols of the above samples. Different quantity of both total flavonoids and polyphenols were found. The CEE from Yeosu had the highest quantity of polyphenols and flavonoids. The CEE from Brazil had the lowest quantity of polyphenols while the CEE from Yangpyeong had the lowest quantity of total flavonoids.

2.2.5 Antitumor activity

El-khawaga *et al*. (2003) reported the antiproliferative activity of crude propolis from Egypt against Ehrlich ascites carcinoma (EAC). Mice were divided into two groups. For the first group, mice were intraperitoneally injected with EAC cells ($2x10^6$ cells/mouse). Then, they were orally treated with 4 mg of propolis. For the second group, mice were intraperitoneally injected with EAC cells ($2x10^6$ cells/mouse) but they had not been untreated with propolis for 7 days. Then, EAC cells from mice in both groups were collected separately. After that, total protein,

DNA, and RNA (µg/10^6 cells) were analysed. The result was shown that more than 30% of mice in group I could survive up to 30 days while mice in group II were dead at day-14. In addition, more amount of total protein, DNA, and RNA of ECA cells of mice in group II was detected than those of mice in group I.

Kouidhi *et al.* (2010) reported the antiproliferative activity of CEE of *A. mellifera* against six cancer cell lines. Normal human fibroblast-like foetal lung cell line (MRC-5) and cancer cell lines of the human epithelial cell line (Hep-2), the human respiratory epithelial cell line (A549), the human intestinal epithelial cell line (HT-29), the murine leukemic macrophage cell line (raw 264.7), and the monkey renal cell line (Vero) were treated with different concentrations of CEE and measured by MTT assay. It was shown that CEE had the highest antiproliferative activity against Hep-2 cell line while had the lowest antiproliferative activity against A549. The IC_{50} values of Hep-2, HT-29, raw 264.7, MRC-5, Vero, and A549 were 15.7 ± 3.4, 18 ± 0.2, 42.5 ± 4.1, 55 ± 5.6, 100 ± 12.3, and 200 ± 22.2 µg/ml, respectively.

Furthermore, Carvalho *et al*. (2011) reported the antiproliferative activity of CEE and crude oil extract of propolis (COE) against leukemia cell line (HL-60), colon cancer cell line (HCT-8), breast cancer cell line (MDA/MB-435), and brain cancer cell line (SF-295). The percentage of cell viability was measured by [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] or MTT assay. It was shown that COE had the medium antiproliferative activity against HL-60, MDA/MB-435, and SF-295 with IC_{50} values of 26.87, 22.19, and 16.63 µg/ml, respectively. The activity was better than the activity of CEE with the IC_{50} values of 25.22, > 40, and > 40 µg/ml, respectively. After COE was purified by sephadex chromatography,

fractions of OLSx 1-6 were active. Only, fractions of OLSx4 and OLSx5 had the medium antiprolifertive activity against HL-60 and HCT-8. The background of MTT could be explained as below:

MTT assay

It is used to determine the cytotoxicity and the proliferation of cells. It is a colorimetric assay. In living cells, succinate dehydrogenase, which is produced by mitochondria, can reduce MTT to formazan crystal that is purple color (Figure 2.5). Then, formazan crystal is dissolved in dimethyl sulfoxide (DMSO) or sodium dodecyl sulfate (SDS). The absorbance can be measured at the wavelength between 500 and 600 nm by a spectrophotometer due to the used solvent (Kajio *et al.,* 1992).

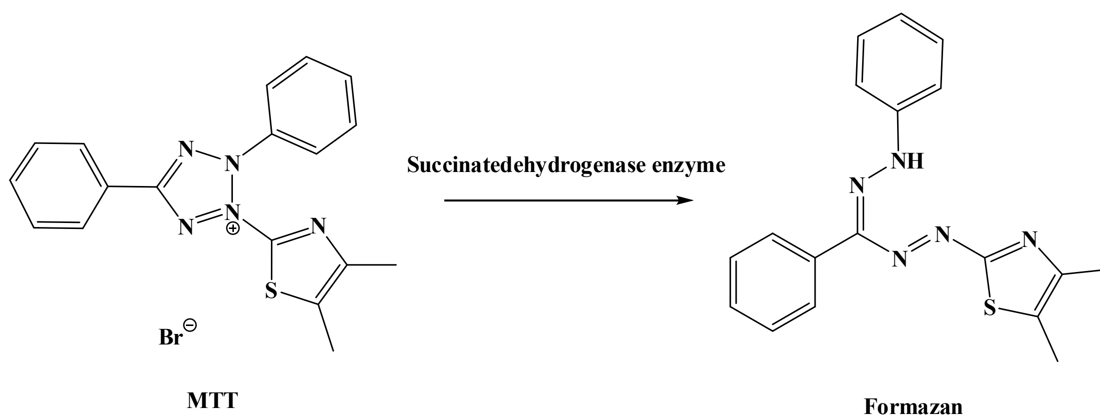


Figure 2.5 The background of MTT assay. MTT is reduced to be formazan crystal by the activity of succinatedehydrogenase. The color will be changed from blue to be yellow.

2.2.6 Apoptosis (programmed cell death)

It is a normal process in development of any organisms. It makes the balance between cell proliferation and cell death. For example, in humans, blood cells about $5x10^{11}$ cell were eliminated daily by programmed cell death and it is produced everyday (Miglani, 2007). Programmed cell death helps to eliminate tissue between the fingers in amphibian. Moreover, programmed cell death provides the body defense by damaging the dangerous cells.

The characters of programmed cell death differ from that of necrosis (acute injury of cells). The apoptosis and the fragmentation of chromosomal DNA are resulted from the nucleosome cleavage, the chromatin condensation, and the burst of nucleus. Eventually, the cell will shrink and burst. Apoptotic cells can send the signal which is phosphatidylserine to the cell surface. That makes phagocytic cells, including macrophages, recognize receptors at the cell surface and will phagocytose apoptotic cells. Thereby, they are removed from living tissues (Cooper and Hausman, 2009).

In contrast, necrotic cells will be swollen and lysed. That leads the contents within a cell leak to the extracellular space as cell debris. Then, it causes the inflammation. The characters of apoptosis and necrosis are shown in Figure 2.6.

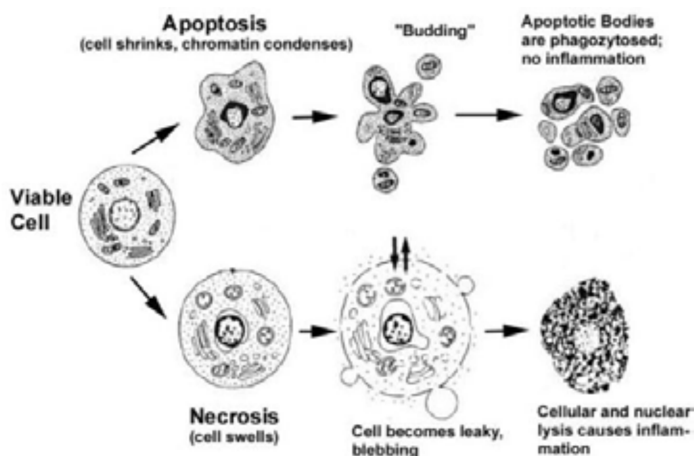


Figure 2.6 Cell death occurred by both apoptosis and necrosis (http://www.celldeath.de/).

2.3 Phenolic compounds

Phenolic compounds or polyphenols are chemical compounds consisting of aromatic hydrocarbon and hydroxyl group. The simple phenolic compound is C_6H_5OH as shown in Figure 2.7. It can be found in plants. Roughly, there are over 8,000 different structures of chemical compounds in this group. These compounds are reported to perform many bioctivities such as anticarcinogenic, anti-inflammatory, anti-atherogenic, and analgesic activities, etc.

In addition, phenolic compounds can be divided into at least 10 types which depend on basic structures: simple phenols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids, and lignins (G´omez-Caravaca *et al*., 2006).

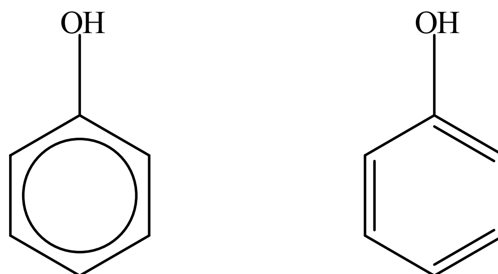


Figure 2.7 The unique structure of the phenolic compound.

Marcucci *et al*. (2001) reported the anti-trypanosomal activity of CME from Mandirituba. After the extract was purified through sephadex LH-20 column and analysed by HPLC. Four chemical compounds of **1** (PHCA), **2** (DCBEN)**, 3** (DHCA), and **4** (DPB) were revealed (Figure 2.8). These compounds are in phenolic group. All chemical compounds were tested for the anti-trypanosomal and antibacterial activities. The compounds at various concentrations ranging from 0.125 to 8 mg/ml were treated with *Trypanosoma cruzi*. The obtained result (ED_{50}) was compared to the result of crystal violet which was a standard drug and crude.

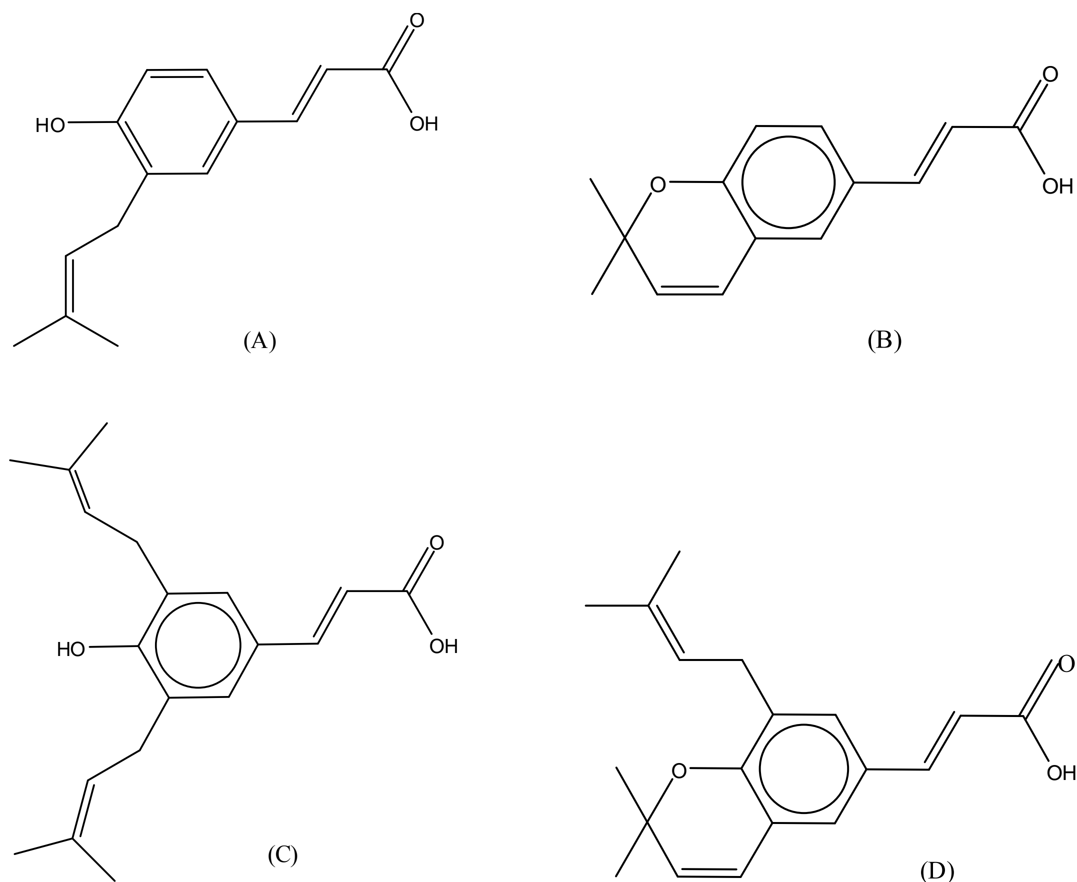
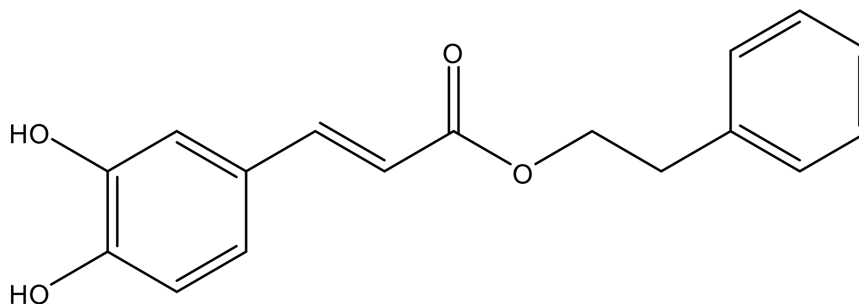


Figure 2.8 The structure of phenolic conpounds in CME from Mandirituba: (A) a structure of (1) as 3-prenyl-4-hydroxycinnamic acid (PHCA); (B) a structure of (2) as 2, 2-dimethyl-6-carboxyethenyl-2H-1-benzopyran (DCBEN); (C) a structure of (3) as 3, 5-diprenyl-4-hydroxycinnamic acid (DHCA); and (D) a structure of (4) as 2, 2-dimethyl-6- carboxyethenyl-8-prenyl-2H-1-benzopyran (DPB).

From this experiment, it was found that compound 1, 3, and 4 had the better anti-trypanosomal activity than MEP and crude. Moreover, the activity obtained from crude was 14x weaker than the activity obtained from MEP. In addition, those 4 chemical compounds were tested for the antibacterial activity against *E. coli* (ATCC

25922), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 13150), and *Streptococcus faecalis* (ATCC 29212). The data was compared to that of standard antibiotics of ampicillin (10 µg), amycacin (30 µg), vancomycin (30 µg), and eritromycin (15 µg). Finally, inhibition zone was measured. It was shown that compound 2 had no inhibition to *P. aeruginosa*. Compound 3 had no inhibition to *E. coli* and *P. aeruginosa*. Compound 4 was the most active against all tested bacteria, especially *S. aureus* with the inhibition zone of ≥ 12 mm. while the inhibition zones from standard antibiotics were 19, 23, 18, and 20 mm, respectively.

Wu *et al*. (2011) reported the antiproliferative activity of caffeic acid phenethyl ester (CAPE) against breast cancer cells (MDA-MB-231, MCF-7, MCF-10A, and MCF-12A cells). The structure of CAPE was presented as in Figure 2.9. They were treated with CAPE ranged from 0-40 µM for 72 h. DMSO was used as cell control. The percentage of cell viability was determined by MTT assay. CAPE had no effect to MCF-10A cells while the growth of MCF-7 and MCF-12A cells were dose dependent. They were significantly inhibited at 25.8% and 74.3%, respectively. MDA-231 cells were inhibited at 30.6% when they were compared to control. The IC_{50} of CAPE against MDA-231 and MCF-7 were 15 µM.



Caffeic acid phenethyl ester (CAPE)

Figure 2.9 The molecular structure of caffeic acid phenethyl ester (CAPE).

In addition, Barbarić *et al.* (2011) reported the antiproliferative activity of phenolic compounds and flavonoids extracted from propolis harvested from 17 areas in Croatia (I–XVII), 2 areas in Bosnia and Herzegovina (XVIII and XIX), and 1 area in Macedonia (XX). All samples were tested on cervix adenocarcinoma cell line (HeLa) (ATCC CCL-2). Ethanol was used as control. After the active samples were analysed, the most common phenolic acids composed in those samples are ferulic and *p*-coumaric acid (Figure 2.10). Chemical compounds belonging to the group of flavonoid were classified into 7 types which were tectochrysin, galangin, pinocembrin, pinocembrin-7-methylether, chrysin, apigenin, and kaempferol. Tectochrysin was found in all of the selected samples. Galangin and pinocembrin-7-methylether were found in 16 samples. Pinocembrin was found in 17 samples. Chrysin and apigenin were found in 8 samples. Kaempferol was found in 3 samples.

After tested with HeLa cell, it was found that all samples of crude propolis had the antiproliferative activity against HeLa cell. At the concentration of 1 mg/ml, best antiproliferative activity from 60 to 85% was obtained. Considering the IC_{50} value, it was shown that propolis harvested from area VII had the highest antiproliferative

activity. It was followed by propolis harvested from area XV, I, and XVIII which presented the IC_{50} values at 75.7 ± 7.8, 79.7 ± 30, 86.8 ± 11.4, and 89.3 ± 9.0 µg/ml, respectively.

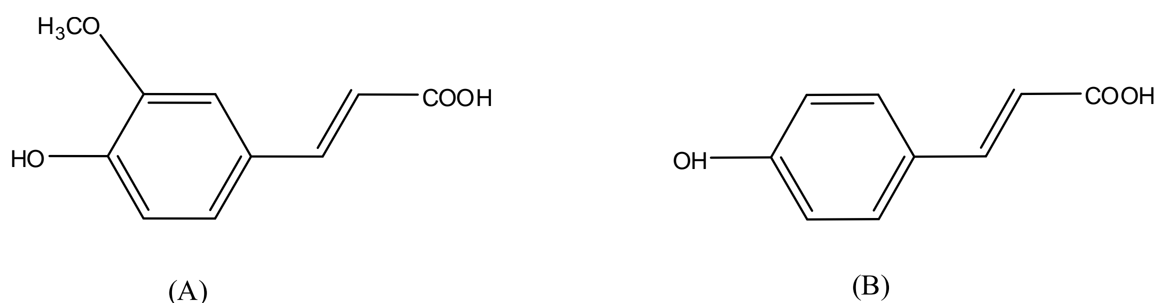


Figure 2.10 The most common phenolic acid found in extracted propolis are ferulic acid (A) and *p*-coumaric acid (B).

2.3.1 Cardol

Cardol is one of phenolic compounds that can be found in several plants such as pistachio, macadamia, and mango (Kubo *et al.*, 2011). In the main structure of cardol, there are aromatic hydrocarbon, 2 positions of hydroxyl group, and the side chain of hydrocarbon. Cardol is known to be a compound causing allergies (Aguilar-Ortigoza *et al.*, 2003).

Kubo *et al.* (2011) reported that cardol ($C_{15:0}$) was cytotoxic to murine B16-F10 (ATCC CRL-6475) melanoma cells. Cardol was tested against murine B16-F10 melanoma cells at the concentration of 0, 2.5, 5, 10, 20, and 40 µM. It was reported

that cardol was cytotoxic to murine B16-F10 melanoma cells with a dose-dependent manner. The IC_{50} values of cardol ($C_{15:0}$) was 24 µM or 7.68 µg/ml but 40 µM of cardol was completely lethal. In addition, the cellular melanin production and total melanin content was obviously suppressed by cardol ($C_{15:0}$) with a dose-dependent manner. The suppression with cardol in the range of 20-40 µM was significantly different. The character of cardol was shown in Figure 2.11 (A).

2.3.2 Cardanol

Cardanol is one of phenolic compounds found in plants in genus *Anacardium, Schinus*, and *Schinopsis*. The compound consists of aromatic hydrocarbon, hydroxyl group, and the side chain of hydrocarbon. However, it was reported to perfrom the allergenic effect (Aguilar-Ortigoza *et al.*, 2003) and the antioxidant activity (Amorati *et al.*, 2001). The character of cardanol was shown in Figure 2.11 (B).

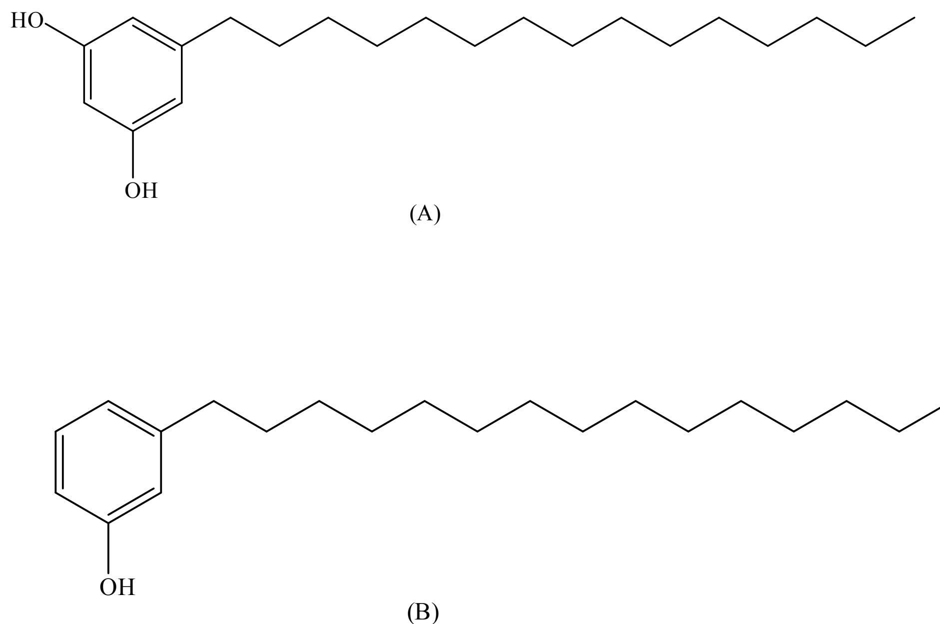


Figure 2.11 A formula structure of phenolic compounds. The structure in (A) presents cardol and the structure in (B) presents cardanol.

2.4 Spectroscopy technique

2.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy or NMR is a technique used to measure the energy from different levels of nucleus in the magnetic field. NMR can reveal a formula structure of organic and inorganic compounds. In addition, NMR can be used for the quantitative analysis. Widely, it is applied for a medical purpose such as the MRI used to detect the condition of brains.

Principle of NMR

Nucleus is a particle that keeps changing all the times. A nucleus of some element will spin around the nuclear axis because of its magnetic property. The spinning of nucleus would provide the magnetic moment around the nuclear axis. The change of magnetic property of nuclei by placing the nuclei into the magnetic field can be observed. Nuclei can be grouped into 2 types because of their limitation in quantum. The first one is the nucleus at the low energy level. It will absorb the energy and move to the higher energy level which the direction is opposite to the magnetic field. The second one is the nucleus at the high energy level. It is stimulated to loose the energy and, then, it will move back to the low energy level which the direction is the same to the magnetic field. The change can be estimated by the formula below in term of "resonance).

$$\Delta E = h\text{v}$$

Where: ΔE refers to the energy difference between two states.

h refers to the Planck's constant value.

v refers to the precessional frequency.

During the operation, radiofrequency generator will send a signal to a detector and an amplifier will send a measured signal as NMR spectrum. Then, the obtained data will be plotted. The Y-axis presents the intensity of signal while the X-axis presents the frequency (Crews *et al*., 1998).

2.4.2 Mass spectroscopy

Mass spectroscopy (MS) is a technique used for chemical analysis. It can reveal the structure and molecular weight by splitting a molecule into ion. Later, the occurring ion will be checked.

Principle of MS

MS is used to separate and measure the mass of ion by the ratio of mass to charge (m/z). In general, an ion has +1 charge ($z = 1$) so the ratio of mass to charge (m/z) equals to the mass of the ion. The graph of MS will be interpreted. The X-axis refers to the ratio of mass to charge (m/z) while the Y-axis refers to the relative abundance.

A pattern of fragmentation of molecules is unique in each compound. It depends on type of compounds, energy, a structure of molecule, and the time between the occurrence and the checking of the ion (Crews *et al*., 1998).

2.5 Cancer

Cancer is a leading cause of death worldwide. It can occur from various causes, both from internal and external factors. An internal factor may be involved in genetic inherits and disorder while an external factor may be from tobacco, alcohol, diet, obesity, infectious agents, environmental pollutants, and radiation. Amazingly, external factors were reported to cause many types of cancers while internal factors caused some types of cancers. There are many ways to treat cancer such as

chemotherapy, radiation therapy, surgery, etc. Although cancer treatments have been continuously developed, there is still the highly increasing percentage of death from cancer every year (Anand *et al*., 2008).

Breast cancer is the most widespread disease in women, especially whom lives in an industrialized country. According to the statistics of the National Cancer Institute of the USA, one of every nine women will be diagnosed with breast cancer before the age of 85 in the USA (Mols *et al*., 2005).

Colorectal cancer is the second most common disease. This disease was almost evenly diagnosed in both men and women. In Europe, over 400,000 new patients who were sick with colon cancer were diagnosed in each year (Jansen *et al*., 2010).

Liver cancer is the sixth most common cancer worldwide and the third most common cause of death. Over 80% of cancer patient deaths are from developing countries. China is mostly affected by liver cancer with the rate of 37.9 per 100,000 for men and 14.2 per 100,000 for females. A mortality rate of liver cancer patients is the second. The mortality rate of men and women was 37.55 and 14.45 per 100,000, respectively. In total, it is counted for about 19.33% among all cancer patients (Chen and Zhang, 2011).

Lung cancer can occur in both males and females. This disease is caused by many risk factors. Although, cigarette smoking is a major risk factor in the development of lung cancer, there are still other risk factors such as indoor air

pollution, exposure to environmental tobacco smoke, high consumption of saturated fat, and family history of cancer (Hu *et al*., 2002).

Due to the data mentioned above, it seems to be that the obtained active chemical compounds depended mainly on regions of harvesting, season, bee species, and so on. Thus, this research was focused on the antiproliferative activity of propolis from Nan province. The extraction and purification of active compounds would be tried in order to get the structure. Selected cancer cell lines in this research were human duetol carcinoma (BT474), human lung undifferentiated (Chago), human liver hepatoblastoma (Hep-G_2), human gastric carcinoma (KATO-III), and human colon adenocarcinoma (SW620). Normal fibroblast cells were also tested in order to reveal the cytotoxicity of the compounds in propolis. By MTT assay, the percentage of cell viability and IC_{50} value were estimated.

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

- Varian Mercury^{+} 400 NMR spectrometer
- Mass spectrometer, VG Trio 2000
- MicroIncubator, M-36, Taitec corporation, Japan
- Benchtop variable transilluminator, BioDoc-IT^{TM} imaging system
- Lamina flow, Renowm Technical Co., Ltd.
- Hemocytometer, Boeco, Germany
- Rotary evaporator, Buchi Rotavapor R-114, Switzerland
- Vacuum pump, Sibata Technology, Ltd., Japan
- CO_2 incubator, Yamato, Scientific Co., Ltd.
- Shaking incubator, Bioer Technology
- Autoclave, Conbraco Ind. Inc., USA
- Microplate reader, Titertek Multiskan® MCC/340
- Microcentifuge, Centrifuge Sorvall® pico D-37520 Osterode, Kendro Laboratory Product, Germany

- Microwave oven, Sharp Carousel R7456, Thailand
- Inverted microscope, Olympus
- Centrifuge, Hettich, Germany
- Separatory funnel (500 and 1,000 ml in size), Buchher®, Germany
- Column (250 ml in size), Schott Duran, Germany
- Vacuum column chromatography, NK Laboratory, Schott Duran, Germany
- Round bottom (50, 500, and 1,000 ml in size), NK Laboratory, Schott Duran, Germany
- Beaker, Pyrex®, Germany
- Test tube (3 ml in size), Pyrex®, Germany
- Filter paper, qualitative circle of 110 mm in Ø, Whatman International, Ltd., England
- Flask (50, 250, 500, and 1,000 ml in size), Schott Duran, Germany
- Measuring cylinder (10, 100, 500, and 1,000 ml in size), Witeg, Germany
- Microtube (1.5 ml in size), Sarstedt, Germany
- Centrifuge tube (15 and 50 ml in size), Sarstedt, Germany

- Semi-microcuvette (1.5 ml in size), Brand, Germany
- Tissue culture plate 96 well, DSI, Thermo Fisher Scientific, Denmark
- Tissue culture flask, Nunclon DSI, Thermo Fisher Scientific, Denmark
- Ultraviolet light, Electronic Money Detector, Thailand
- Automatic micropipettes (P10, P20, P100, P200, and P1,000), Gilson, France
- Pipette tips (200 and 1,000 µl in size), BioScience, Inc., USA
- Pipette tips (10 µl in size), Axygen Scientific, Inc., USA
- Centrifuge and vortex mixer, model: Centrifuge FVL-2400, Biosan, Latvia
- Electrophoresis chamber set, model: Mupid, Advance Co., Ltd., Japan

3.2 Chemicals

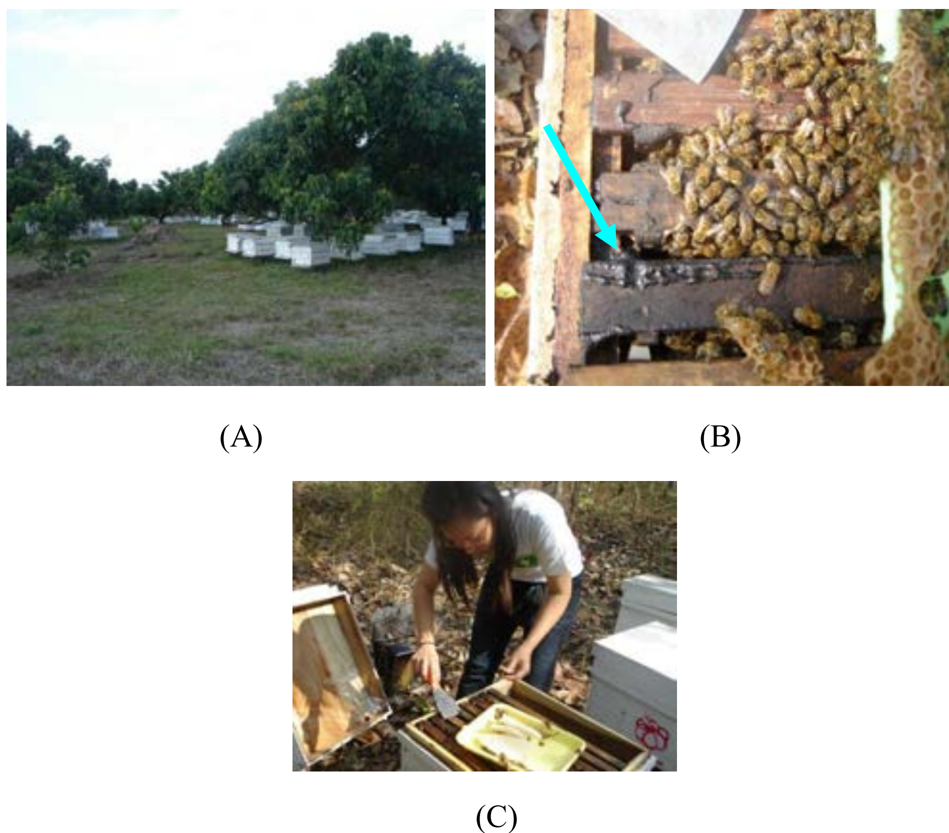
- Absolute ethanol (C_2H_6O, M = 46.07 g/mol), Merck KGaA Darstadt, Germany
- Hexane ($C_6H_{14}C_6H_{12}$), TSL Chemical, Thailand
- Methylene chloride (CH_2Cl_2), TSL Chemical, Thailand
- Methanol (CH_3OH), TSL Chemical, Thailand

- Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$, MW = 156.01), Univar, Ajex Finechem, Australia
- Di-sodium hydrogen orthophosphate (Na_2HPO_4, MW = 141.96), Univar, Ajex Finechem, Australia
- Dimethyl sulfoxide, Sigma-Aldrich Laborchemikalien GmbH, USA
- Fetal Bovine Serum, PAA Laboratories GmbH, Germany
- RPMI 1640 medium, Biochrome, Germany
- Basal Iscove medium, Biochrom, Germany
- Trypsin, Sigma, USA
- 0.1 M glycine (normal saline, Biochemical, England
- 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Bio Basic Inc., Canada
- Silica gel 60 for column chromatography, 0.063-0.200 mm in Ø (O_2Si, M = 60.08 g/mol), Merck KGaA, Germany
- Silica gel 60 G for thin layer chromatography (M= 60.08 g/mol), Merck KGaA, Germany
- TLC silica gel 60 F_{254}, Merck KGaA, Germany
- QIAamp ®DNA mini kit, cat. no. 51304, Qiagen, Germany

- Agarose, low EEO, Research Organics Inc., USA
- λ DNA/*Hin*dIII marker, Fermentas, USA
- 100 bp DNA ladder marker, SibEnzyme, Russia
- Ethidium bromide ($C_{21}H_{20}BrN_3$, M.W. = 934.32), Bio Basic, Inc., USA
- Chloroform ($CHCl_3$, M = 120.38 g/mol), Germany)

3.3 Propolis collection

Propolis of *Apis mellifera* was collected from an apiary in Pua district, Nan province, Thailand during January 28 – February 1, 2010 (Figure 3.1). Propolis was kept in the dark by wrapping with aluminium foil until used. In beekeeping management, proplis is scrapped out of bee hives very often otherwise it will be very difficult to open the cover of bee hives. By doing this, it can be confident that propolis that we collected was from plants in Nan province since bee hives are sometimes moved out of Nan province in order that bees can forage food from available plants.



(A) (B)

(C)

Figure 3.1. Propolis of *Apis mellifera*. Bee hives of *A. mellifera* were located in a fruit garden in Pua district (A). Within a hive, propolis as indicated by an arrow was located on top of frames (B). A spatula was used to scrap propolis out of the frames (C).

3.4 Extraction

It was followed by Najafi *et al*. (2007). Propolis was cut into small pieces. Then, 90 g of propolis was mixed with 400 ml of 96% (v/v) ethanol (EtOH) and incubated at 15°C for 18 h. The mixture was spun at 7,000 rpm, 20°C for 15 min. The supernatant was kept and the pellet was re-extracted with 100 ml of 96% (v/v) EtOH. The supernatant from both steps was pooled. The EtOH extract (200 ml) was

separated for extraction with water. The rest was evaporated by rotary evaporator. Then, the crude ethanol extract (CEE) was obtained and kept in the dark at -20°C. The EtOH extract (200 ml) was further stirred with 200 ml of 20 mM phosphate buffer (pH 7.0) at 20°C for 20 min. The mixture was spun at 7,000 rpm, 20°C for 15 min. The supernatant of water extract was evaporated by rotary evaporator. Then, the crude water extract (CWE) was obtained and kept in the dark at -20°C. Both crude extracts were tested for the antiproliferation by MTT assay as described in 3.7.3.

3.5 Bioassay-guided isolation (partition)

It was followed by Umthong *et al*. (2011). The 40 ml of CEE which provided the better antiproliferation was mixed with 2x vol. of 80% (v/v) methanol (MeOH) or until the CEE became not sticky. Next, it was transferred into a separating funnel. The equal vol. (120 ml) of hexane was added to the sample. The mixture was shaken and incubated until the mixture was separated into two phase. The upper phase (120 ml) of hexane part was separated and evaporated by rotary evaporator. Then, the crude hexane extract (CHE) was obtained and kept in the dark at -20°C. Later, the lower phase (120 ml) of MeOH part [80% (v/v)] was mixed with equal vol. of water to make 40% (v/v) MeOH part. Then, 240 ml of dichloromethane (CH_2Cl_2) was added into the 40% (v/v) MeOH part. The mixture was shaken and incubated until the mixture was separated into two phases. The upper phase of MeOH part and the lower phase of CH_2Cl_2 part were separately collected. Both were evaporated by rotary evaporator. Finally, the crude MeOH extract (CME) and the crude CH_2Cl_2 extract

(CDE) were obtained and kept in the dark at -20°C until used. All three crude extracts (CHE, CME, and CDE) were tested for the antiproliferation by MTT assay as described in 3.7.3.

3.6 Chromatographies

3.6.1. Quick column chromatography

The sintered glass (250 ml) was filled with silica gel 60 G (0.063 - 0.2 mm in size). A vacuum pump was used in order to make the packing tighter. After that, CHE was mixed with silica gel 60 and left until silica gel dry. Then, the sample was sprinkled onto the packed column. Then, a piece of filter paper (110 mm in Ø) was put on top of the sample. It was followed by being covered by cotton. The solvent of hexane, 25% (v/v) CH_2Cl_2-hexane, 50% (v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, 100% CH_2Cl_2, and 30% (v/v) MeOH-CH_2Cl_2 was separately used as a mobile phase for elution. Five hundred ml of each solvent was loaded to the packed column for 3 times. Fractions (500 ml of each) were collected. The purity of each fraction was determined by thin layer chromatography (TLC) as described in 3.6.3. The fractions presenting the same pattern of chemical compounds were pooled together and evaporated. Then, they were tested for the antiproliferation by MTT assay as described in 3.7.3

3.6.2. Adsorption chromatography

Hexane (200 ml) was mixed with silica gel 60 (90 g) and packed into a column (250 ml) at the appropriate height. Simultaneously, the solvent was released out of the column to prevent the excess amount of solvent. Fractions which had the good antiproliferation were dissolved in the appropriate solvent and mixed with silica gel 60 (5-7 g). The sample was incubated at RT until it was dry. Later, it was placed on top of the column. The release of surplus solvent made the sample better absorbed into the silica gel layer. More solvent was added. Also, more silica gel was placed on top in order to make the layer containing silica gel and fractions smooth. Then, cotton was placed on top and the solvent was released again. The solvent (500 ml) of hexane, 50% (v/v) CH_2Cl_2-hexane, 100% CH_2Cl_2, and MeOH were poured to the column, respectively. Fractions (2.5 ml of each) were collected and the purity was checked by TLC as mentioned in 3.6.3. Any fractions presenting the same pattern of chemical compounds were pooled together and evaporated. Then, they were tested for the antiproliferation by MTT assay as described in 3.7.3

3.6.3. Thin layer chromatography (TLC)

A TLC plate (a silica coated plate) was cut into the size of 5×5 cm^2. The first line was drawn far from the bottom line at 0.5 cm by a light pencil and the second line was drawn far from the top line at 0.5 cm by a dark pencil. Each fraction was spotted for three times on a TLC plate by using a small capillary tube. The sample could be dissolved shortly in its former solvent if it was too sticky. The solvent of hexane, 50%

(v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, 100% CH_2Cl_2, and 5% (v/v) MeOH-CH_2Cl_2 were used as the mobile phase. Then, the TLC plate was dipped into the mobile phase. The position of sample spots had to be above the level of the mobile phase. The plate was dipped until the solvent permeated to the top line of the TLC plate. Then, the TLC plate was left at RT until it was dry. The result was visualized under ultraviolet light.

3.7 An antiproliferation against cancer cell lines

3.7.1 Cancer cell lines

Selected cancer cell lines used in this research were human duetol carcinoma (BT474, ATCC No. HTB 20), human lung undifferentiated (Chago I, National Cancer Institute), human liver hepatoblastoma (Hep-G_2, ATCC No. HB8065), human gastric carcinoma (KATO-III, ATCC No. HTB 103), and human colon adenocarcinoma (SW620, ATCC No. CCL 227). Fibroblast cells (Hs27, ATCC No. CRL 1634)were used as control. All cancer cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn Unversity. They were cultured in RPMI 1640 medium containing 5% (v/v) fetal calf serum (FCS) while fibroblast cells were cultured in Basal Iscove medium containing 5% (v/v) FCS. The culture was treated at $37^{o}C$ with 5% CO_2 as mentioned in Najafi *et al*. (2007).

3.7.2 Cell count

Cells in a culture flask was added with trypsin (1-1.5 ml) and incubated at RT for 1-2 min. After that, trypsin was removed and the RPMI media (1.5-2 ml) was added to dissociate cells which were previously attached to the bottom of the culture flask. After cells were trysinized, they were sucked and transferred into a 15 ml tube. Cancer cell lines were ten-fold diluted (Exp. 10 µl of cancer cells were mixed with 90 µl of RPMI 1640 medium) in order that cells would not be overlapped on a grid of hematocytometer. The ten-fold suspension of cells in the volume of 10 µl was released on a counting chamber. Cells positioning at four large corner squares of the hematocytometer were counted. The number of cells was calculated by the formula below:

Concentration of cells (cells/ml) = (Number of cells/4) x dilution factor (10) x 10^4 cells/ml

3.7.3 [3- (4, 5-dimethyl-thiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] or MTT assay

It was followed by Santos *et al*. (2002) and Hernandez *et al*. (2007). For each cancer cell line, cells at the concentration of 5×10^3 cells in 200 µl of RPMI 1640 medium containing 5% (v/v) FCS were transferred into a well of 96 well tissue culture plate. They were incubated in a 37°C incubator containing 5% CO_2 for 24 h. Then, they were treated with 2 µl/well of an interesting extract of various concentrations. Also, 2 µl/well of DMSO was used as control. They were further

incubated at 37°C containing 5% CO_2 for 72 h. After that, cells in each well were added by 10 µl of 5 mg/ml MTT in order to measure cell viability and they were incubated at 37°C containing 5% CO_2 for another 4 h. The supernatant was removed. The mixture containing 150 µl of DMSO and 25 µl of 0.1 M glycine were added to dissolve formazan crystal (blue crystal). The absorbance was measured at 540 nm by a microplate reader. Three replication of each was performed. The obtained absorbance was further used to estimate the inhibition concentration at 50% (IC_{50}).

3.7.4 Estimation of the inhibition concentration at 50% (IC_{50})

The absorbance at 540 nm of treated cancer cells and control was used to calculate the percentage of cell viability. The percentage of cell viability of control was set to be 100%. The percentage of cell viability of treated groups was calculated by the following formula:

$$\text{The percentage of cell viability} = \frac{\text{(Abs of sample)} \times 100}{\text{(Abs of control)}}$$

Where:Abs of sample is defined as the absorbance at 540 nm of treated cancer cells

Abs of control is defined as the absorbance at 540 nm of control.

After the calculation, all data were plotted on a graph. The concentration of an extract was plotted on an X axis while the percentage of cell viability was plotted on a Y axis. A line was drawn from a Y axis at 50% until it reached the curve line. Then, the line was drawn downward to an X axis where it indicated the concentration of the extract. The obtained concentration presented the IC_{50} value. The data was reported as in mean ± S.E. Then, the IC_{50} of those cell lines were compared and statistically analysed by using the Kruskal-Wallis One Way Analysis of Variance.

3.8 Chemical structure analysis by spectroscopy

3.8.1 Nuclear Magnetic Resonance (NMR)

After the purified active fraction was evaporated, 2-3 mg of purified active fraction was dissolved in 500 µl of deuterated solvent (deuterated chloroform, $CDCl_3$) and transferred into an NMR tube. The sample was analysed and recorded by a Varian Mercury$^+$ 400 NMR spectrometer operated at 400 MHz for ^1H and 2D NMR (COSY, HSQC, HMBC, and 100 MHz for ^{13}C nuclei) in order to search for functional groups. This operation was performed at Department of Chemistry, Faculty of Science, Chulalongkorn University. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual protons in deuterated solvents and TMS was used as an internal standard in some cases.

3.8.2 Mass Spectroscopy (MS)

An evaporated purified fraction (1-2 mg) was dissolved in ethylacetate (1 ml) and sent to National Science and Technology Development Agency (NSTDA) for the service to search for molecular weight. Electrospray ionization mass spectroscopy (ESIMS) was a selected mode used to run MS.

3.9 DNA fragmentation

3.9.1. DNA extraction

The SW620 cancer cells ($5x10^5$ cells/ flask) were cultured in 6 ml of RPMI 1640 medium containing 5% FCS and incubated at 37°C containing 5% CO_2 for 24 h. Then, they were treated with the evaporated purified fraction at the concentration of IC_{50}. While the cells had been incubated at 37°C containing 5% CO_2 for 72 h (3 days), they were photographed everyday. The morphology of the SW620 cancer cells was compared to the morphology of fibroblast cells (control). The SW620 cancer cells were trypsinized (as mentioned previously in 3.7.2). Cells were transferred into a 1.5 ml microcentrifuge tube. The cell suspension was spun at 2,000x g at 15-25°C for 5 min. The precipitated SW620 cancer cells were dissolved in 200 µl of PBS. Then, 20 µl of proteinase K was mixed. Genomic DNA of the SW620 cancer cells was extracted by QIAMP mini kit (Qiagen, cat. no. 51304). Genomic DNA was kept at -20°C until used. Genomic DNA was spectrometrically measured at the absorbances of

260 and 280 nm. The concentration and the purity of genomic DNA were estimated from the formulas below:

The concentration of genomic DNA (μg/μl) = (Abs260)(dilution factor)(50)

The purity of genomic DNA = Abs260/Abs280

Where: Abs260 is defined as the absorbance of genomic DNA at 260 nm.

Abs 280 is defined as the absorbance of genomic DNA at 280 nm.

3.9.2. Agarose gel electrophoresis

Genomic DNA (1 μg) was mixed with 2 μl of 6x loading dye (Fermentas) and loaded into a well of 1.8% (w/v) agarose gel. λ *Hin*dIII (1.25 μg) and 100 bp DNA ladder (0.5 μg) were used as DNA markers. The electrophoresis was performed in 1x TBE (50 mM Tris aminomethane, 0.65 mM EDTA, and 50 mM Boric acid) as running buffer at 100 V for 45 min. After electrophoresed, the gel was stained with 10 μg/ml of ethidium bromide (EtBr) for 10 min and destained in distilled water for 20 min. The genomic DNA was visualized under ultraviolet light.

CHAPTER IV

RESULT

4.1 Crude solvent extract of propolis from *Apis mellifera*

4.1.1 Crude ethanol extract of propolis (CEE)

After propolis was extracted by EtOH (as mentioned in 3.4), CEE at the weight of 59.8 g was obtained. It looked brown, sticky, and smelled uniquely.

4.1.2 Crude water extract of propolis (CWE)

After 200 ml of supernatant of EtOH extract of propolis was further extracted by 200 ml of 20 mM phosphate buffer (as mentioned in 3.4), CWE at the weight of 48.5 g was obtained. It looked muddy yellow and smelled uniquely.

4.1.3 Crude hexane extract of propolis (CHE)

After CEE was partitioned by hexane, CH_2Cl_2, and MeOH (as mentioned in 3.5), CHE at the weight of 22.5 g was obtained. It looked dark brown, sticky, and smelled uniquely.

4.1.4 Crude CH_2Cl_2 extract of propolis (CDE)

After CEE was partitioned with by hexane, CH_2Cl_2, and MeOH (as mentioned in 3.5), CDE at the weight of 1.32 g was obtained. It looked yellow-brown and sticky. In addition, it smelled like wax.

4.1.5 Crude MeOH extract of propolis (CME)

After CEE was partitioned by hexane, CH_2Cl_2, and MeOH (as mentioned in 3.5), CME at the weigh of 740 mg was obtained. It looked hazel. Comparing to CHE, it was less viscous. In addition, it smelled like caramel.

4.1.6 Fraction I-V after adsorption chromatography

Since CHE performed the best antiproliferative activity by MTT assay, it was further purified by quick column chromatography (as mentioned in 3.6.1), five fractions were obtained and recorded as in Table 4.1.

Table 4.1 The weight and character of five fractions after quick column chromatography.

Fraction	Weight (mg)	Character
I	80	Clear wax and plastic-like smell
II	20	Clear yellow oil and plastic-like smell
III	320	Yellow oil and unique smell
IV	270	Yellow powder and unique smell
V	4,300	Dark brown oil and unique smell

4.2 Antiproliferative activity

4.2.1 Effect of CEE and CWE on difference cancer cell lines

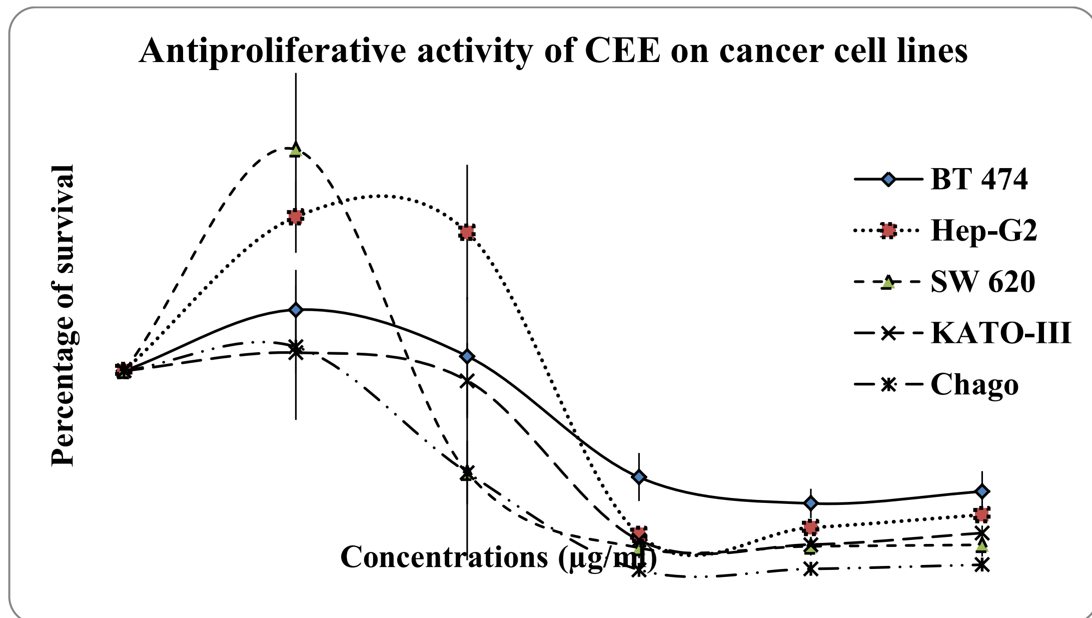
After CEE and CWE at various concentrations of 0.125, 0.25, 0.50, 1.0, and 2.0 mg/ml were tested against different cancer cell lines, it was revealed that CEE had the antiproliferative activity against those selected cancer cell lines in the concentration-dependent manner [Figure 4.1 (A)]. The highest to the lowest IC_{50} values of CEE were found to be on BT474 at 520.56 ± 54.25 µg/ml, on Hep-G_2 at 464.48 ± 20.53 µg/ml, on KATO-III at 395.77 ± 28.04 µg/ml, on SW620 at 389.45 ± 33.38 µg/ml, and on Chago at 323.13 ± 30.74 µg/ml, respectively (Table 4.2). Alternatively, it could be reported that the most sensitivity to the least sensitivity of cancer cell lines to CEE was Chago, SW620, KATO-III, Hep-G_2, and BT474, respectively. In contrast, it was obvious that CWE at the concentration of 0.125, 0.25, 0.50, 1.0, and 2.0 mg/ml did not have the antiproliferative activity so the IC_{50} values could not be determined [Table 4.2 and Figure 4.1 (B)].

Table 4.2 The IC_{50} values of CEE and CWE on the selected cancer cell lines.

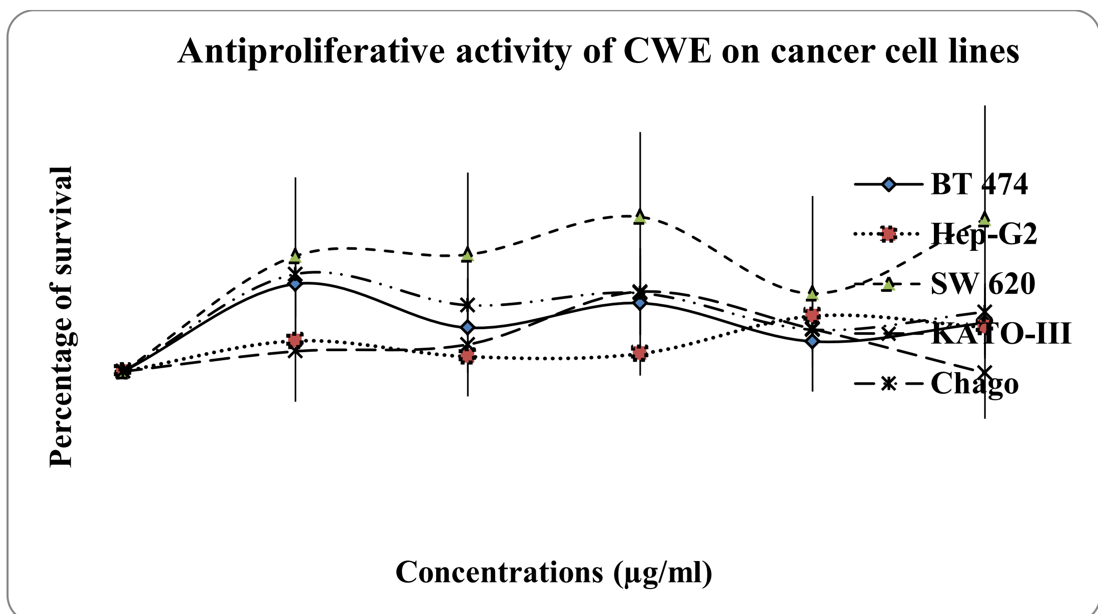
Cancer cell lines	IC_{50} (µg/ml)	
	CEE	**CWE**
BT474	520.56 ± 54.25	ND
Chago	323.13 ± 30.74	ND
KATO-III	395.77 ± 28.04	ND
SW620	389.45 ± 33.38	ND
Hep-G_2	464.48 ± 20.53	ND

Remark: ND indicated no data of IC_{50} values due to no antiproliferative activity.

All reported data came from the triplication in each experiment.



(A)



(B)

Figure 4.1 Effect of propolis extract on different cancer cell lines. Effect of CEE on different cancer cells was shown in (A) while effect of CWE on the same cancer cells was shown in (B). The percentage of survival was expressed as mean ± SE (µg/ml).

4.2.2 Effect of CHE, CDE, and CME on different cancer cell lines

Since CEE had the best antiproliferative activity on cancer cell lines, it continued to be partitioned (as mentioned in 3.5). Different cancer cell lines were treated with CHE. It was revealed that CHE had the antiproliferative activity on cancer cell lines with the concentration-depending manner as shown in Figure 4.2 (A). The highest to the lowest IC_{50} values of CHE was on Hep-G_2 at 52.41 ± 3.7 µg/ml, on BT474 at 48.33 ± 1.6 µg/ml, on SW620 at 45.33 ± 0.33 µg/ml, on KATO-III at 42.5 ± 6.61µg/ml, and on Chago at 41.25 ± 3.75 µg/ml, respectively. Alternatively, it could be presented that CHE had the most to the least antiproliferative activity against Chago, KATO-III, SW620, BT474, and Hep-G_2, respectively. The mean ± SE of IC_{50} values of CHE, CDE, and CME on different cancer cell lines were shown in Table 4.3.

The CDE also had the antiproliferative activity against different cancer cell lines [Figure 4.2 (B)]. The highest to the lowest IC_{50} values of CDE was on Hep-G_2 at 53.5 ± 0.5 µg/ml, on BT474 at 52.6 ± 3.7 µg/ml, on SW620 at 46 ± 0.57 µg/ml, on Chago at 44.66 ± 0.33 µg/ml, and on KATO-III at 43.75 ± 6.5 µg/ml, respectively. Alternatively, it could be reported that the most to the least sensitive cancer cell lines to CDE were KATO-III, Chago, SW620, BT474, and Hep-G_2.

Furthermore, CME had the antiproliferative activity against different cancer cell lines [Figure 4.2 (C)]. The highest to the lowest IC_{50} values of CME were on Hep-G_2 at 605 ± 39.05 µg/ml, on KATO-III at 600 ± 50 µg/ml, on Chago at 580 ± 20 µg/ml, on SW620 at 555 ± 7.5 µg/ml, and on BT474 at 500 ± 50 µg/ml, respectively.

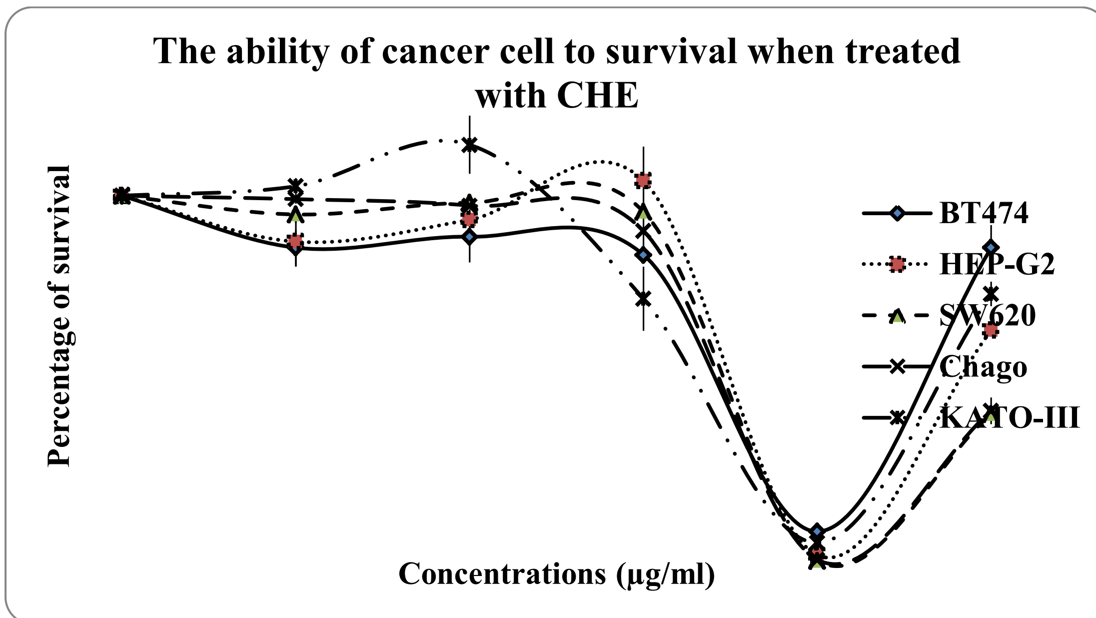
Alternatively, it could be reported that CME had the most to the least antiproliferative activity against BT474, SW620, Chago, KATO-III, and Hep-G_2.

The IC_{50} values of those extracts were compared by using the Kruskal Wallis one way analysis of variance. It was revealed that the IC_{50} values of both CHE and CDE had no significantly different antiproliferative activity ($p \leq 0.05$). In contrast, the IC_{50} value of CME was significantly different from the IC_{50} value of CHE and CDE ($p \leq 0.05$).

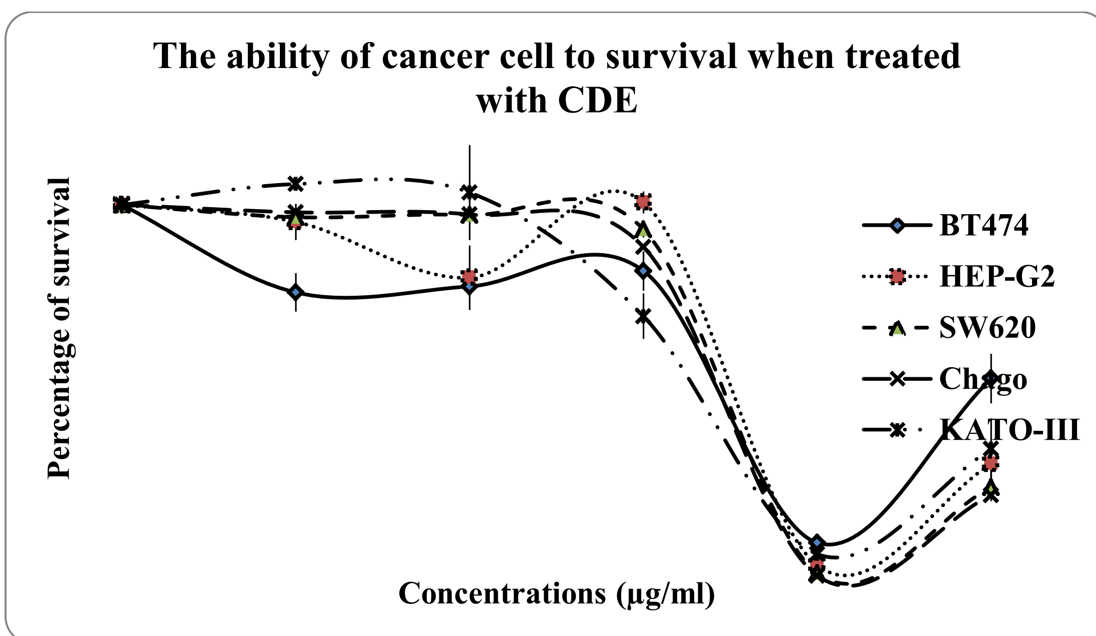
Table 4.3 The IC_{50} values of CHE, CDE, and CME on selected cancer cell lines.

Cancer cell lines	**IC_{50} (µg/ml)**		
	CHE	**CDE**	**CME**
BT474	48.33 ± 1.6^a	52.6 ± 3.7^a	500 ± 50^b
Chago	41.25 ± 3.75^a	44.66 ± 0.33^a	580 ± 20^b
KATO-III	42.5 ± 6.61^a	43.75 ± 6.5^a	600 ± 50^b
SW620	45.33 ± 0.33^a	46 ± 0.57^a	555 ± 7.5^b
Hep-G2	52.41 ± 3.7^a	53.5 ± 0.5^a	605 ± 39.05^b

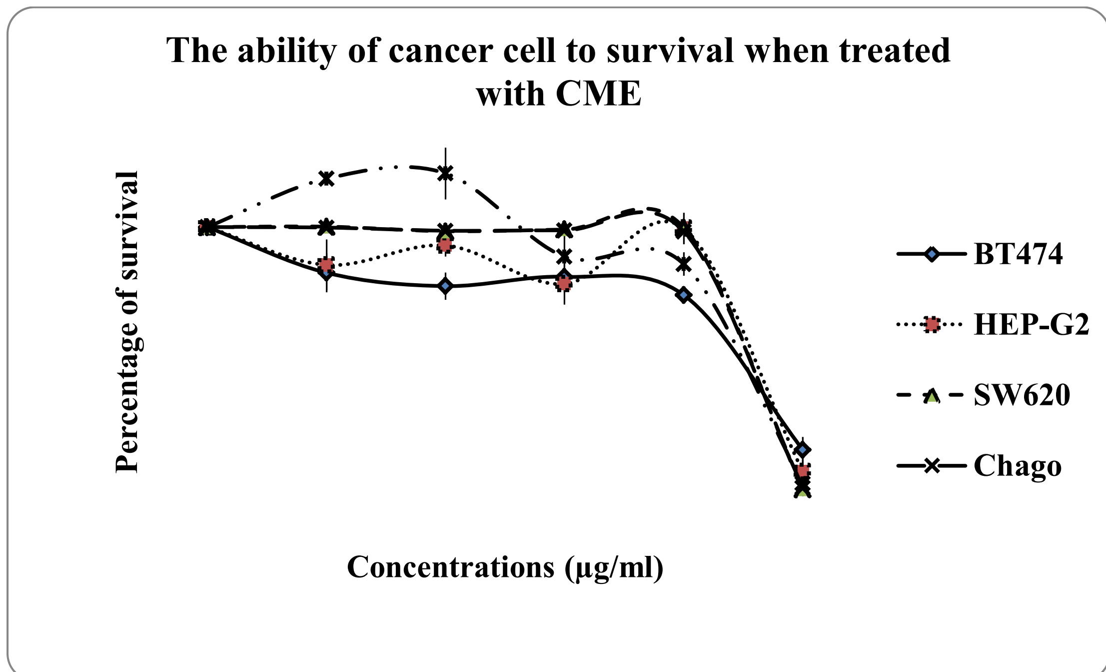
Remark: All reported data came from the triplication in each experiment. The different lowercase letters above the IC_{50} values presented the significant difference when p value was equal to or less than 0.05.



(A)



(B)



(C)

Figure 4.2 Effect of partitioned CEE on different cancer cell lines: (A) Effect of CHE; (B) Effect of CDE; and (C) Effect of CME on different cancer cells. The percentage of survival was expressed as mean ± SE (µg/ml).

4.3 Effect of fraction I-V on different cancer cell lines

Since CHE had the best antiproliferative activity against different cancer cells, it was further purified by quick column chromatography. Five fractions of fraction I-V were determined for the antiproliferative activity against those selected cancer cell lines. The result was presented that all fractions, except fraction I and II, had the antiproliferative activity against the selected cancer cell lines. The IC_{50} values of the active fractions were shown in both Figure 4.3 (A) and (B).

Considering fraction III, the most to the least active antiproliferative activity was on KATO-III at 13.69 ± 1.44 μg/ml, on Hep-G_2 at 19.37 ± 0.36 μg/ml, and on SW620 at 19.94 ± 1.83 μg/ml, respectively. In contrast, it had no antiproliferative activity against BT474 and Chago [Figure 4.3 (C)].

Furthermore, fraction IV had the antiproliferative activity on KATO-III at 40.16 ± 2.66 μg/ml and on SW620 at 44.56 ± 1.89 μg/ml. In contrast, it had no antiproliferative activity against Chago, Hep-G_2, and BT474 [Figure 4.3 (D)].

Among active fractions, fraction V had the highest antiproliferative activity against the selected cancer cell lines [Figure 4.3 (E)]. The most to the least sensitive cancer cell lines to fraction V was SW620 at 7.37 ± 0.23 μg/ml, Chago at 12.75 ± 0.68 μg/ml, KATO-III at 15.205 ± 2.13 μg/ml, Hep-G_2 at 22.22 ± 0.69 μg/ml, and BT474 at 29.36 ± 1.36 μg/ml, respectively.

The IC_{50} values were compared by using the Kruskal Wallis one way analysis of variance. Comparing among fraction III, IV and V, it was revealed that the

antiproliferative activity against KATO-III of fraction III was not significantly different from that of fraction V ($p \leq 0.05$). However, the activity of both fractions was higher than that of fraction IV. Considering the IC_{50} values for SW620, the antiproliferative activity of fraction III, IV, and V were significantly different ($p \leq 0.05$). The best to the least activity was from fraction V, III, and IV, respectively. The IC_{50} values (as mean ± SE) of fraction I-V for different cancer cell lines were shown in Table 4.4.

Table 4.4 The IC_{50} values of different fractions from CHE after quick column chromatography.

Cancer cell lines	**IC_{50} values (µg/ml)**				
	Fraction 1	**Fraction 2**	**Fraction 3**	**Fraction 4**	**Fraction 5**
BT474	ND	ND	ND	ND	29.36 ± 1.36
Chago	ND	ND	ND	ND	12.75 ± 0.68
KATO-III	ND	ND	13.69 ± 1.44[a]	40.16 ± 2.66[b]	15.205 ± 2.13[a]
SW620	ND	ND	19.94 ± 1.83[b]	44.56 ± 1.89[c]	7.37 ± 0.23[a]
Hep-G_2	ND	ND	19.37 ± 0.36	ND	22.22 ± 0.69

Remark: ND indicated no data for IC_{50} values since there was no antiproliferative activity. All reported data came from the triplication in each experiment. The different lowercase letters above the IC_{50} values presented the significant difference when p value was equal to or less than 0.05.

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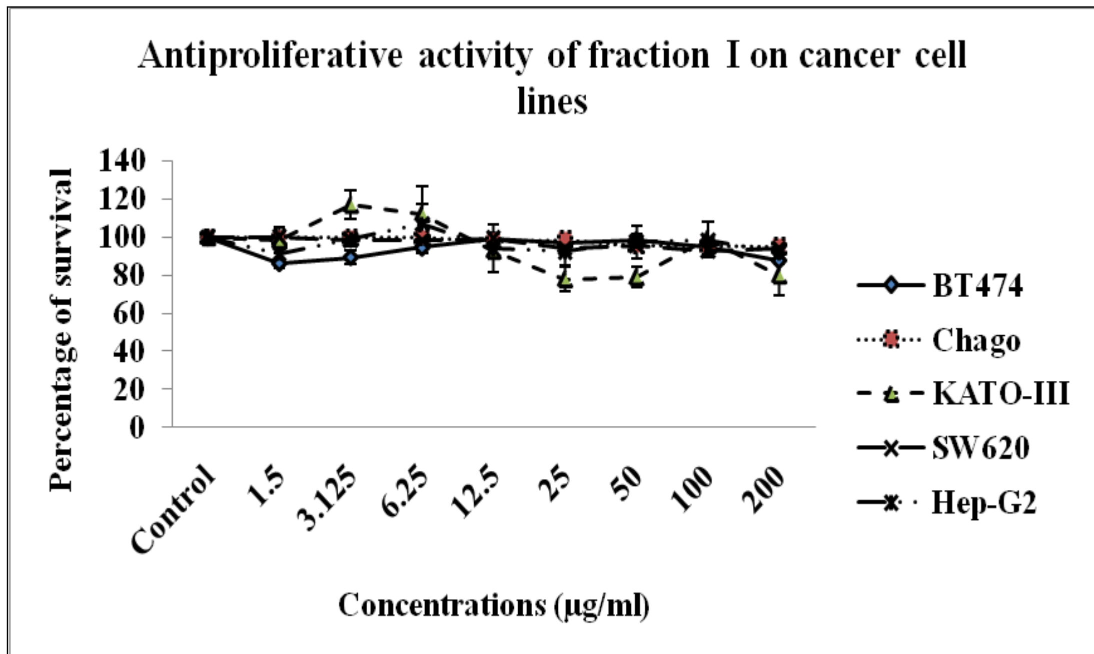
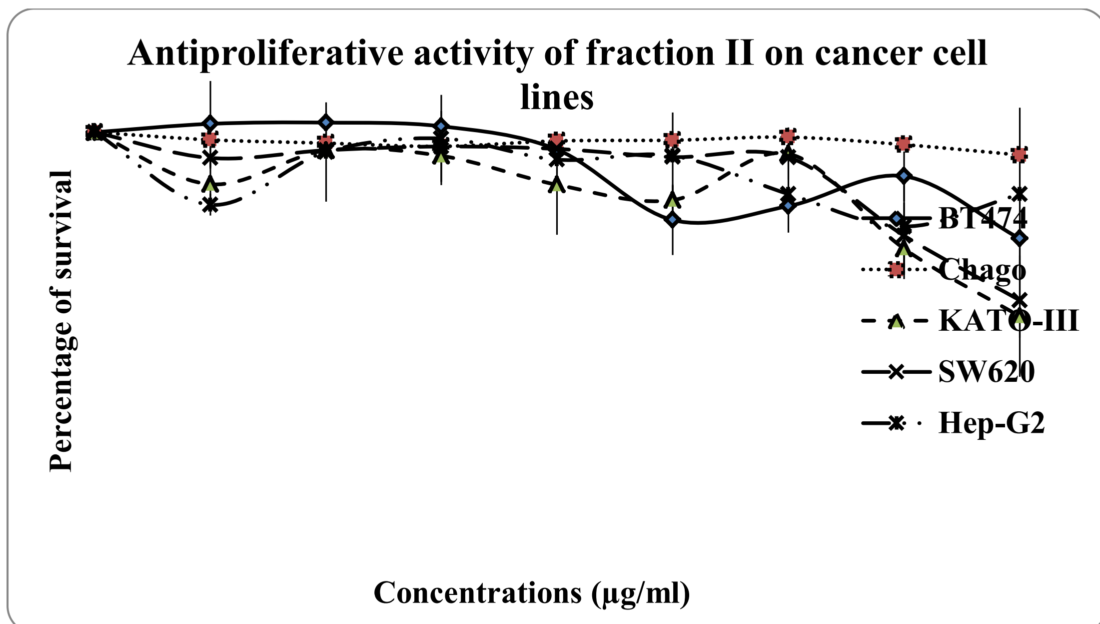
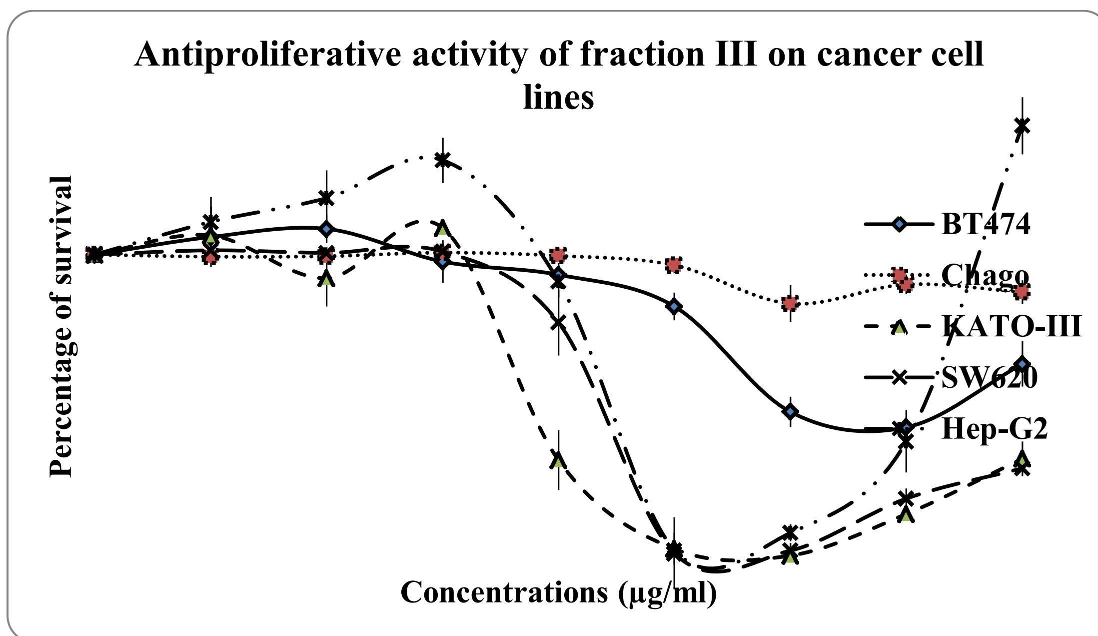


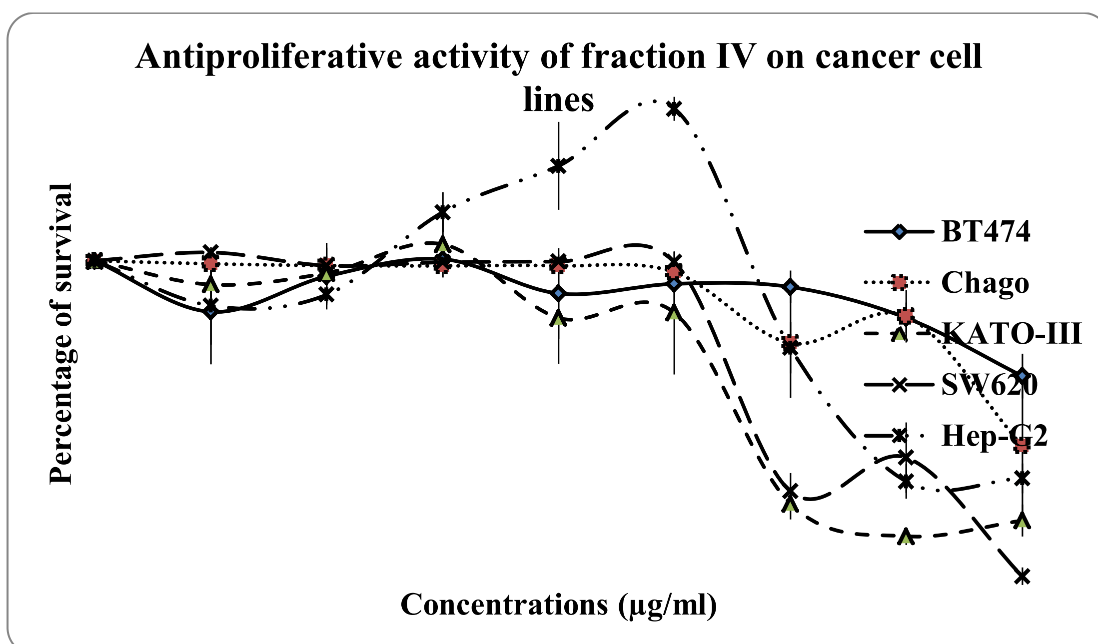
(A)



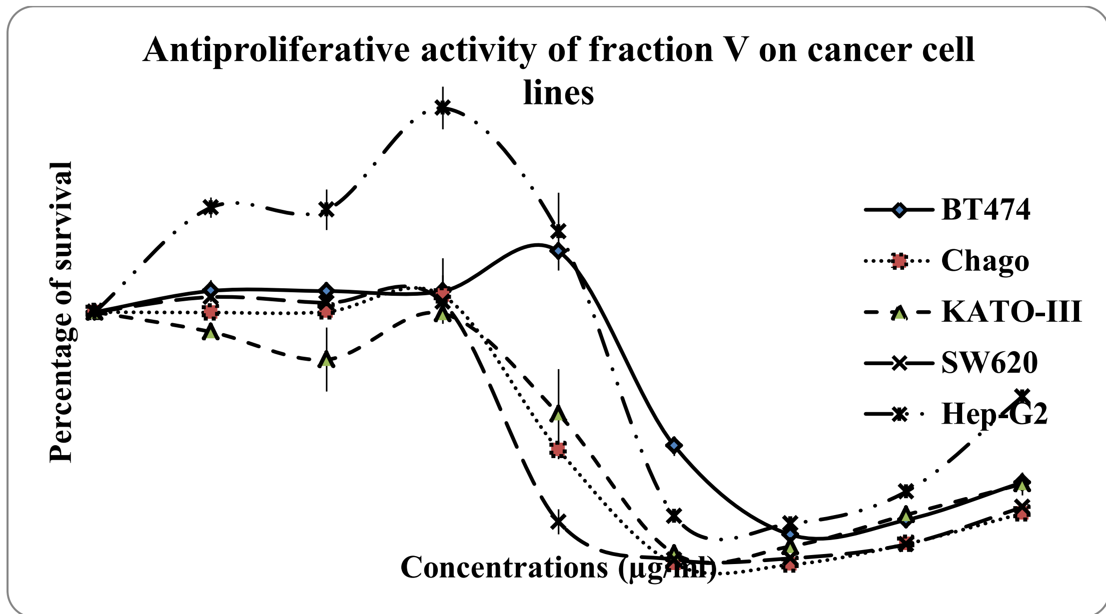
(B)



(C)



(D)



(E)

Figure 4.3 Effect of CHE on different cancer cell lines: (A) Effect of fraction I on different cancer cells: (B) Effect of fraction II extract on different cancer cells: (C) Effect of fraction III on different cancer cells: (D) Effect of fraction IV on different cancer cells: (E) Effect of fraction V on different cancer cells. The percentage of survival were expressed as the mean ± SE (µg/ml).

4.4 Effect of compound 1 and compound 2 on different cancer cell lines

Since fraction III and V performed the best antiproliferative activity, both were further purified by adsorption chromatography. Many fractions were obtained. The pattern of chemical compounds was observed by TLC. Two dominant spots were obviously seen. The first spot was from fraction III (obtained after adsorption chromatography) while the latter spot was from fraction V (obtained after adsorption chromatography). Later, it could be discovered that compound 1 was located at the first spot while compound 2 was located at the latter spot. After that, compound 1 was divided into two parts. The first part was tested on different cancer cell lines as well as fibroblast (Hs27). Also, the second part was analysed for the chemical structure. It was revealed that compound 1 had the interesting antiproliferative activity against those different cancer cell lines (Figure 4.4 A). The IC_{50} values of compound 1 on different cancer cell lines were compared to that on Hs27. It was revealed that the IC_{50} values for SW620, KATO-III, and BT474 were less than that for Hs27. In contrast, the IC_{50} values for Hep-G_2 and Chago were higher than that for Hs27. Alternatively, it could be presented that compound 1 could inhibit the proliferation of SW620, KATO-III, and BT474 better than that of Hep-G_2 and Chago. At the same time, the cytotoxicity of compound 1 on Hs27 should still be concerned and aware.

At the same time, compound 2 was also divided into two parts. The first part was tested on those different cancer cell lines and Hs27. Also, the second part was analysed for the chemical structure. The result was indicated that compound 2 had the

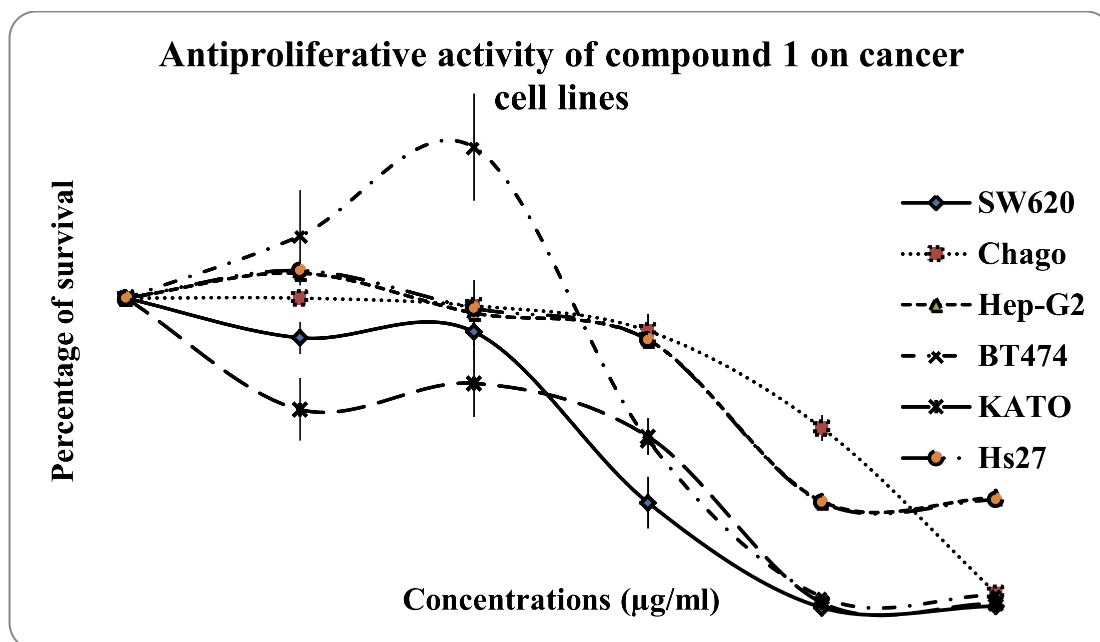
antiproliferative activity against different cancer cell lines (Figure 4.4 B). The IC_{50} values of compound 2 for those mentioned different cancer cell lines were compared to that for Hs27. It was revealed that the IC_{50} values for SW620, KATO-III, and BT474 were less than that of Hs27. In contrast, the IC_{50} values for Chago and Hep-G_2 were close to that for Hs27. This could be presented that the antiproliferative activity against SW620, KATO-III, and BT474 was better than that against Chago and Hep-G_2.

Considering the IC_{50} values of compounds 1 and 2 for Hep-G_2 and Hs27, it was found that the IC_{50} values of both compounds for Hep-G_2 were similar to those for Hs27. In addition, the IC_{50} value of compound 1 on Chago was higher than that on Hs27. Both cases were good to remind us the cytotoxicity left over in our both target compounds. Nevertheless, the IC_{50} values (as mean ± SE) of compounds 1 and 2 on different cancer cell lines were shown Table 4.5.

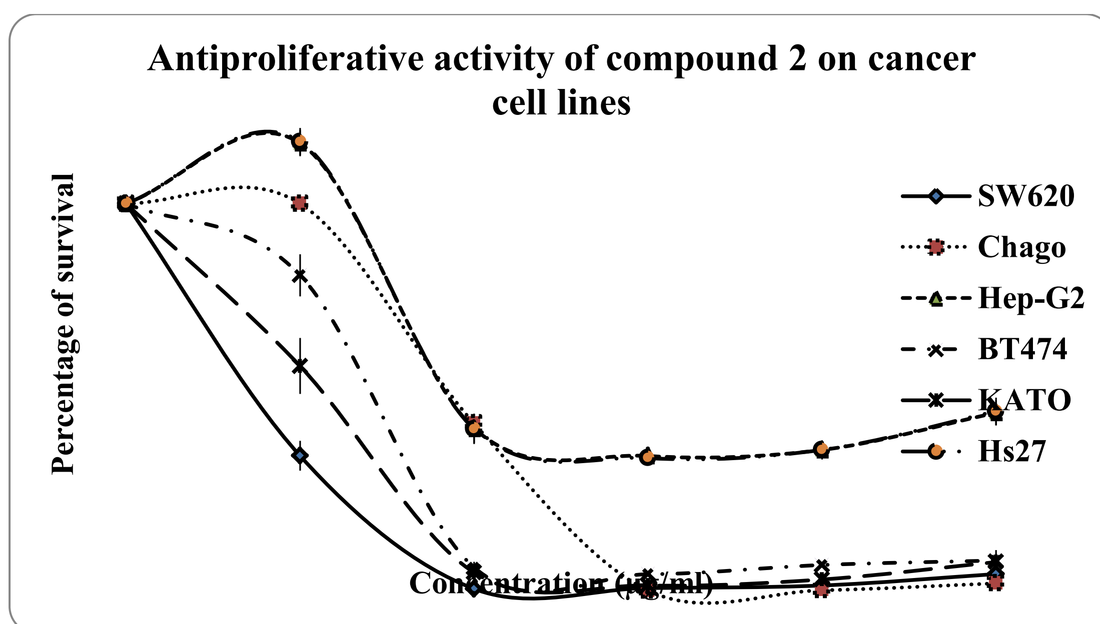
Table 4.5 The IC_{50} values of compounds 1 and 2 after adsorption chromatography.

Cancer cell lines	**IC_{50} value (µg/ml)**	
	Compound 1	**Compound 2**
BT474	13.95 ± 0.9	4.41 ± 0.15
Chago	29.30 ± 1.08	5.78 ± 0.07
KATO-III	13.71 ± 1.42	4.03 ± 0.13
SW620	10.76 ± 0.92	< 3.125
Hep-G_2	21.53 ± 0.35	5.97 ± 0.15
Hs27	21.35 ± 0.52	5.97 ± 0.15

Remark: All reported data came from the triplication in each experiment.



(A)



(B)

Figure 4.4 Effect of compound 1 and 2 on different cancer cell lines: (A) Effect of compound 1 on different cancer cells and (B) Effect of compound 2 on

different cancer cells. The percentage of survival was expressed as the mean ± SE (μg/ml).

In order to make sure that our target compound was only cytotoxic to cancer cells only, not normal cells. As mentioned in 3.7.4, while drawing a line to find the IC_{50} value of compound 1 on SW620 cell (10.76 ± 0.92 μg/ml), the line was continued drawing in the direction paralleling to the Y axis until it reached the Hs27 line. The percentage of survival of Hs27 cell was determined. In our research, it was approximately 85-90%. The same method was applied to compound 2 which its IC_{50} value on SW620 was < 3.125 μg/ml. it was revealed that the percentage of survival of Hs27 was approximately 110-120%. This indicated that compound 1 was little cytotoxic to Hs27 which was normal cells while compound 2 was not cytotoxic to Hs27.

As mentioned in Results, the IC_{50} value of compound 1 on KATO-III was 13.71 ± 1.42 μg/ml. By doing the same method as compound 1 on SW620, it was found that the percentage of survival of Hs27 was approximately 80-90%. The IC_{50} value of compound 2 on KATO-III was 4.03 ± 0.13 μg/ml. That led to the percentage of survival of Hs27 was approximately 95-100%. This indicated that compound 1 had the effects on Hs27 because the cell number was decreased about 10-20%. In contrast, compound 2 had no effect on Hs27 because the cell number was decreased only about 0-5%.

As mentioned in Results, the IC_{50} value of compound 1 on BT474 was 13.95 ± 0.9 μg/ml. By doing the same method as compound 1 on SW620, it was found that the

percentage of survival of Hs27 was approximately 80-90%. The IC_{50} value of compound 2 on BT474 was 4.41 ± 0.15 μg/ml. That led to the percentage of survival of Hs27 was approximately 80-85%. This indicated that both compounds 1 and 2 had the effects on Hs27 because the cell number was decreased about 10-20% and 15-20%, respectively.

As mentioned in Results, the IC_{50} value of compound 1 on Hep-G_2 was 21.53 ± 0.35 μg/ml. By doing the same method as compound 1 on SW620, it was found that the percentage of survival of Hs27 was approximately 50%. The IC_{50} value of compound 2 on Hep-G_2 was 5.97 ± 0.15 μg/ml. That led to the percentage of survival of Hs27 was approximately 50%, as well. This indicated that both compounds 1 and 2 had the effects on Hs27 because the cell number was decreased about 50%

As mentioned in Results, the IC_{50} value of compound 1 on Chago was 29.30 ± 1.08 μg/ml. By doing the same method as compound 1 on SW620, it was found that the percentage of survival of Hs27 was approximately 35-40%. The IC_{50} value of compound 2 on Chago was 5.78 ± 0.07 μg/ml. That led to the percentage of survival of Hs27 was approximately 50%. This indicated that both compounds 1 and 2 had the effects on Hs27 because the cell number was decreased about 60-65% and 50%, respectively.

From IC_{50} values of each cancer cells, percentage of survival of Hs27 were analyzed by drawing a line to find the IC_{50} value of compound 1 and 2 on each cancer cell, the line was continued drawing in the direction paralleling to the Y axis until it

reached the Hs27 line follow to Figure 4.5. The percentage of survival of Hs27 cell was determined and summarized in the table 4.6.

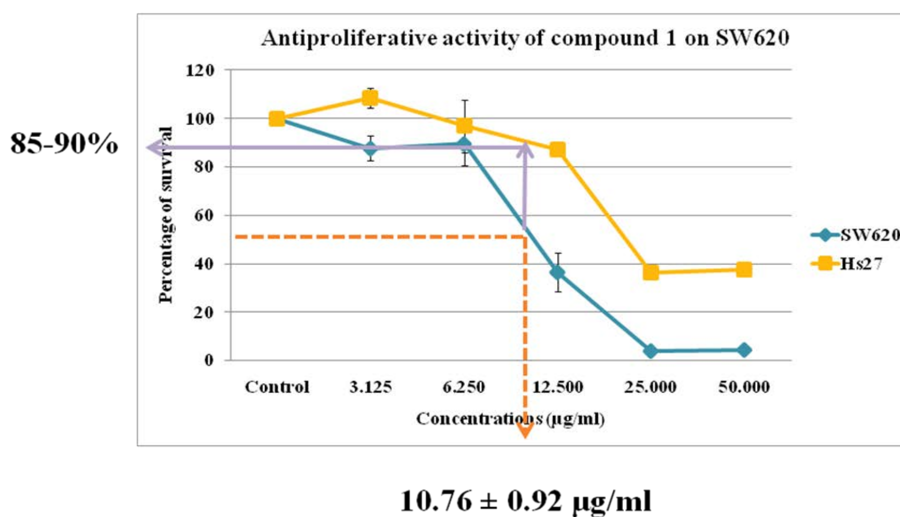


Figure 4.5 The percentage of survival of Hs27 after the IC_{50} value of SW620 was calculated.

Table 4.6 The percentage of survival of Hs27 after the selected cancer cell lines were treated with compound 1 and 2.

The IC_{50} values of cancer cells	The percentage of survival of Hs27	
	Compound 1	Compound 2
SW620	85-90	110-120
Chago	35-40	50
Hep-G_2	50	50
BT474	80-90	80-85
KATO-III	80-90	95-100

4.5 Morphology of SW620 and Hs27 cells

4.5.1 SW620 cancer cell line

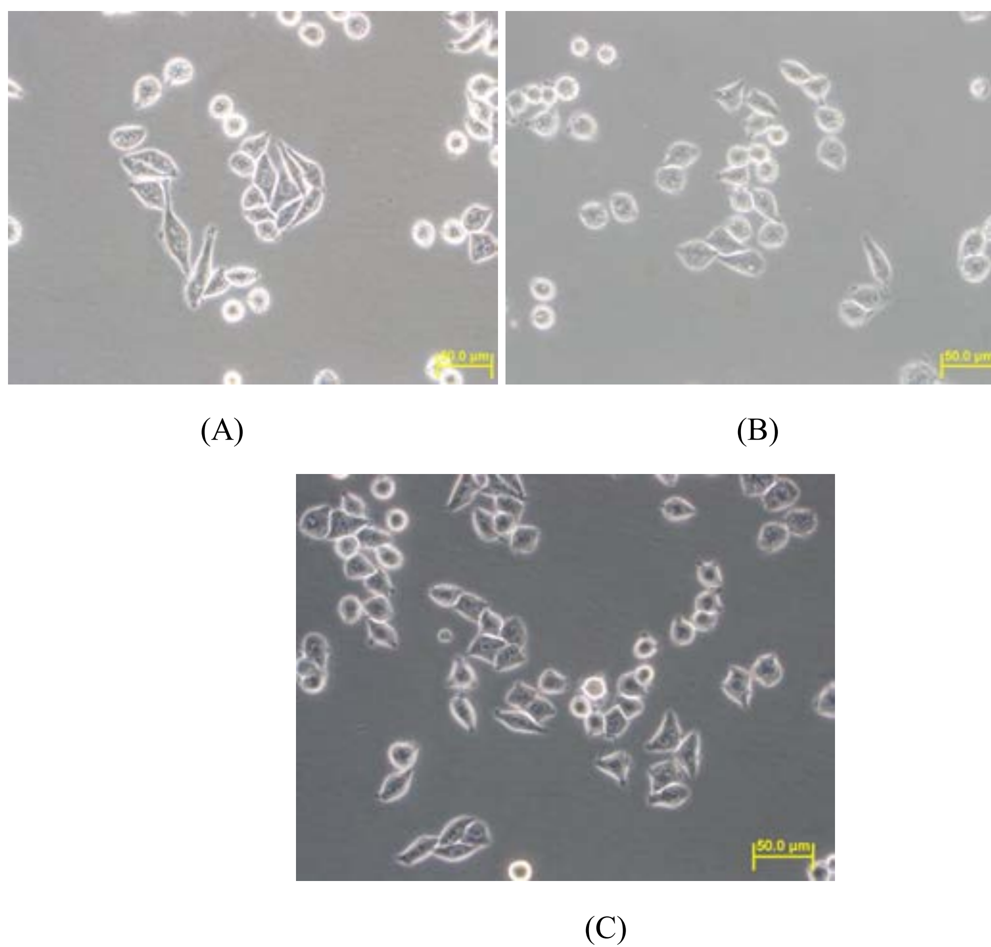
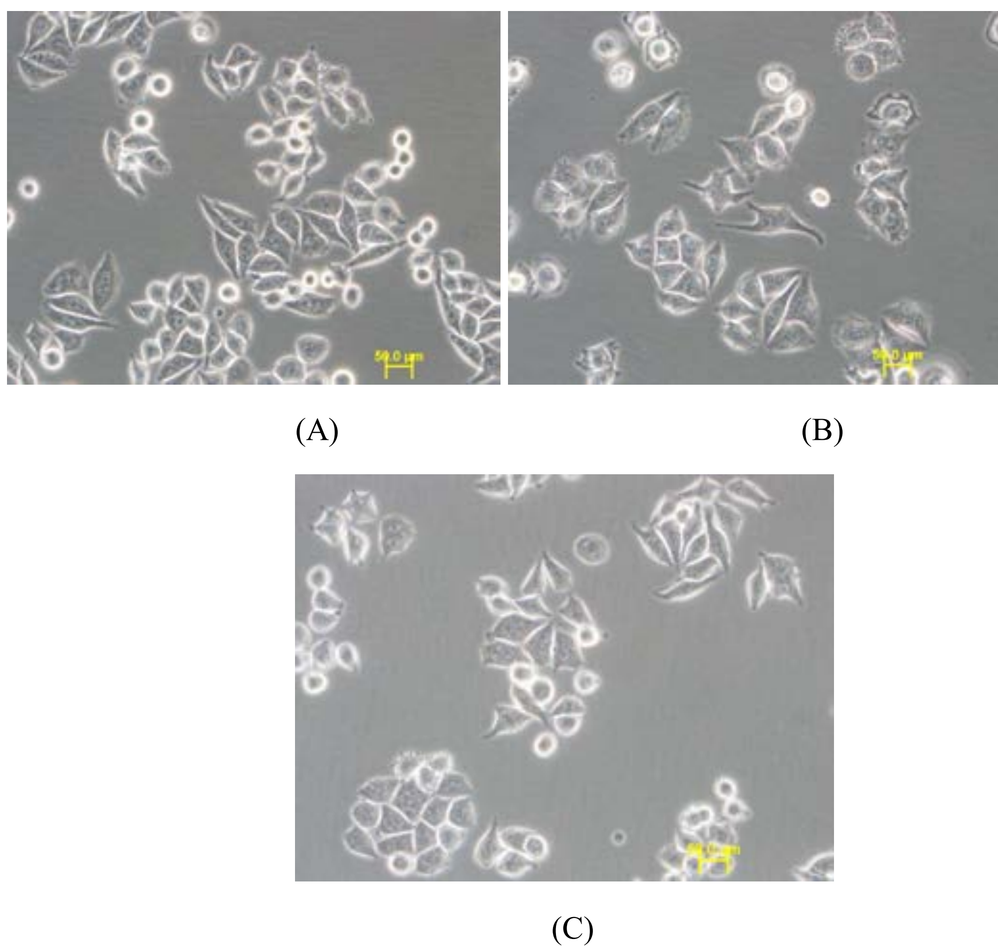


Figure 4.6 The shape of SW620 cancer cell line at 0 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B),

and treated with compound 2 at 3.0 μg/ml (C). All images were magnified at 40x.

Due to Figure 4.6, it could be seen that there was no morphology change in all samples at 0 h. In overall, cells looked flat and in a spindle shape.



(A) (B)

(C)

Figure 4.7 The shape of SW620 cancer cell line at 24 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 μg/ml (B), and treated with compound 2 at 3.0 μg/ml (C). All images were magnified at 40x.

From Figure 4.7, after the SW620 cells was treated with compound 1 at 10.76 µg/ml and compound 2 at 3.0 µg/ml for 24 h., the morphology of cells were observed. No change in morphology was still observed in all samples. The cells in all samples still looked flat and in a spindle shape.

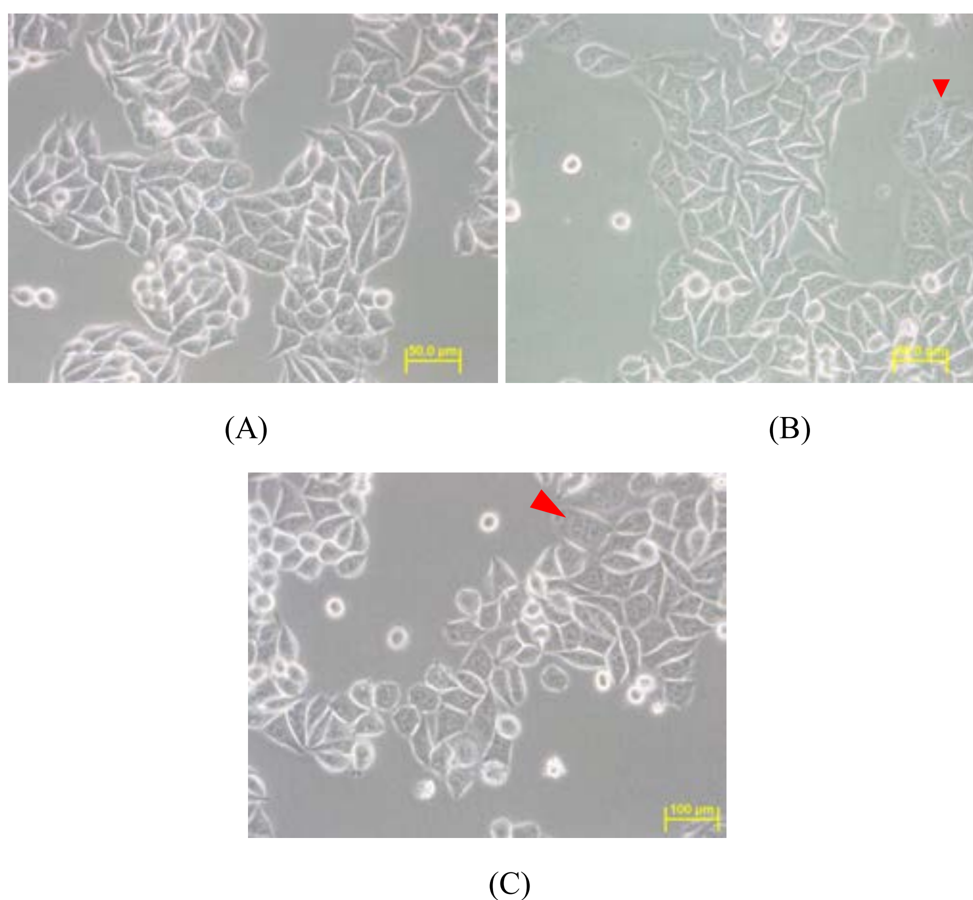


Figure 4.8 The shape of SW620 cancer cell line at 48 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B), and treated with compound 2 at 3.0 µg/ml (C). All images were magnified at 40x.

From Figure 4.8, after the SW620 cells were treated with compound 1 at 10.76 μg/ml and compound 2 at 3.0 μg/ml for 48 h., the morphology of cells were again observed. No change in shape was still observed in (A). In contrast, in some SW620 cells, the vacuolation could be visible inside the cells (B). The morphological change indicated by a red arrow was noticed in both (B) and (C).

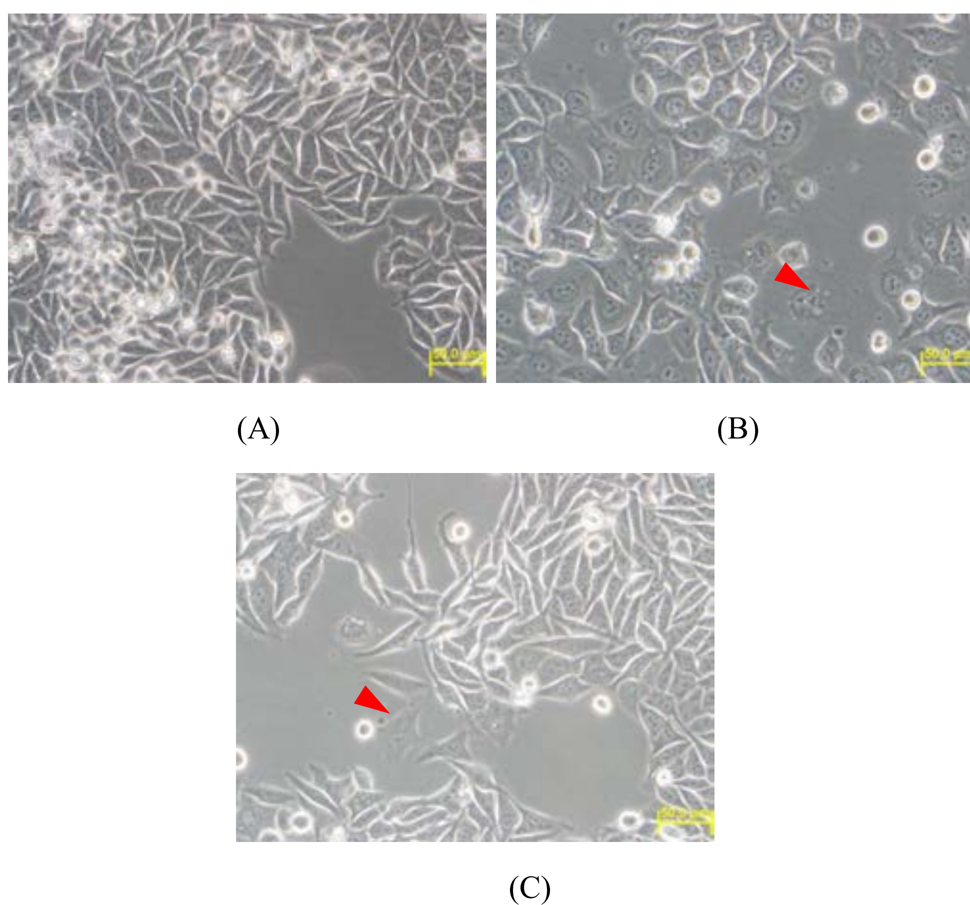


Figure 4.9 The shape of SW620 cancer cell line at 72 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 μg/ml (B), and treated with compound 2 at 3.0 μg/ml (C). All images were magnified at 40x.

From Figure 4.9, after 72 h of cell culture, the morphology of cells was observed again. Cells under control condition did not show the sign of morphological change. They still looked flat and in a spindle shape (A). Considering (B), in some SW620 cells, the DNA condensation within a nucleus could be noticed. In addition, the morphological change and cell debris indicated by a red arrow was visible. Moreover, the morphological change could be observed in some SW620 cells in (C). It was indicated by a red arrow.

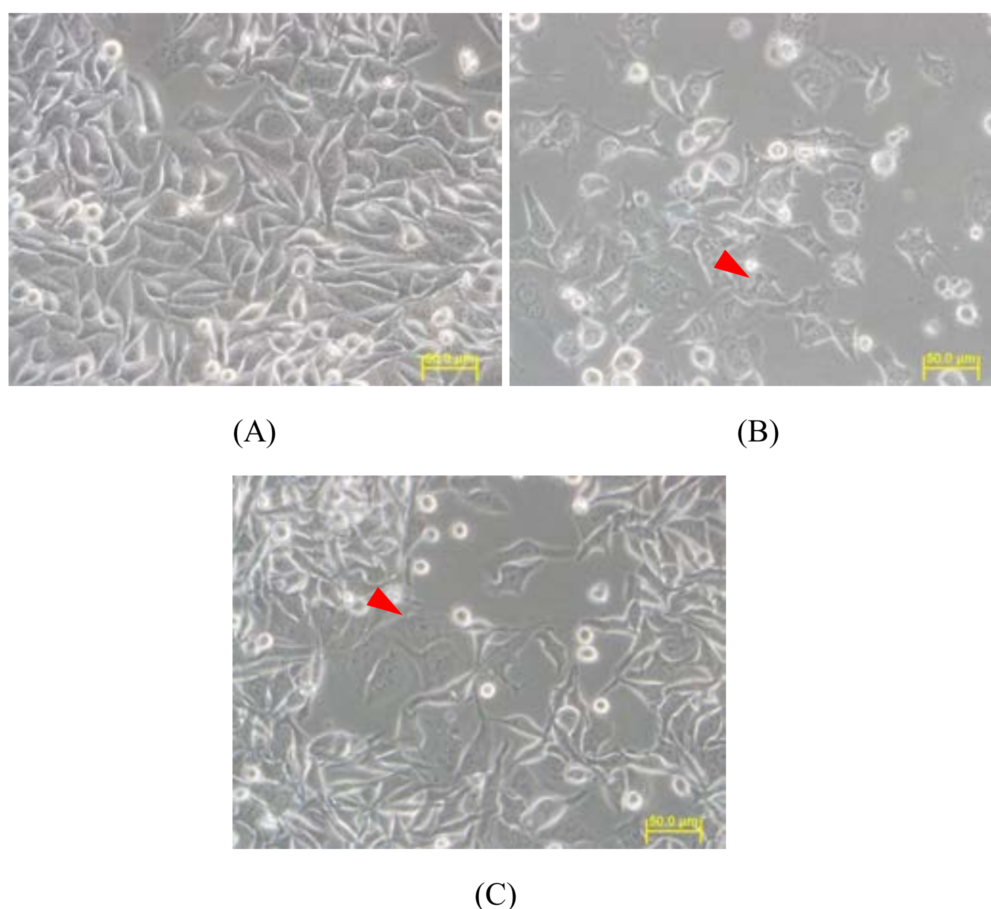
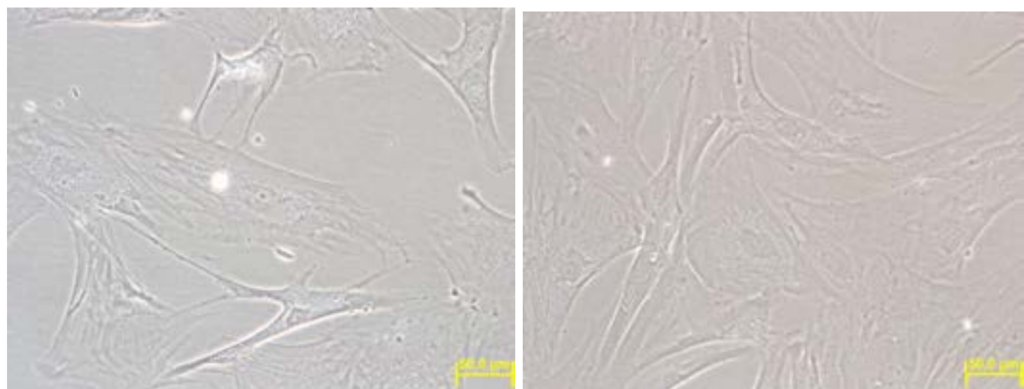


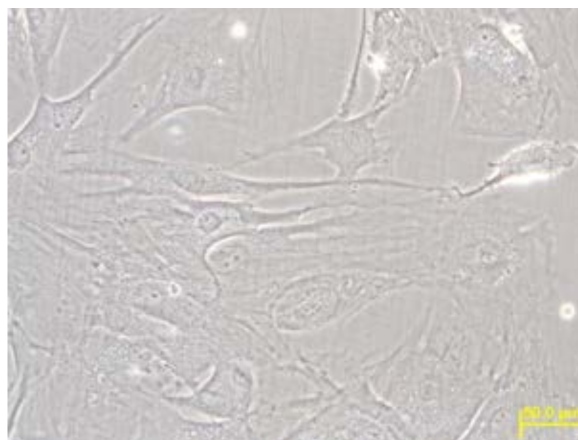
Figure 4.10 The shape of SW620 cancer cell line at 96 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B), and treated with compound 2 at 3.0 µg/ml (C). All images were magnified at 40x.

From Figure 4.10, after 96 h of cell culture, the morphology of cells was one more time observed. No change in morphology of cells under control condition was still observed in (A) while the DNA condensation within a nucleus indicated by a red arrow was visible in some SW620 cells (B). Furthermore, not only the morphological change and cell debris could be seen but the loss of cell adhesion which was indicated by a red arrow could be noticed in (C). Obviously, the density of cells in both (B) and (C) was less than that in control

4.5.2 Hs27 cells



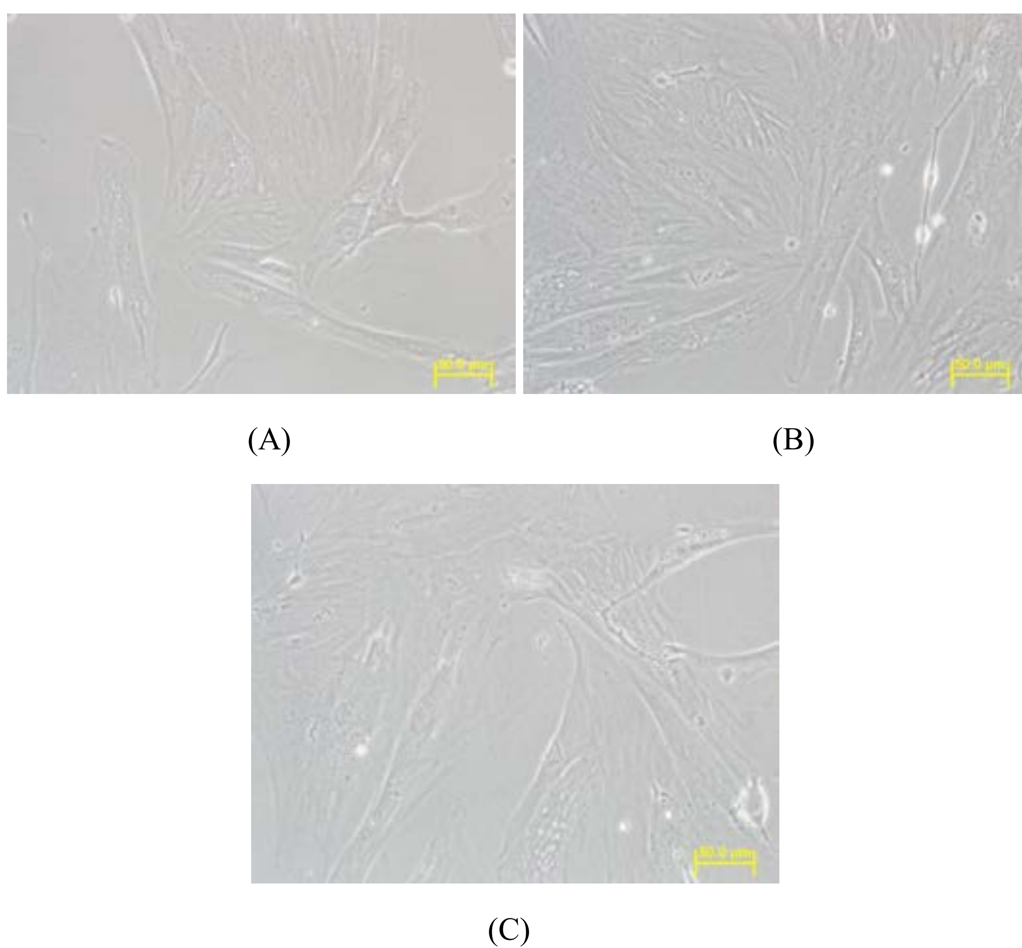
(A) (B)



(C)

Figure 4.11 The shape of Hs27 cells at 0 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B), and treated with compound 2 at 3.0 µg/ml (C). All images were magnified at 40x.

From Figure 4.11, the morphology of Hs27 cells in all conditions was the same. They looked flat and were attached to the substratum.

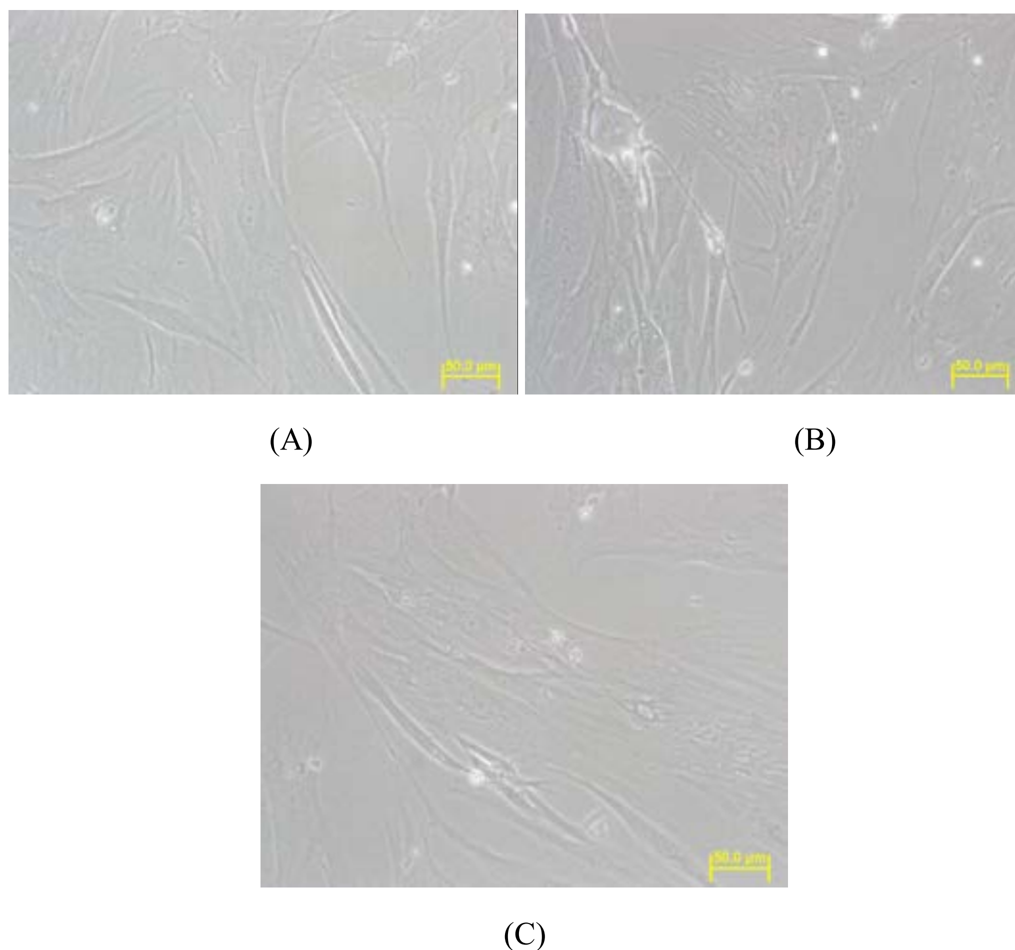


(A) (B)

(C)

Figure 4.12 The shape of Hs27 cells at 24 h of culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B), and treated with compound 2 at 3.0 µg/ml (C). All images were magnified at 40x.

From Figure 4.12, after the Hs27 cells were cultured under various conditions for 24 h, the morphology of cells was observed. The Hs27 cells in all conditions remained the same as they used to be previously.



(A) (B)

(C)

Figure 4.13 The shape of Hs27 cells at 48 h culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B), and treated with compound 2 at 3.0 µg/ml (C). All images were magnified at 40x.

From Figure 4.13, after 48 h culture of the Hs27 cells under various conditions, the morphology of cells was observed again. The Hs27 cells in all conditions remained the same as they used to be previously.

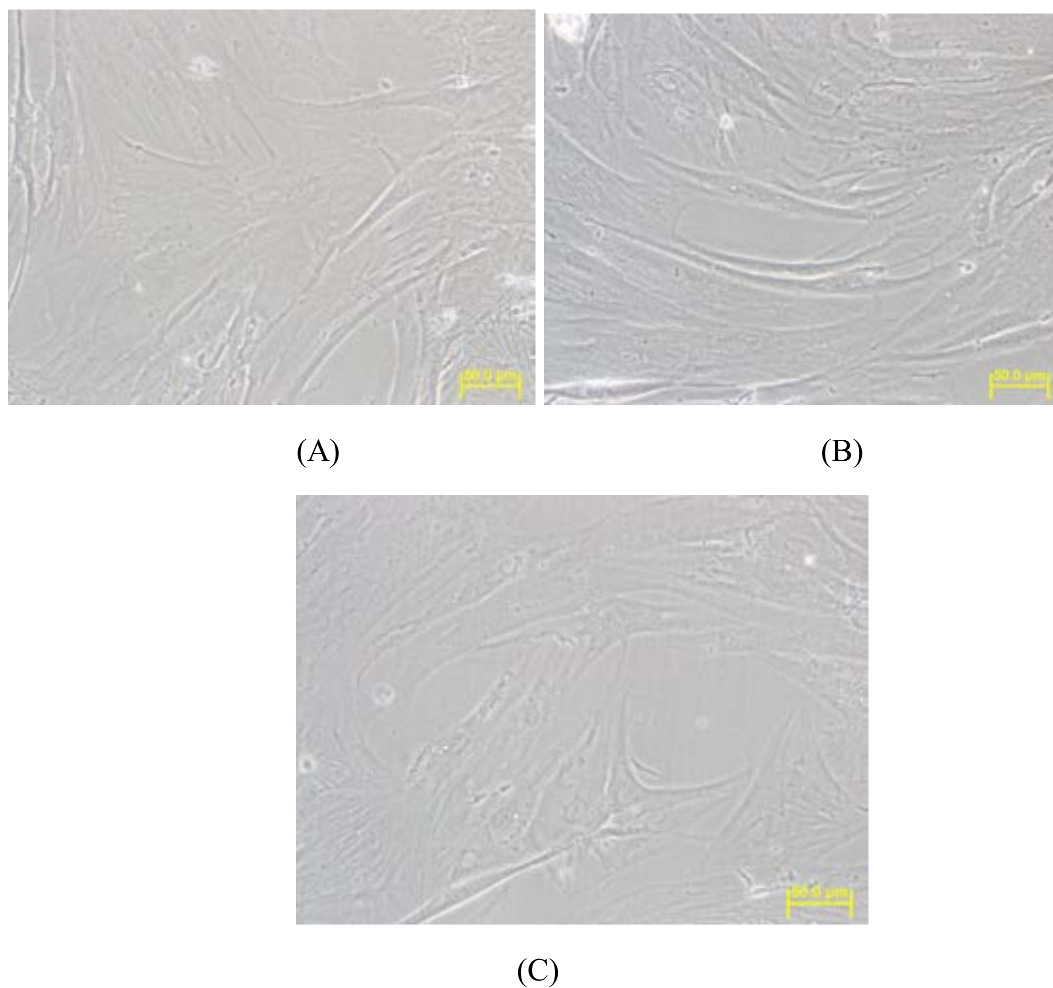


Figure 4.14 The shape of Hs27 cells at 72 h culture. The cells were untreated as control (A), treated with compound 1 at 10.76 µg/ml (B), and treated with compound 2 at 3.0 µg/ml (C). All images were magnified at 40x.

From Figure 4.14, after 72 h culture of the Hs27 cells under various conditions, the morphology of cells was observed again. The Hs27 cells in all conditions remained the same as they used to be previously.

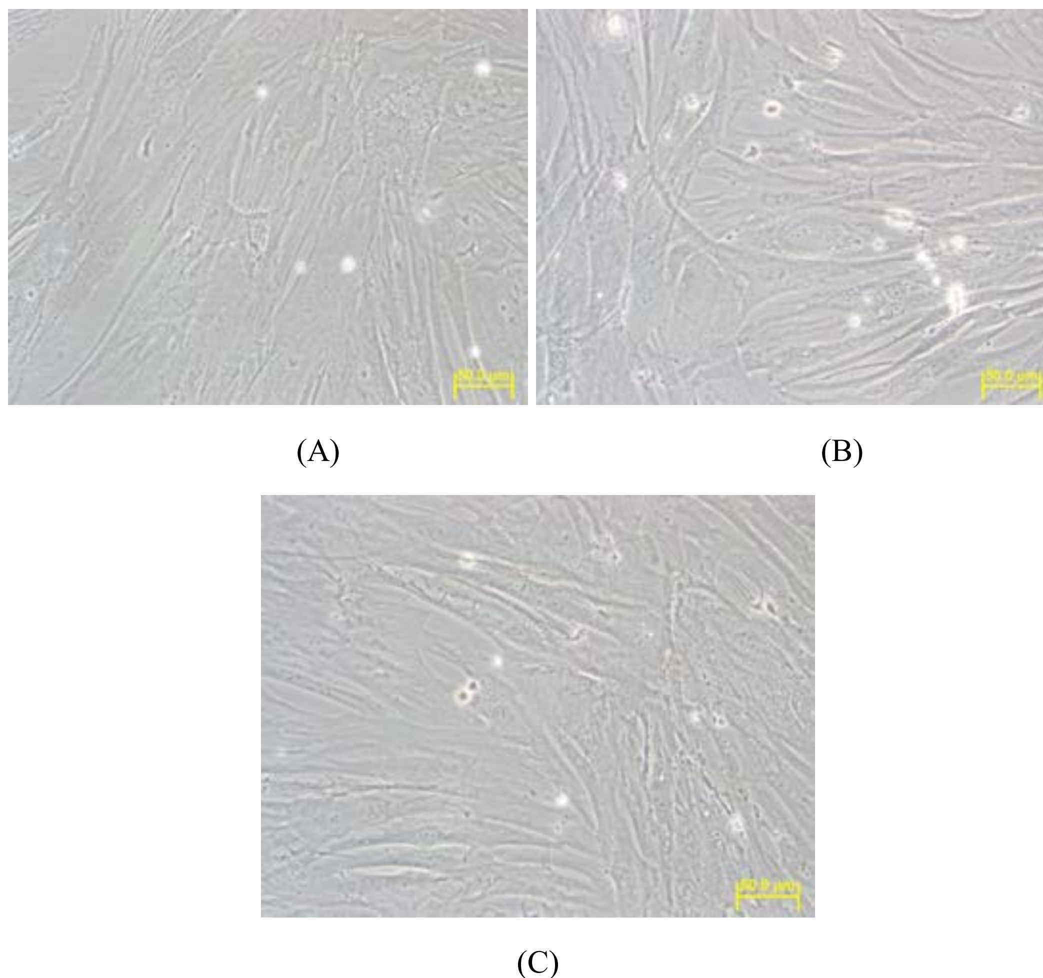


Figure 4.15 The shape of Hs27 cells at 96 h culture. The cells were untreated as control (A), treated with compound 1 at 10.76 μg/ml (B), and treated with compound 2 at 3.0 μg/ml (C). All images were magnified at 40x.

From Figure 4.15, after 96 h culture of the Hs27 cells under various conditions, the morphology of cells was observed again. The Hs27 cells in all conditions remained the same as they used to be previously.

In overall, comparing the results in Figure 4.11-4.15 to the results in Figure 4.6-4.10, it could be presented that our samples could change the morphology of

cancer cell lines but not normal cells. This leads to the possibility to develop our samples to be an anti-cancer agent in the future.

4.6 DNA Fragmentation

In order to find out whether our active compounds could damage the DNA of cancer cells or not, nuclear DNA was extracted as mentioned in Materials and Methods. If they play no roles in damaging, the DNA will be in a good condition. The high molecular weight and sharp band will be observed. If they do, smear of fragmented DNA will be seen.

4.6.1 DNA fragmentation of colon cancer cell line (SW620)

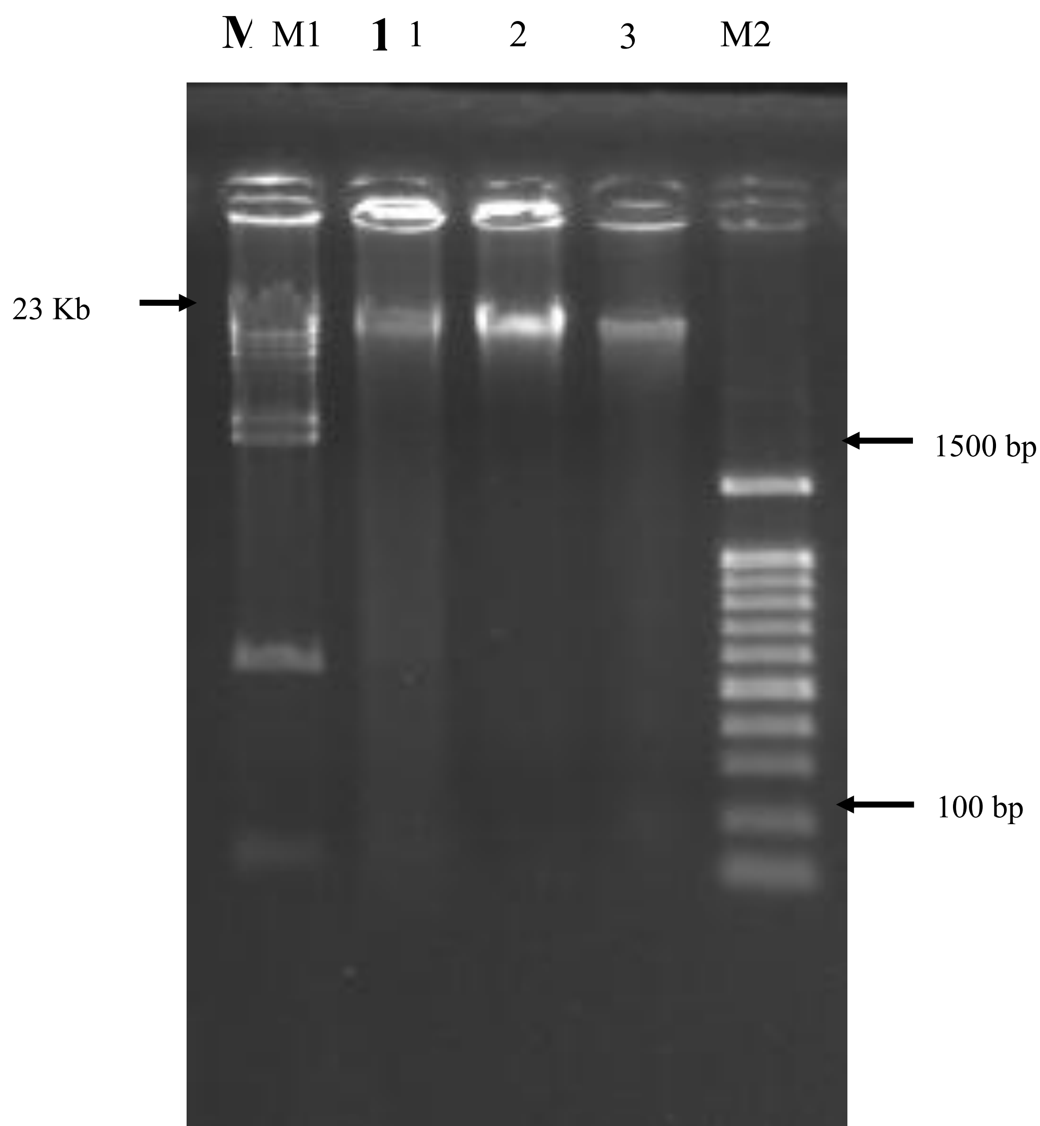


Figure 4.16 Agarose gel electrophoresis (1.8%) of extracted DNA from SW620. Lane M1 and M2 contained λ*Hin*d III and 100 bp ladder as DNA markers. Lane 1 represented the extracted DNA of untreated SW620. Lane 2 represented the extracted DNA of SW620 treated with compound 1 at its IC_{50} value (10.76 μg/ml) while lane 3 represented

the extracted DNA of SW620 treated with compound 2 at its IC_{50} value (3.0 µg/ml).

After the morphology of SW620 was observed, DNA was extracted and run on 1.8% (w/v) agarose gel electrophoresis. After EtBr staining and under U.V. light, no DNA fragmentation was observed in all samples (Figure 4.16).

4.6.2 DNA fragmentation of Hs27

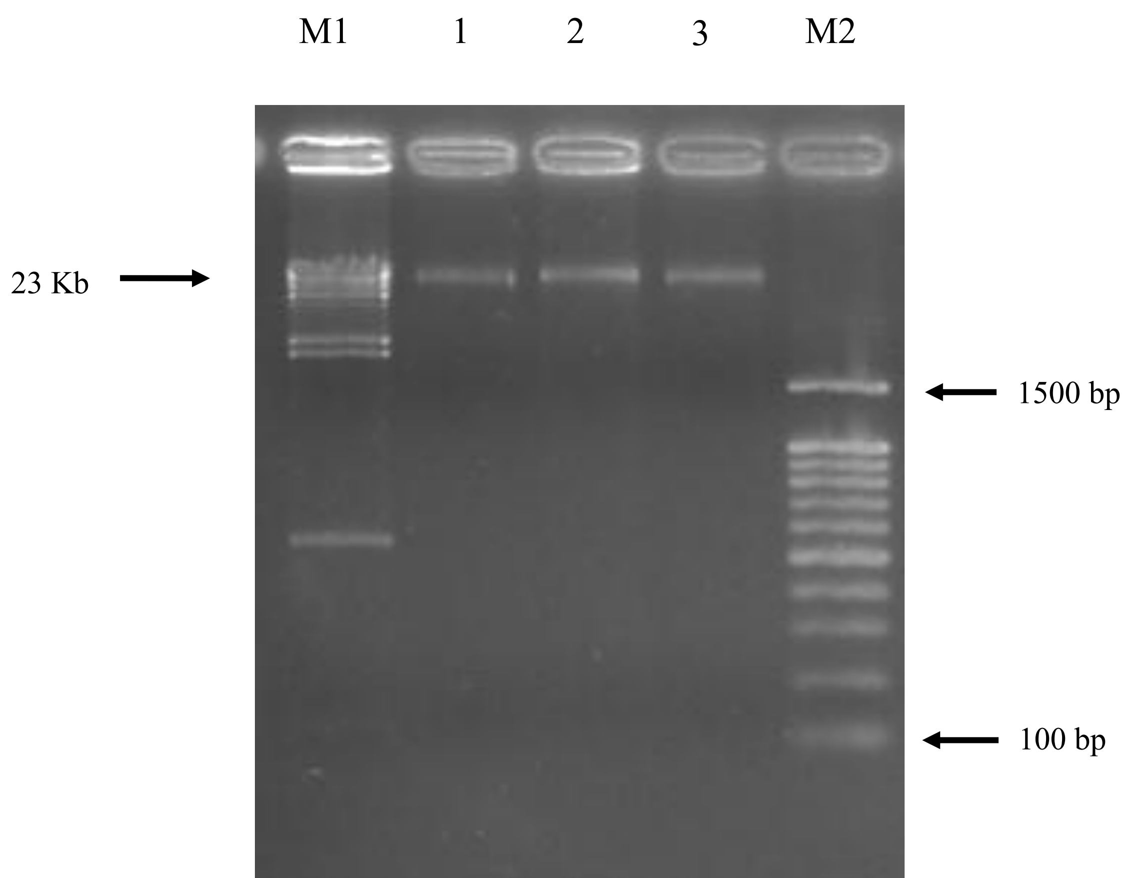


Figure 4.17 Agarose gel electrophoresis (1.8%) of extracted DNA from Hs27 cells. Lane M1 and M2 contained λ*Hin*d III and 100 bp ladder as DNA markers. Lane 1 represented the extracted DNA of untreated Hs27.

Lane 2 represented the extracted DNA of Hs27 treated with compound 1 at its IC_{50} value (10.76 µg/ml) while lane 3 represented the extracted DNA of Hs27 treated with compound 2 at its IC_{50} value (3.0 µg/ml).

After the morphology of Hs27 was observed, the DNA was extracted and run on 1.8% (w/v) agarose gel electrophoresis. After EtBr staining and under U.V. light, no DNA fragmentation was observed in all samples (Figure 4.17).

Considering Figures 4.16 and 4.17, it could be said that our compounds did not kill cancer cells by apoptosis since DNA damage could not be found. However, it was likely that our compounds killed cancer cells by necrosis since the shape of cells was changed after treated. Fortunately, the change in morphology of control cells were not seen (Figures 4.6-4.15). Thus, it is promising that we may find new antiproliferative agents from propolis from Nan province, Thailand.

4.7 NMR and MS analysis

4.7.1 Compound 1

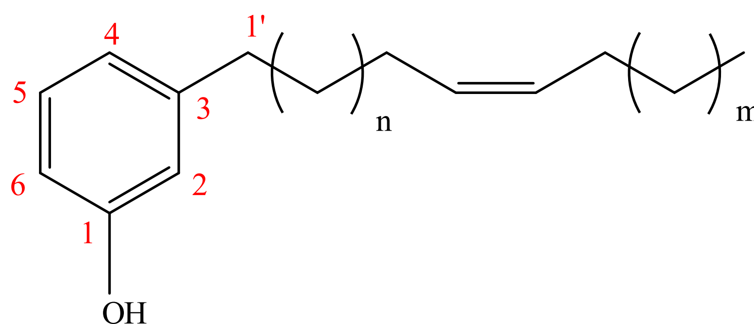
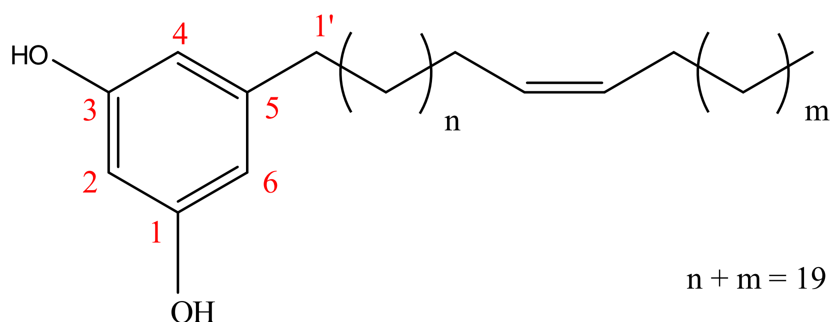


Figure 4.18 The chemical structure of compound 1 by NMR and MS analysis.

Compound 1: yellow oil and specific smell; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.05 (1H, t, *J* = 7.6 Hz, H-5), 6.67 (1H, d, *J* = 7.6 Hz, H-6), 6.58 (1H, s, H-2), 6.57 (1H, d, *J* = 8.0 Hz, H-4), 5.36 (1H, br s, OH), 5.28 (2H, m, olefinic proton), 2.46 (2H, t, *J* = 7.6 Hz, H-1'), 1.95 (4H, br s), 1.48-1.52 (2H, m), 1.18-1.25 (30H, br s), 0.82 (3H, t, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 155.4, 145.0, 130.0, 129.9, 129.4, 120.9, 115.3, 112.5, 35.9, 32.0, 31.4, 29.8, 29.7, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 27.2, 26.9, 22.4, 14.1; ESIMS *m/z* $[M+H]^+$ in the range of 400-500 and could not be calculated the exact molecular weight.

4.7.2 Compound 2



Compound 2

Figure 4.19 The chemical structure of compound 2 by NMR and MS analysis.

Compound 2: brown resin; DT46-3; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 6.17 (2H, d, *J* = 2.0 Hz, H-4, and H-6), 6.10 (1H, s, H-2), 5.28 (2H, m, olefinic proton), 2.39 (2H, t, *J* = 7.6 Hz, H-1'), 1.95 (4H, br s), 1.48 (2H, br s), 1.18-1.25 (38H, br s), 0.82 (3H, t, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 156.5, 146.2, 129.9, 129.8, 108.0, 100.1, 35.8, 31.9, 31.1, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.3, 27.2, 26.9, 22.3, 14.0; ESIMS *m/z* $[M+H]^+$ 459 corresponding to molecular formula of $C_{31}H_{54}O_2$.

CHAPTER V

DISCUSSIONS

In this research, propolis of *Apis mellifera* was used to determine the antiproliferative activity on cancer cells since *A. mellifera* is economic. It is native to the continents of Europe and Africa. Although there are many bee species those can produce propolis, especially stingless bees, *A. mellifera* was chosen due to the best well-managed bees in an apiary. The bees are known to be not aggressive and diligent in foraging (Oldroyd and Wongsiri, 2006). It was reported that propolis bioactivities depended mainly on plant sources, geography, climate, bee species, etc (Choi *et al*., 2006). Propolis used in this research was collected from Nan province where it is located in the northern part of Thailand. There is a big gap of temperature in winter and summer. This region is rich in forests and mountains. High diversity of plants is known. That is possible for us to find a new compound with the antiproliferative activity.

At the beginning, collected propolis was extracted by EtOH and water according to Najafi *et al*. (2007). The obtained crude extracts were tested against cancer cell lines by MTT assay according to Santos *et al*. (2002) and Hernandez *et al*. (2007). The result showed that CEE had the better proliferative activity against the selected cancer cells. Due to Table 4.2, the IC_{50} values of different cancer cells were

in the range of 300- 500 µg/ml. The obtained result was coincided to Kouidhi *et al*. (2010), Alencar *et al*. (2007), and Barbarić *et al*. (2011). In 2010, Kouidhi *et al*. reported that CEE of *A. mellifera* from the states of Monastir (Tunisia) had the antiproliferative activity against cancer human intestinal epithelial cells (HT-29), human epithelial cells (Hep-2), human respiratory epithelial cell line (A549), and human fibroblast-like foetal lung cell line (MRC-5) as normal cells with the IC_{50} values of 18±0.2, 15.7±3.4, 200±22.2, and 55±5.6 µg/ml respectively. Moreover, the CEE of the same bees from Brazil showed the cytotoxic activity on HeLa tumor cells with the IC_{50} value of 7.45 µg/ml (Alencar *et al*., 2007).

However, there were many researches supporting that water could be a suitable solvent to extract propolis. For example, Najafi *et al*. (2007) used CWE to treat McCoy and HeLa cancer cells. The result was shown that it could provide the active antiproliferative activity at 1 and 2 mg/ml, respectively. At 1 mg/ml of CWE, it could inhibit the growth of McCoy cancer cells near 70% and could inhibit the growth of HeLa cancer cells near 30%. In addition, at 2 mg/ml of CWE, the percentage of growth inhibition on HeLa cancer cells was increased to be near 40%.

After being partitioned with hexane, CH_2Cl_2, and MeOH, it was found that both CHE and CDE had the good antiproliferative activity against those selected cancer cells. The result of this research was a little bit different from Lim *et al*. (2009). They reported the comparison of the antiproliferative activity among different solvents (n-hexane, $CHCl_3$, EtOAc, C_4H_9OH and aqueous residue) used to extract *Citrus grandis* Osbeck fruits. It was shown that only CHE had the best activity against the same cancer cells used in our research. Roughly, it could be said that active

compounds in propolis were not highly polar. Considering the IC_{50} values of CHE in our research and in Lim *et al*. (2009) against the same types of cancer cells, it was surprisingly found that the IC_{50} values of our work were lower than the other. The IC_{50} value of CHE against human liver hepatoblastoma (Hep-G_2) was 52.41 ± 3.7 µg/ml in our work and was 131.45 µg/ml in the other. The IC_{50} value of CHE against breast cancer cells was 48.33 ± 1.6 µg/ml for BT474 in our work and was 143.76 µg/ml for MCF-7 in the other. The IC_{50} value of CHE against colon cancer cells was 45.33 ± 0.33 µg/ml for SW620 in our work and was 86.55 µg/ml for HCT-15 in the other. The IC_{50} value of CHE against gastric carcinoma was 42.5 ± 6.61 µg/ml for KATO-III in our work and was 90.0 µg/ml for SNU-16 in the other. The IC_{50} value of CHE against lung cancer cells was 41.25 ± 3.75 µg/ml for Chago in our work and was 72.67 µg/ml for NCI-H460 in the other. From this data, it was promising to find a new and better antiproliferative agent from Thai propolis.

Moreover, in 2009, Castro *et al*. reported the best antiproliferative activity against HeLa tumor cells was from CHE of Brazilian propolis (IC_{50} = 0.1756 µM).

Later, quick column chromatography was performed in order to separate the active compounds based on their polarities as mentioned in 3.6.1. Five fractions were obtained and tested for the cytotoxicity against the selected cancer cells. It was found that fraction III showed the good antiproliferative activity against KATO-III, SW620, and Hep-G_2 but not BT474 and Chago. Fraction IV showed the antiproliferative activity against KATO-III and SW620 but not Hep-G_2, BT474, and Chago. Fraction V showed the antiproliferative activity against all cancer cells with the IC_{50} values in the range of 7-30 µg/ml (Table 4.4). From the above result, it could be seen that three

fractions had the effect to KATO-III and SW620. Fraction III performed the best activity against KATO-III. Then, it was followed by fractions V and IV. For the growth inhibition of SW620, the best activity was from fraction V. Then, it was followed by fractions III and IV. Interesingly, it was found that all cancer cells were sensitive to fraction V. After fraction III was further purified by adsorption chromatography, one purified fraction was very effective to inhibit the growth of all cancer cells. Then, compound 1 was found to be in that fraction. Considering fraction III, it was impure because it consisted of many compounds providing the antagonism effect which led to the insensitivity of some cancer cells.

Considering the cytotoxicity between compounds 1 and 2 on SW620, KATO-III, BT474, Hep-G_2, Chago, and Hs27, compound 1 had the antiproliferative activity with the IC_{50} values at 10.76 ± 0.92, 13.71 ± 1.42, 13.95 ± 0.9, 21.53 ± 0.35, 29.30 ± 1.08, and 21.35 ± 0.52 µg/ml, respectively (Table 4.6) while the IC_{50} values of compound 2 were < 3.125, 4.03 ± 0.13, 4.41 ± 0.15, 5.97 ± 0.15, 5.78 ± 0.07, and 5.97 ± 0.15 µg/ml, respectively (Table 4.6). In overall, it was likely that SW620 was the most sensitive to compounds 1 and 2. Comparing the IC_{50} values of compounds 1 and 2, compound 2 looked to be a promising agent for anti-cancer.

In 2005, Wang *et al*. reported the success in purifying caffeic acid phenethyl ester (CAPE) from propolis. It could perform the good antiproliferative activity on human colorectal cancer (CRC) cell line. Also, it was found that CAPE inhibited the growth of CRC cells in the dose- and time-dependent manner. The IC_{50} value of CAPE after 72 h treatment was 6.47 µg/ml. Moreover, CAPE showed the strong inhibitory effect on matrix metalloproteinase (MMP-9) which was related to the

invasion and metastasis of hepatocellular carcinoma (Jin *et al*., 2005). Comparing compound 2 from our research and CAPE, the IC_{50} value of compound 2 on SW620 was lower than the IC_{50} value of CAPE. This indicated that compound 2 could be the better antiproliferative agent against SW620. However, the cytotoxicity on normal cells needs to be concerned.

The CAPE was capable of inhibiting the growth of many cancer cell lines such as C6 glioma cells (Kuo *et al*., 2006), human leukemia (HL-60) (Chen *et al*., 1996), etc. It was also cytotoxic to neck metastasis of gingiva carcinoma (GNM) and tongue squamous cell carcinoma (TSCCa) cells (Lee *et al*., 2000). In addition, CAPE also had an effect on breast cancer cells (MDA-231 and MCF-7). The IC_{50} value of CAPE on MDA-231 and MCF-7 were approximately 15 µM or 4.26 µg/ml (Wu *et al*., 2011). Comparing the IC_{50} value, it was similar between the value of CAPE and compound 2 from our work while the value of compound 1 was higher.

Other than the cytotoxicity test of compounds 1 and 2 on those selected cancer cells, the change in morphology of SW620 and Hs27 treated with compounds 1 and 2 were also observed (as mentioned in 3.9). The used concentration of both compounds was at the concentration of IC_{50} values on SW620. The obtained result was presented that the change in morphology of SW620 could be observed. In addition, the number of cells was noticeably decreased. In contrast, for the treated Hs27, no change in morphology and the same number of cells could be visible. The obtained data from this part was very supportive that a new antiproliferative agent should be found in Thai propolis.

Nevertheless, we could not conclude that both compounds 1 and 2 caused the cell death by the mean of apoptosis. That was because the cell morphology was not clear and the chromatin condensation was not clearly shown in the nucleus of treated cells. Unlike our work, Vatansever *et al*. (2010) reported that CEE from Turkey could lead the death of human breast cancer cell line (MCF-7) by apoptosis. Although the morphology of MCF-7 was not changed, the number of cells was decreased. In addition, Umthong *et al*. (2009) found that CWE and CME from *Trigona laeviceps* (stingless bee) in Samut Songkram provine, Thailand had the effect on SW620. Their result was similar to our work in term that the change in morphology of SW620 could be observed. The SW620 lost their cell adhesion and died. Considering DNA fragmentation, our result was opposite to Umthong *et al*. (2009). In their work, it was shown that the extracts caused DNA fragmentation. Moreover, unlike our work, Chen *et al*. (2007) reported that propolin A and propolin B extracted from Taiwanese propolis could induce an apoptosis on human melanoma A2058 cells. Also, they could induce the morphological change of cells, chromatin condensation, and cell shrinkage.

After the formula structure of compounds 1 and 2 was analyzed by spectroscopy as mentioned in 3.8. Compound 1 showed the characteristic signals of m-disubstituted benzene [δ_H 7.05 (1H, H-5), 6.67 (1H, H-6), 6.58 (1H, H-2), 6.57 (1H, H-4)] and showed the characteristic resonances of the hydroxyl group from the chemical shift of carbon at δ_C 155.4 ppm. In addition, resonances at δ_H 5.28 (2H, m) was suggested the presence of olefinic proton. Z-geometry of two olefinic protons, which were located at alkyl side chain, was assigned from the chemical shift of allylic

carbons (δ_C 27.2 and 26.9). The presence of alkyl group (R-) indicated the signal of methylenes ($-CH_2-$) in the range of 1.2-2.5 ppm in addition to termal methyl [0.82 (3H, t, J = 6.8 Hz)]. The chain length could not be exactly determined due to the lack of calculated molecular mass.

The molecular formular of compound 2 was revealed to be $C_{31}H_{54}O_2$ by ESIMS [m/z $(M+H)^+$]. The characteristic signals of m-trisubstituted benzene was revealed [δ_H 6.17 (2H, H-4, and H-6), 6.10 (1H, H-2)] and demonstrated the characteristic resonances of the hydroxyl group from the chemical shift of carbon at δ_C 156.5 ppm due to the symmetry. There has only one chemical shift value. In addition, the resonances at δ_H 5.28 (2H, m) were suggested the presence of olefinic proton. Z-geometry of two olefinic protons, which were located at the alkyl side chain, was assigned from the chemical shift of allylic carbons (δ_C 27.2 and 26.9). The presence of alkyl group (R-) was indicated the signal of methylenes ($-CH_2-$) in the range of 1.1-2.6 ppm in addition to thermal methyl [0.82 (3H, t, J = 6.8 Hz)].

From the result of NMR and MS, compound 1 was analysed to be a chemical compound in cardanol group. It was previously reported that cardanol group could inhibit the growth of bacteria (Begum *et al.*, 2002). Compound 2 was a chemical compound in cardol group. Also, it was previously reported that cardol group had the anticancer activity (Kubo *et al.*, 2011) and showed the lysis of zoospores of phytopathogenic *Aphanomyces cochliodes* (Begum *et al.*, 2002). Moreover, both compounds were reported to perform the allergenic effect (Aguilar-Ortigoza *et al.*, 2003). It could be supportive that the characteristic of chemical structure of phenolic

compounds causing the allergenic activity depended on the number and the position of hydroxyl group and double bonds on the hydrocarbon side chain (Aguilar-Ortigoza *et al*., 2003).

Considering the chemical structure of these active compounds, they were phenolic lipid so they contained the amphiphilic character (Przeworska *et al*., 2001). That meant one molecule contained both hydrophilic (hydroxyl group) and hydrophobic (long chain hydrocarbon) parts (Stasiuk and Kozubek, 2010). These compounds were worldwide found in tropical plants of family Anacardiaceae, both in the native and cultivated culture (Trevisan *et al*., 2006). Economic plants in this family were cashew nut, mango, and ginkgo (Gellerman *et al*., 1976).

Thus, both compounds from our research were phenolic lipid which had high diversity of compounds such as anacardic acid, catechol, resorcinol, and gingkolic acid (Stasiuk and Kozubek, 2010). They were previously reported to perform many bioactivitiees such as antibacterial (Muroi and Kubo, 1993; Kubo *et al*., 1999), antiplasmodial (Lee *et al*., 2009), antioxidant (Lodovici *et al*., 2001; Kubo *et al*., 2006; Trevisan *et al*., 2006), and antifungal activities (Aguilar-Ortigoza *et al*., 2003).

From the data mentioned above, it could be highly possible that two new compounds from Thai propolis could be an alternative source for an antiproliferative agent. In the future, it may be possible to be developed to be an anti-cancer drug.

CHAPTER VI

CONCLUSIONS

1. At the beginning, propolis of *Apis mellifera* from Nan province, Thailand was extracted by EtOH and water. After different cancer cell lines were treated by CEE and CWE, it was found that, by MTT assay, CEE had the better antiproliferative activity against those cancer cell lines. The IC_{50} values of different cancer cells were in the range of 320-520 µg/ml.

2. After CEE was partitioned by hexane, CH_2Cl_2, and MeOH, three crude extracts of CHE, CDE, and CME were obtained. Both CHE and CDE had the good antiproliferative activity against the selected cancer cell lines.

3. Since the obtained amount of CDE was not enough for being purified by quick column chromatography so CHE only was purified by this chromatography. Five fractions were obtained. By MTT assay, fraction III and V had the antiproliferative activity.

4. Adsorption chromatography was selected to purify fraction III and V. The purified fraction III (compound 1) and fraction V (compound 2) were again tested against different cancer cells. It was found that compound 2 (in the

range of 3-6 µg/ml) had the better antiproliferative activity than compound 1 (in the range of 10-22 µg/ml).

5. After compound 1 and 2 (0, 3.125, 6.25, 12.5, 25.0, and 50 µg/ml) were tested against SW620 cancer cells and Hs27 cells for 96 h, the cell morphology was observed. The change in morphology of SW620 cancer cells could be visible and the cell number was decreased, unlike Hs27 cells.

6. The DNA of SW620 cancer cells and Hs7 cells treated with compound 1 and 2 was extracted. The result no DNA fragmentation in both samples.

7. Due to the result of NMR and MS spectroscopy, compound 1 showed the characteristic signals of m-disubstitution-benzene and a hydroxyl group. In addition, it showed the signal of hydrocarbon side chain containing a position of double bonds in *cis* form. Therefore, compound 1 was in the cardanol group. Compound 2 showed the characteristic signal of m-trisubstitution-benzene and two hydroxyl groups. In addition, it showed the signal of hydrocarbon side chain containing a position of double bonds in *cis* form. Thus, compound 2 was in the cardol group.

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APPENDICES

APPENDIX A

The absorbance values at 540 nm of different cancer cell after being tested by CEE and CWE

Cancer cell lines	CEE (mg/ml)						CWE (mg/ml)					
	Control	0.125	0.25	0.50	1.0	2.0	Control	0.125	0.25	0.50	1.0	2.0
SW620	0.77	1.30	0.14	0.13	0.14	0.14	0.77	0.86	0.87	1.03	0.82	1.07
	0.41	1.15	0.60	0.13	0.13	0.14	0.41	0.95	0.98	1.07	0.98	1.19
	0.68	1.26	0.28	0.12	0.13	0.13	0.68	1.02	1.00	1.07	0.72	0.88
Average	0.62	1.24	0.34	0.13	0.13	0.14	0.62	0.95	0.95	1.05	0.84	1.05
Percentage of survival	100.00	199.25	54.32	20.61	21.31	21.95	100.00	152.17	152.82	169.83	135.27	168.76
BT474	0.23	0.38	0.37	0.17	0.13	0.15	0.23	0.39	0.30	0.35	0.37	0.36
	0.36	0.40	0.34	0.17	0.13	0.14	0.36	0.43	0.45	0.38	0.40	0.41
	0.35	0.41	0.28	0.14	0.13	0.14	0.35	0.49	0.38	0.50	0.30	0.38
Average	0.31	0.40	0.33	0.16	0.13	0.14	0.31	0.44	0.37	0.41	0.35	0.38
Percentage of survival	100.00	127.38	106.52	52.30	40.53	45.88	100.00	139.57	119.79	130.91	113.58	121.93
Chago	1.09	0.39	0.30	0.35	0.37	0.36	1.09	1.63	1.45	1.55	1.26	1.48
	0.97	0.43	0.45	0.38	0.40	0.41	0.97	1.62	1.49	1.62	1.50	1.44
	1.32	0.49	0.38	0.50	0.30	0.38	1.32	1.63	1.46	1.41	1.27	1.38
Average	1.13	0.44	0.37	0.41	0.35	0.38	1.13	1.62	1.47	1.53	1.34	1.43
Percentage of survival	100.00	139.57	119.79	130.91	113.58	121.93	100.00	143.99	129.96	135.39	118.91	126.85
KATO-III	0.71	0.44	0.69	0.14	0.13	0.18	0.71	0.54	0.58	0.84	0.72	0.48
	0.44	0.73	0.56	0.14	0.13	0.15	0.44	0.68	0.71	0.79	0.59	0.61
	0.53	0.64	0.36	0.13	0.11	0.13	0.53	0.61	0.60	0.66	0.69	0.59
Average	0.56	0.61	0.54	0.13	0.12	0.15	0.56	0.61	0.63	0.76	0.67	0.56
Percentage of survival	100.00	108.15	95.54	24.05	22.08	27.26	100.00	109.11	112.20	136.07	119.23	99.35
Hep-G2	0.36	0.54	0.64	0.11	0.11	0.13	0.36	0.41	0.36	0.38	0.45	0.47
	0.35	0.70	0.74	0.10	0.12	0.14	0.35	0.41	0.42	0.45	0.54	0.48
	0.46	0.72	0.50	0.11	0.12	0.14	0.46	0.51	0.46	0.43	0.46	0.44
Average	0.39	0.65	0.63	0.10	0.11	0.14	0.39	0.44	0.41	0.42	0.48	0.46
Percentage of survival	100.00	169.03	162.12	26.57	29.68	35.46	100.00	113.81	106.73	108.02	125.02	120.02

APPENDIX B

The absorbance values at 540 nm of different cancer cells after being tested by CHE, CDE, and CME (continued)

	SW620						KATO-III					
	control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	10^3 µg/ml	control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	10^3 µg/ml
CHE	1.63	1.49	1.64	1.59	0.15	0.71	1.16	1.23	1.47	1.04	0.13	0.94
	1.64	1.59	1.60	1.55	0.14	0.74	1.17	1.16	1.28	0.82	0.16	0.87
	1.64	1.60	1.58	1.57	0.12	0.77	1.15	1.18	1.18	0.72	0.16	0.81
Average	1.64	1.56	1.61	1.57	0.14	0.74	1.16	1.19	1.31	0.86	0.15	0.87
Percentage of survival	100.00	95.31	98.25	95.98	8.44	45.29	100.00	102.39	112.79	74.14	12.93	75.37
CDE	1.63	1.62	1.61	1.55	0.17	0.54	1.16	1.23	1.25	0.75	0.17	0.55
	1.64	1.58	1.56	1.48	0.15	0.50	1.17	1.21	0.95	0.98	0.17	0.57
	1.64	1.57	1.62	1.58	0.17	0.47	1.15	1.22	1.38	0.81	0.17	0.29
Average	1.64	1.59	1.60	1.54	0.16	0.51	1.16	1.22	1.20	0.84	0.17	0.47
Percentage of survival	100.00	96.98	97.53	93.99	9.93	30.96	100.00	105.09	103.02	72.76	14.60	40.32
CME	1.63	1.67	1.64	1.65	1.64	0.28	1.16	1.35	1.39	1.25	1.10	0.27
	1.64	1.63	1.62	1.62	1.63	0.31	1.17	1.37	1.19	0.81	0.96	0.20
	1.64	1.62	1.58	1.60	1.63	0.24	1.15	1.30	1.49	1.09	1.01	0.19
Average	1.64	1.64	1.61	1.62	1.63	0.28	1.16	1.34	1.36	1.05	1.02	0.22
Percentage of survival	100.00	100.12	98.70	99.21	99.86	16.90	100.00	115.32	117.01	90.60	88.28	18.85

(continued)

APPENDIX B

The absorbance values at 540 nm of different cancer cells after being tested by CHE, CDE, and CME (continued)

	SW620						KATO-III					
	control	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml	10^3 μg/ml	control	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml	10^3 μg/ml
CHE	1.63	1.49	1.64	1.59	0.15	0.71	1.16	1.23	1.47	1.04	0.13	0.94
	1.64	1.59	1.60	1.55	0.14	0.74	1.17	1.16	1.28	0.82	0.16	0.87
	1.64	1.60	1.58	1.57	0.12	0.77	1.15	1.18	1.18	0.72	0.16	0.81
Average	1.64	1.56	1.61	1.57	0.14	0.74	1.16	1.19	1.31	0.86	0.15	0.87
Percentage of survival	100.00	95.31	98.25	95.98	8.44	45.29	100.00	102.39	112.79	74.14	12.93	75.37
CDE	1.63	1.62	1.61	1.55	0.17	0.54	1.16	1.23	1.25	0.75	0.17	0.55
	1.64	1.58	1.56	1.48	0.15	0.50	1.17	1.21	0.95	0.98	0.17	0.57
	1.64	1.57	1.62	1.58	0.17	0.47	1.15	1.22	1.38	0.81	0.17	0.29
Average	1.64	1.59	1.60	1.54	0.16	0.51	1.16	1.22	1.20	0.84	0.17	0.47
Percentage of survival	100.00	96.98	97.53	93.99	9.93	30.96	100.00	105.09	103.02	72.76	14.60	40.32
CME	1.63	1.67	1.64	1.65	1.64	0.28	1.16	1.35	1.39	1.25	1.10	0.27
	1.64	1.63	1.62	1.62	1.63	0.31	1.17	1.37	1.19	0.81	0.96	0.20
	1.64	1.62	1.58	1.60	1.63	0.24	1.15	1.30	1.49	1.09	1.01	0.19
Average	1.64	1.64	1.61	1.62	1.63	0.28	1.16	1.34	1.36	1.05	1.02	0.22
Percentage of survival	100.00	100.12	98.70	99.21	99.86	16.90	100.00	115.32	117.01	90.60	88.28	18.85

(continued)

APPENDIX B

The absorbance values at 540 nm of different cancer cells after being tested by CHE, CDE, and CME (continued)

	Chago					
	control	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
CHE	1.70	1.66	1.64	1.65	0.15	0.70
	1.66	1.69	1.64	1.54	0.14	0.74
	1.70	1.67	1.64	1.42	0.15	0.89
Average	1.69	1.67	1.64	1.54	0.15	0.78
Percentage of survival	100.00	99.15	97.53	91.16	8.68	46.07
CDE	1.70	1.69	1.66	1.52	0.15	0.45
	1.66	1.63	1.66	1.49	0.16	0.49
	1.70	1.64	1.62	1.52	0.16	0.53
Average	1.69	1.65	1.65	1.51	0.16	0.49
Percentage of survival	100.00	98.18	97.65	89.67	9.34	29.04
CME	1.70	1.72	1.67	1.69	1.68	0.36
	1.66	1.67	1.66	1.65	1.63	0.31
	1.70	1.66	1.67	1.67	1.68	0.21
Average	1.69	1.68	1.67	1.67	1.66	0.30
Percentage of survival	100.00	99.84	98.85	98.97	98.62	17.51

APPENDIX C

The absorbance values at 540 nm of different cancer cells after being tested by fraction I

Fraction 1	Control	1.5 µg/ml	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
BT474	1.09	0.89	0.97	1.02	1.12	1.07	1.04	1.04	0.98
	1.13	1.02	1.08	1.12	1.13	1.08	1.08	1.03	0.96
	1.12	0.97	0.94	1.02	1.06	1.08	1.18	1.11	0.99
Average	1.11	0.96	1.00	1.05	1.10	1.08	1.10	1.06	0.98
Percentage of survival	100.00	86.15	89.41	94.46	98.80	96.65	98.74	95.03	87.58
SE	0.00	2.50	3.62	2.57	2.52	0.66	3.10	2.27	1.42
Chago	1.64	1.63	1.65	1.62	1.63	1.66	1.61	1.61	1.51
	1.65	1.65	1.65	1.67	1.60	1.63	1.52	1.54	1.60
	1.64	1.64	1.63	1.66	1.61	1.58	1.59	1.56	1.59
Average	1.65	1.64	1.64	1.65	1.61	1.63	1.58	1.57	1.56
Percentage of survival	100.00	99.53	99.64	99.96	98.02	98.70	95.71	95.38	95.04
SE	0.00	0.16	0.37	0.71	0.84	1.51	1.86	1.30	1.48
KATO-III	1.12	1.01	1.48	1.59	1.06	1.02	1.00	1.26	1.06
	1.24	1.15	1.39	1.27	1.07	0.88	0.91	1.04	1.09
	1.28	1.42	1.39	1.23	1.26	0.94	0.97	1.32	0.76
Average	1.21	1.19	1.42	1.36	1.13	0.95	0.96	1.21	0.97
Percentage of survival	100.00	98.27	117.08	112.38	92.89	77.87	78.97	99.42	79.90
SE	0.00	6.60	7.37	14.47	3.73	6.58	5.18	8.58	10.87
SW620	1.64	1.62	1.62	1.60	1.61	1.50	1.51	1.42	1.50
	1.60	1.60	1.52	1.53	1.60	1.46	1.57	1.55	1.51
	1.60	1.61	1.60	1.63	1.60	1.59	1.53	1.53	1.54
Average	1.61	1.61	1.58	1.59	1.60	1.51	1.54	1.50	1.52
Percentage of survival	100.00	100.14	98.20	98.47	99.36	94.06	95.51	92.90	94.18
SE	0.00	0.56	1.60	1.87	0.63	2.71	1.77	3.25	1.34
Hep-G_2	0.86	0.82	0.80	0.91	0.60	0.69	0.70	0.85	0.74
	0.73	0.64	0.77	0.91	0.74	0.77	0.80	0.72	0.75
	0.81	0.71	0.79	0.73	0.90	0.74	0.84	0.78	0.70
Average	0.80	0.73	0.79	0.85	0.75	0.74	0.78	0.78	0.73
Percentage of survival	100.00	91.17	98.83	106.95	93.81	92.34	97.53	98.37	91.67
SE	0.00	2.58	3.61	10.14	12.54	7.22	8.63	0.93	5.65

The absorbance values at 540 nm of different cancer cells after being tested by fraction II

Fraction 2	Control	1.5 µg/ml	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
BT474	1.08	1.10	1.07	1.02	1.07	0.99	0.94	1.08	0.91
	1.13	0.96	1.13	1.17	1.11	0.98	0.96	1.04	0.94
	1.21	1.41	1.29	1.27	1.11	0.81	0.98	0.98	0.80
Average	1.14	1.16	1.16	1.15	1.10	0.93	0.96	1.03	0.88
Percentage of survival	100.00	101.78	102.05	101.26	96.31	81.37	84.32	90.67	77.45
SE	0.00	9.14	2.48	3.25	2.42	7.48	1.65	5.29	5.75
Chago	1.67	1.64	1.63	1.63	1.66	1.65	1.66	1.63	1.62
	1.67	1.63	1.63	1.61	1.64	1.63	1.64	1.63	1.61
	1.65	1.63	1.61	1.60	1.60	1.62	1.63	1.59	1.51
Average	1.66	1.63	1.62	1.61	1.63	1.63	1.64	1.62	1.58
Percentage of survival	100.00	98.39	97.69	97.01	98.15	98.31	98.96	97.37	95.18
SE	0.00	0.38	0.24	0.26	0.57	0.36	0.32	0.33	1.63
KATO-III	1.42	1.42	1.40	1.50	1.34	1.30	1.47	1.15	0.33
	1.46	1.20	1.38	1.40	1.52	1.19	1.43	1.17	0.98
	1.49	1.27	1.43	1.26	1.02	1.26	1.27	0.97	0.80
Average	1.46	1.30	1.40	1.38	1.29	1.25	1.39	1.10	0.89
Percentage of survival	100.00	88.97	96.13	94.99	88.85	85.60	95.42	75.30	60.94
SE	0.00	5.64	1.28	6.16	10.60	3.12	5.55	5.24	12.89
SW620	1.62	1.46	1.52	1.58	1.52	1.51	1.54	1.49	0.92
	1.62	1.56	1.60	1.54	1.58	1.57	1.56	1.18	1.30
	1.62	1.58	1.55	1.60	1.59	1.53	1.51	1.13	0.91
Average	1.62	1.53	1.56	1.57	1.56	1.54	1.53	1.27	1.04
Percentage of survival	100.00	94.55	96.17	96.98	96.46	94.73	94.71	78.07	64.35
SE	0.00	2.25	1.46	1.12	1.20	0.95	0.99	7.01	8.01
Hep-G_2	0.81	0.72	0.70	0.90	0.84	0.66	0.72	0.82	1.00
	0.99	0.83	0.86	0.81	0.92	0.92	0.73	0.62	0.73
	0.78	0.63	0.91	0.83	0.67	0.87	0.79	0.63	0.51
Average	0.86	0.73	0.82	0.85	0.81	0.82	0.75	0.69	0.75
Percentage of survival	100.00	84.61	95.81	98.68	94.18	95.00	86.89	79.99	86.82
SE	0.00	2.31	10.55	9.26	5.10	9.18	8.17	11.13	18.36

The absorbance values at 540 nm of different cancer cells after being tested by fraction III

Fraction 3	Control	1.5 µg/ml	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
BT474	1.08	1.32	1.21	1.19	0.99	0.85	0.52	0.51	0.64
	1.13	1.13	1.25	1.04	1.14	1.04	0.66	0.64	0.91
	1.21	1.14	1.20	1.12	1.10	1.03	0.74	0.62	0.83
Average	1.14	1.20	1.22	1.12	1.08	0.97	0.64	0.59	0.79
Percentage of survival	100.00	104.94	107.20	98.07	94.38	85.52	56.10	51.80	69.46
SE	0.00	8.40	3.92	5.95	3.23	3.84	4.18	2.87	6.34
Chago	1.67	1.66	1.68	1.67	1.68	1.55	1.27	1.45	1.57
	1.67	1.65	1.63	1.68	1.65	1.63	1.50	1.60	1.39
	1.65	1.64	1.65	1.66	1.64	1.65	1.53	1.52	1.51
Average	1.66	1.65	1.65	1.67	1.66	1.61	1.43	1.52	1.49
Percentage of survival	100.00	99.44	99.56	100.56	99.70	97.05	86.33	91.69	89.56
SE	0.00	0.10	0.73	0.35	0.42	2.12	5.12	2.69	3.26
KATO-III	1.42	1.54	1.41	1.57	0.57	0.18	0.21	0.37	0.69
	1.46	1.54	1.14	1.53	0.85	0.20	0.26	0.39	0.49
	1.49	1.53	1.53	1.59	0.44	0.40	0.23	0.46	0.71
Average	1.46	1.53	1.36	1.57	0.62	0.26	0.23	0.40	0.63
Percentage of survival	100.00	105.38	93.41	107.46	42.57	17.81	15.95	27.58	42.98
SE	0.00	1.74	7.83	1.97	8.32	4.46	0.79	1.48	4.82
SW620	1.62	1.63	1.64	1.62	1.14	0.75	0.24	0.53	0.63
	1.62	1.65	1.62	1.65	1.61	0.30	0.26	0.43	0.65
	1.62	1.65	1.63	1.64	1.19	0.24	0.35	0.59	0.68
Average	1.62	1.64	1.63	1.63	1.31	0.27	0.28	0.52	0.65
Percentage of survival	100.00	101.21	100.49	100.88	80.99	16.57	17.36	31.89	40.34
SE	0.00	0.33	0.35	0.58	9.19	10.04	2.09	2.79	0.86
Hep-G_2	0.81	0.95	0.81	1.12	0.73	0.14	0.20	0.43	1.14
	0.99	0.96	1.23	1.16	1.00	0.16	0.18	0.33	1.21
	0.78	0.91	0.95	0.97	0.66	0.15	0.20	0.48	1.16
Average	0.86	0.94	1.00	1.09	0.80	0.15	0.19	0.41	1.17
Percentage of survival	100.00	109.15	115.78	126.37	92.56	17.45	22.30	47.89	136.02
SE	0.00	6.99	7.78	6.34	4.54	0.97	2.35	8.71	8.02

The absorbance values at 540 nm of different cancer cells after being tested by fraction IV

Fraction 4	Control	1.5 µg/ml	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
BT474	0.95	0.76	0.90	0.94	0.86	0.84	1.01	0.93	0.65
	0.89	0.83	0.87	0.96	0.82	1.04	0.87	0.77	0.61
	0.93	1.19	1.11	0.89	1.19	0.89	0.85	0.79	0.81
Average	0.92	0.79	0.88	0.93	0.84	0.86	0.86	0.78	0.63
Percentage of survival	100.00	85.97	95.72	100.43	91.01	93.66	92.80	84.29	68.31
SE	0.00	8.40	3.92	5.95	3.23	3.84	4.18	2.87	6.34
Chago	1.64	1.66	1.63	1.63	1.64	1.62	1.55	1.60	1.42
	1.65	1.62	1.62	1.64	1.63	1.59	1.49	1.39	0.66
	1.65	1.62	1.63	1.60	1.61	1.58	0.78	1.20	0.34
Average	1.65	1.63	1.63	1.62	1.62	1.60	1.27	1.40	0.81
Percentage of survival	100.00	99.15	98.66	98.58	98.60	96.86	77.38	84.74	49.13
SE	0.00	1.03	0.26	0.66	0.57	0.69	15.07	7.15	19.55
KATO-III	1.15	1.16	1.31	1.41	1.20	1.39	0.47	0.32	0.42
	1.35	1.22	1.18	1.35	1.21	1.00	0.35	0.35	0.29
	1.34	1.20	1.22	1.24	0.82	0.90	0.46	0.27	0.40
Average	1.28	1.19	1.23	1.34	1.08	1.10	0.43	0.31	0.37
Percentage of survival	100.00	93.45	96.61	104.51	84.30	85.68	33.26	24.36	28.64
SE	0.00	3.75	8.24	9.10	12.54	16.87	4.30	2.42	4.38
SW620	1.57	1.65	1.56	1.55	1.58	1.58	0.50	0.42	0.13
	1.55	1.57	1.55	1.58	1.58	1.56	0.73	0.85	0.25
	1.60	1.60	1.54	1.55	1.53	1.55	0.50	0.90	0.24
Average	1.57	1.61	1.55	1.56	1.57	1.56	0.58	0.72	0.21
Percentage of survival	100.00	102.27	98.66	99.58	99.75	99.58	36.68	45.92	13.34
SE	0.00	1.54	1.03	1.44	1.88	1.18	5.04	9.69	2.41
Hep-G_2	0.70	0.50	0.61	0.84	0.79	1.01	0.47	0.33	0.31
	0.67	0.57	0.66	0.69	0.78	0.90	0.48	0.26	0.22
	0.63	0.68	0.53	0.73	0.94	0.91	0.57	0.20	0.28
Average	0.66	0.58	0.60	0.75	0.84	0.94	0.51	0.26	0.27
Percentage of survival	100.00	87.66	90.67	113.30	126.04	141.70	76.07	39.39	40.24
SE	0.00	10.63	4.11	5.46	12.04	3.31	7.04	4.75	4.07

The absorbance values at 540 nm of different cancer cells after being tested by fraction V

Fraction 5	Control	1.5 µg/ml	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
BT474	0.95	1.09	1.02	0.88	1.12	0.55	0.18	0.25	0.35
	0.89	0.91	0.98	1.17	1.11	0.41	0.19	0.21	0.35
	0.93	0.98	0.98	0.93	1.15	0.46	0.17	0.22	0.36
Average	0.92	0.99	0.99	0.99	1.13	0.48	0.18	0.23	0.35
Percentage of survival	100.00	107.62	107.58	107.55	121.92	51.50	19.43	24.74	38.32
SE	0.00	3.91	1.53	11.84	2.02	3.53	0.87	0.89	0.56
Chago	1.64	1.63	1.66	1.98	0.92	0.15	0.15	0.24	0.44
	1.65	1.64	1.64	1.63	0.75	0.16	0.14	0.23	0.42
	1.65	1.66	1.65	1.62	0.81	0.13	0.14	0.33	0.49
Average	1.65	1.64	1.65	1.75	0.83	0.14	0.14	0.26	0.45
Percentage of survival	100.00	99.82	100.08	105.99	50.10	8.74	8.54	16.03	27.11
SE	0.00	0.49	0.37	7.34	3.05	0.60	0.18	1.95	1.25
KATO-III	1.15	1.06	0.93	1.15	0.33	0.17	0.18	0.31	0.42
	1.35	1.31	0.86	1.35	1.01	0.16	0.20	0.33	0.42
	1.34	1.19	1.39	1.31	1.08	0.16	0.20	0.38	0.61
Average	1.28	1.19	1.06	1.27	0.81	0.16	0.19	0.34	0.48
Percentage of survival	100.00	92.83	82.81	99.43	63.07	12.52	15.15	26.60	37.77
SE	0.00	2.35	11.60	0.66	16.33	1.01	0.39	1.28	4.11
SW620	1.57	1.67	1.63	1.64	0.50	0.18	0.18	0.28	0.49
	1.55	1.65	1.61	1.60	0.26	0.16	0.17	0.23	0.45
	1.60	1.65	1.62	1.63	0.37	0.15	0.16	0.24	0.44
Average	1.57	1.65	1.62	1.63	0.38	0.16	0.17	0.25	0.46
Percentage of survival	100.00	105.40	103.33	103.55	24.07	10.34	10.83	15.93	29.31
SE	0.00	1.08	0.76	0.78	4.47	0.51	0.55	0.86	0.98
Hep-G_2	0.70	1.01	0.86	1.14	1.00	0.19	0.17	0.24	0.48
	0.67	0.91	0.97	1.14	0.68	0.17	0.15	0.23	0.46
	0.63	0.83	0.90	1.18	0.90	0.16	0.15	0.23	0.44
Average	0.66	0.92	0.91	1.15	0.86	0.17	0.16	0.23	0.46
Percentage of survival	100.00	137.78	136.98	173.81	129.10	26.09	23.43	35.02	69.34
SE	0.00	3.60	7.37	7.68	14.17	0.29	0.59	1.02	0.49

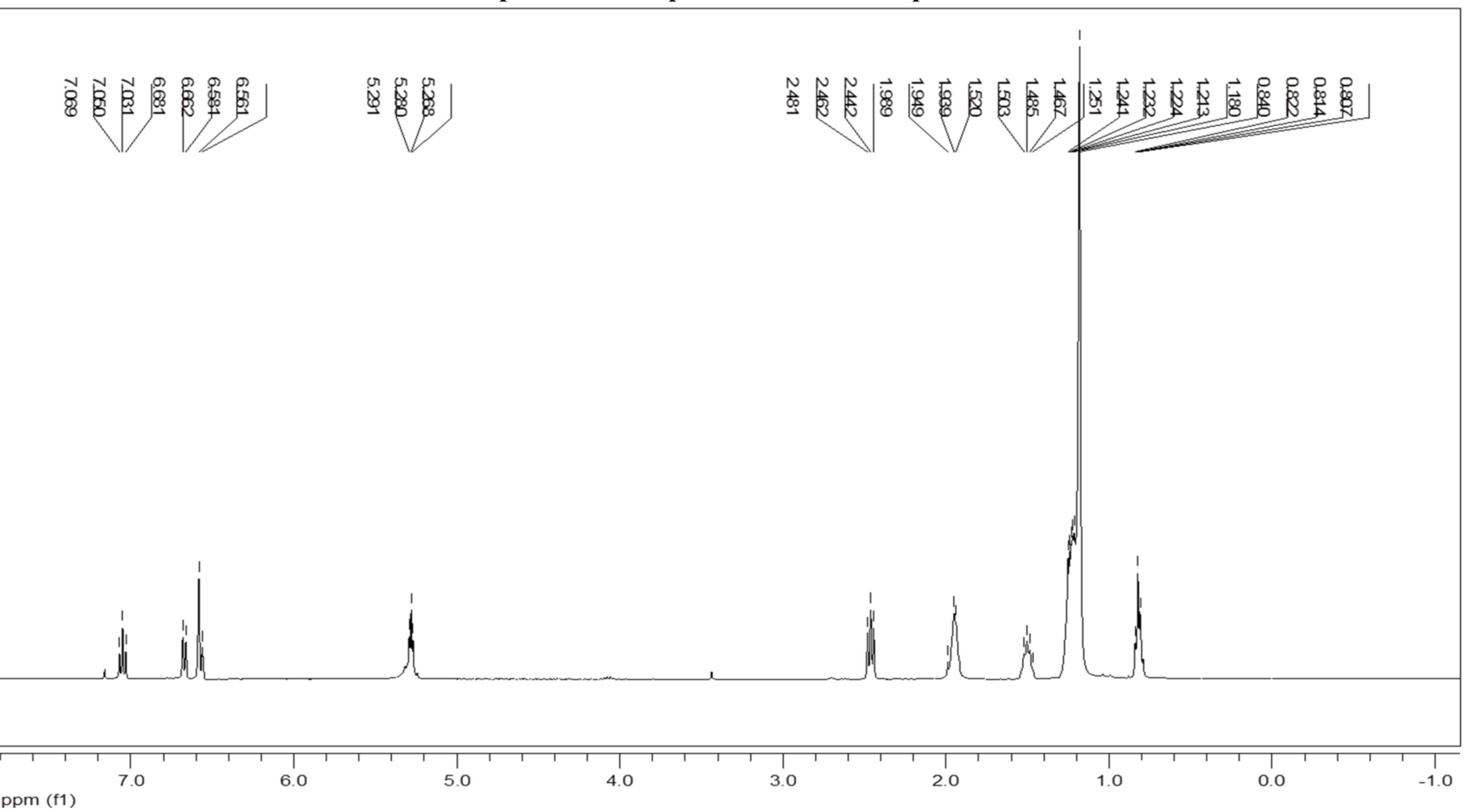
APPENDIX D

The absorbance values at 540 nm of different cancer cells after being tested by compound 1 and 2

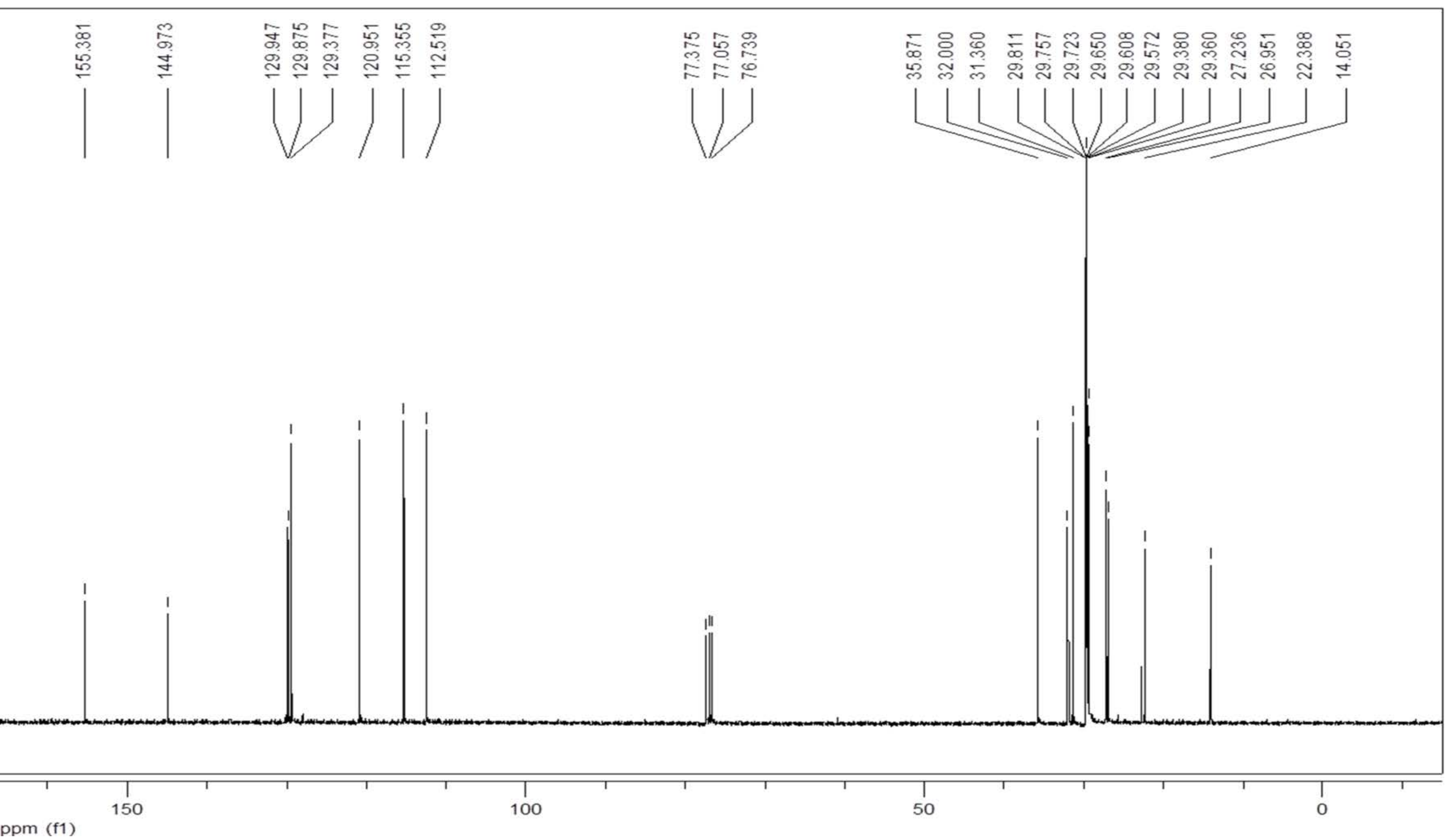
Cancer cells	Compound 1						Compound 2					
	Control	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	Control	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50µg/ml
SW620	3.45	2.71	2.51	1.05	0.12	0.14	3.45	1.04	0.11	0.13	0.14	0.23
	3.11	2.98	3.03	1.63	0.13	0.15	3.11	1.21	0.13	0.13	0.16	0.26
	3.10	2.79	3.11	0.84	0.13	0.13	3.10	1.32	0.12	0.12	0.15	0.23
Average	3.22	2.82	2.88	1.17	0.13	0.14	3.22	1.19	0.12	0.13	0.15	0.24
Percentage of survival	100.00	87.67	89.53	36.47	3.88	4.35	100.00	36.99	3.74	3.93	4.58	7.43
Chago	4.00	4.00	3.72	3.29	2.12	0.20	4.00	4.00	1.85	0.11	0.12	0.19
	4.00	4.00	4.00	3.49	2.68	0.33	4.00	4.00	1.81	0.13	0.14	0.22
	4.00	4.00	4.00	4.00	2.35	0.47	4.00	4.00	1.75	0.16	0.14	0.21
Average	4.00	4.00	3.91	3.59	2.38	0.33	4.00	4.00	1.80	0.13	0.14	0.20
Percentage of survival	100.00	100.00	97.64	89.81	59.60	8.29	100.00	100.00	45.05	3.31	3.38	5.10
Hep-G_2	0.28	0.32	0.33	0.24	0.11	0.12	0.28	0.31	0.14	0.11	0.12	0.15
	0.33	0.33	0.30	0.29	0.12	0.12	0.33	0.40	0.15	0.12	0.13	0.16
	0.31	0.35	0.25	0.28	0.11	0.11	0.31	0.36	0.12	0.11	0.11	0.13
Average	0.31	0.33	0.29	0.27	0.11	0.12	0.31	0.36	0.14	0.11	0.12	0.15
Percentage of survival	100.00	107.78	95.36	87.04	36.83	38.01	100.00	115.12	43.84	36.93	38.44	47.73
BT474	1.46	1.87	2.31	0.81	0.11	0.11	1.46	1.35	0.11	0.11	0.16	0.21
	1.88	1.78	2.21	0.96	0.11	0.14	1.88	1.41	0.15	0.13	0.16	0.12
	1.46	2.08	2.53	0.91	0.13	0.13	1.46	1.17	0.18	0.12	0.14	0.20
Average	1.60	1.91	2.35	0.89	0.11	0.13	1.60	1.31	0.15	0.12	0.16	0.17
Percentage of survival	100.00	119.30	146.83	55.84	7.17	7.86	100.00	82.10	9.09	7.38	9.69	10.82
KATO-III	1.98	1.31	1.46	1.16	0.14	0.13	1.98	1.40	0.19	0.11	0.16	0.29
	3.38	1.73	1.98	1.63	0.14	0.14	3.38	1.61	0.21	0.13	0.16	0.26
	2.44	2.07	2.30	1.66	0.14	0.13	2.44	1.63	0.22	0.12	0.16	0.26
Average	2.60	1.70	1.91	1.48	0.14	0.13	2.60	1.55	0.20	0.12	0.16	0.27
Percentage of survival	100.00	65.39	73.53	57.01	5.37	5.16	100.00	59.42	7.83	4.57	6.07	10.43
Hs27	0.29	0.33	0.34	0.24	0.11	0.12	0.29	0.31	0.14	0.11	0.12	0.15
	0.33	0.33	0.31	0.29	0.12	0.12	0.33	0.40	0.15	0.12	0.13	0.16
	0.31	0.35	0.25	0.27	0.10	0.11	0.31	0.35	0.12	0.11	0.11	0.13
Average	0.31	0.33	0.30	0.27	0.11	0.12	0.31	0.36	0.13	0.11	0.12	0.15
Percentage of survival	100.00	108.56	96.86	87.00	36.51	37.38	100.00	115.49	43.66	36.40	38.46	48.10

APPENDIX E

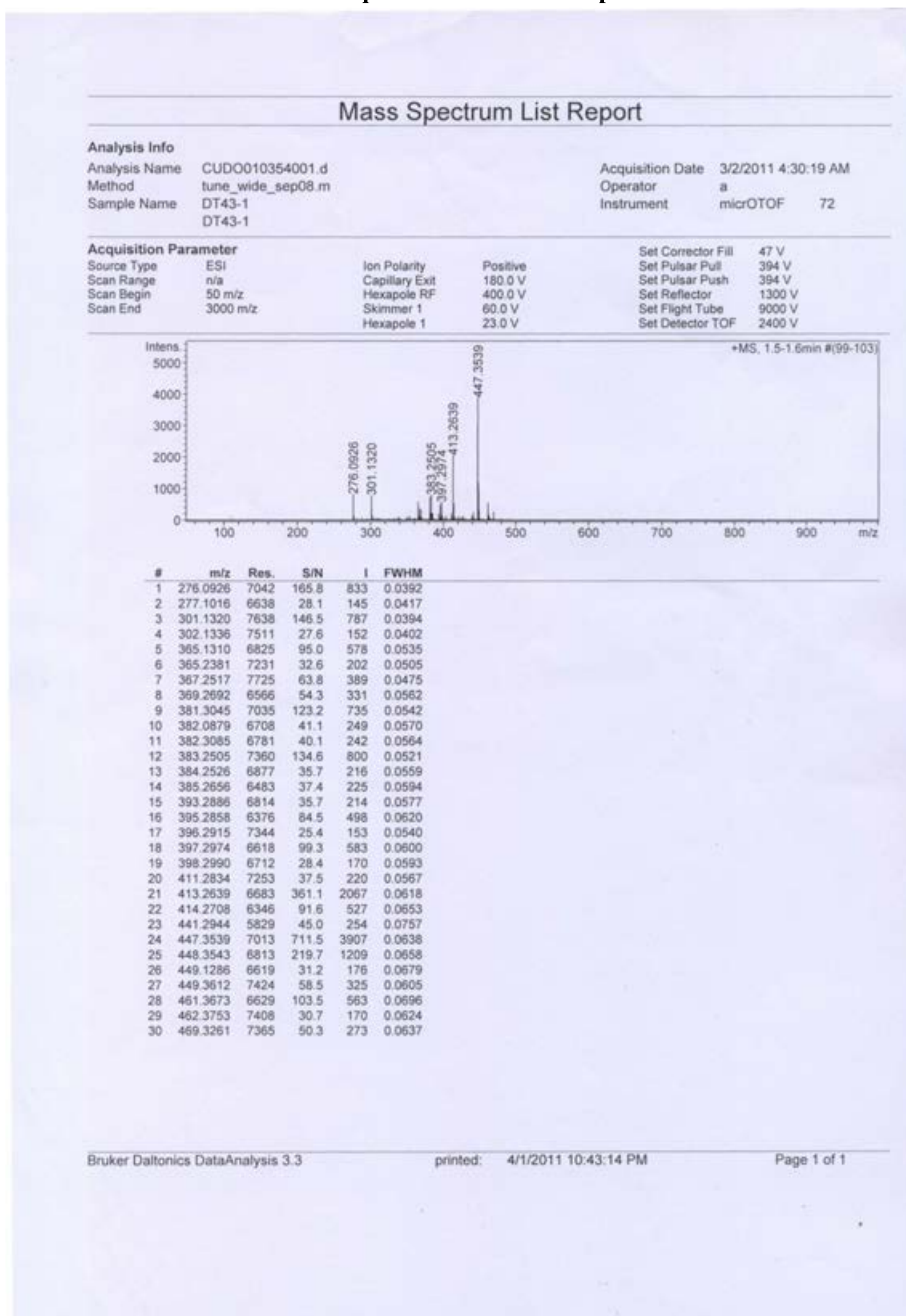
The proton NMR spectrum data of compound 1



The carbon NMR spectrum of compound 1



The mass spectrum data of compound 1



Mass Spectrum List Report

Analysis Info

Analysis Name CUDO010354001.d
Method tune_wide_sep08.m
Sample Name DT43-1
DT43-1

Acquisition Date 3/2/2011 4:30:19 AM
Operator a
Instrument micrOTOF 72

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	47 V
Scan Range	n/a	Capillary Exit	180.0 V	Set Pulsar Pull	394 V
Scan Begin	50 m/z	Hexapole RF	400.0 V	Set Pulsar Push	394 V
Scan End	3000 m/z	Skimmer 1	60.0 V	Set Reflector	1300 V
		Hexapole 1	23.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2400 V

#	m/z	Res.	S/N	I	FWHM
1	276.0926	7042	165.8	833	0.0392
2	277.1016	6638	28.1	145	0.0417
3	301.1320	7638	146.5	787	0.0394
4	302.1336	7511	27.6	152	0.0402
5	365.1310	6825	95.0	578	0.0535
6	365.2381	7231	32.6	202	0.0505
7	367.2517	7725	63.8	389	0.0475
8	369.2692	6566	54.3	331	0.0562
9	381.3045	7035	123.2	735	0.0542
10	382.0879	6708	41.1	249	0.0570
11	382.3085	6781	40.1	242	0.0564
12	383.2505	7360	134.6	800	0.0521
13	384.2526	6877	35.7	216	0.0559
14	385.2656	6483	37.4	225	0.0594
15	393.2886	6814	35.7	214	0.0577
16	395.2858	6376	84.5	498	0.0620
17	396.2915	7344	25.4	153	0.0540
18	397.2974	6618	99.3	583	0.0600
19	398.2990	6712	28.4	170	0.0593
20	411.2834	7253	37.5	220	0.0567
21	413.2639	6683	361.1	2067	0.0618
22	414.2708	6346	91.6	527	0.0653
23	441.2944	5829	45.0	254	0.0757
24	447.3539	7013	711.5	3907	0.0638
25	448.3543	6813	219.7	1209	0.0658
26	449.1286	6619	31.2	176	0.0679
27	449.3612	7424	58.5	325	0.0605
28	461.3673	6629	103.5	563	0.0696
29	462.3753	7408	30.7	170	0.0624
30	469.3261	7365	50.3	273	0.0637

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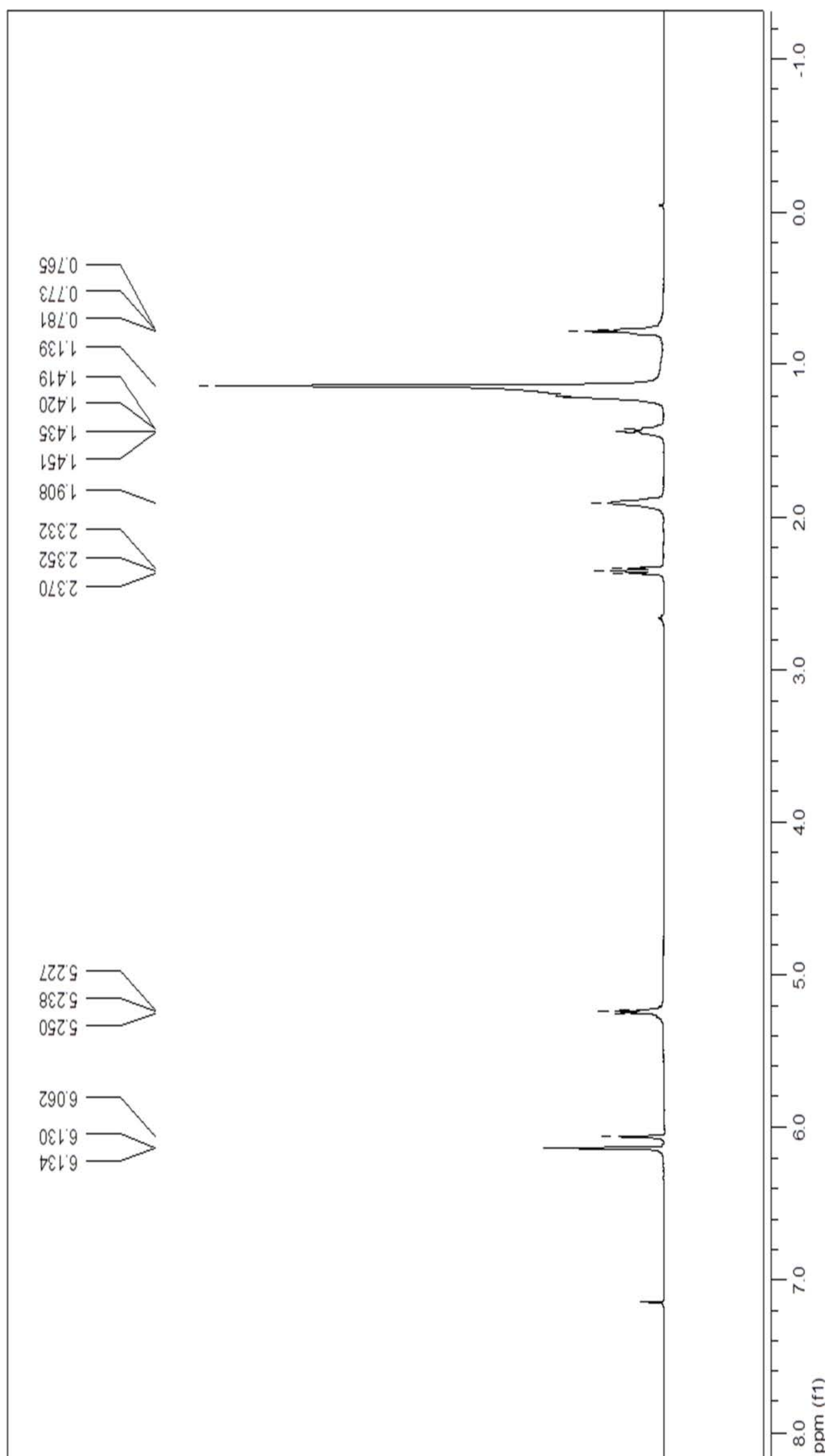
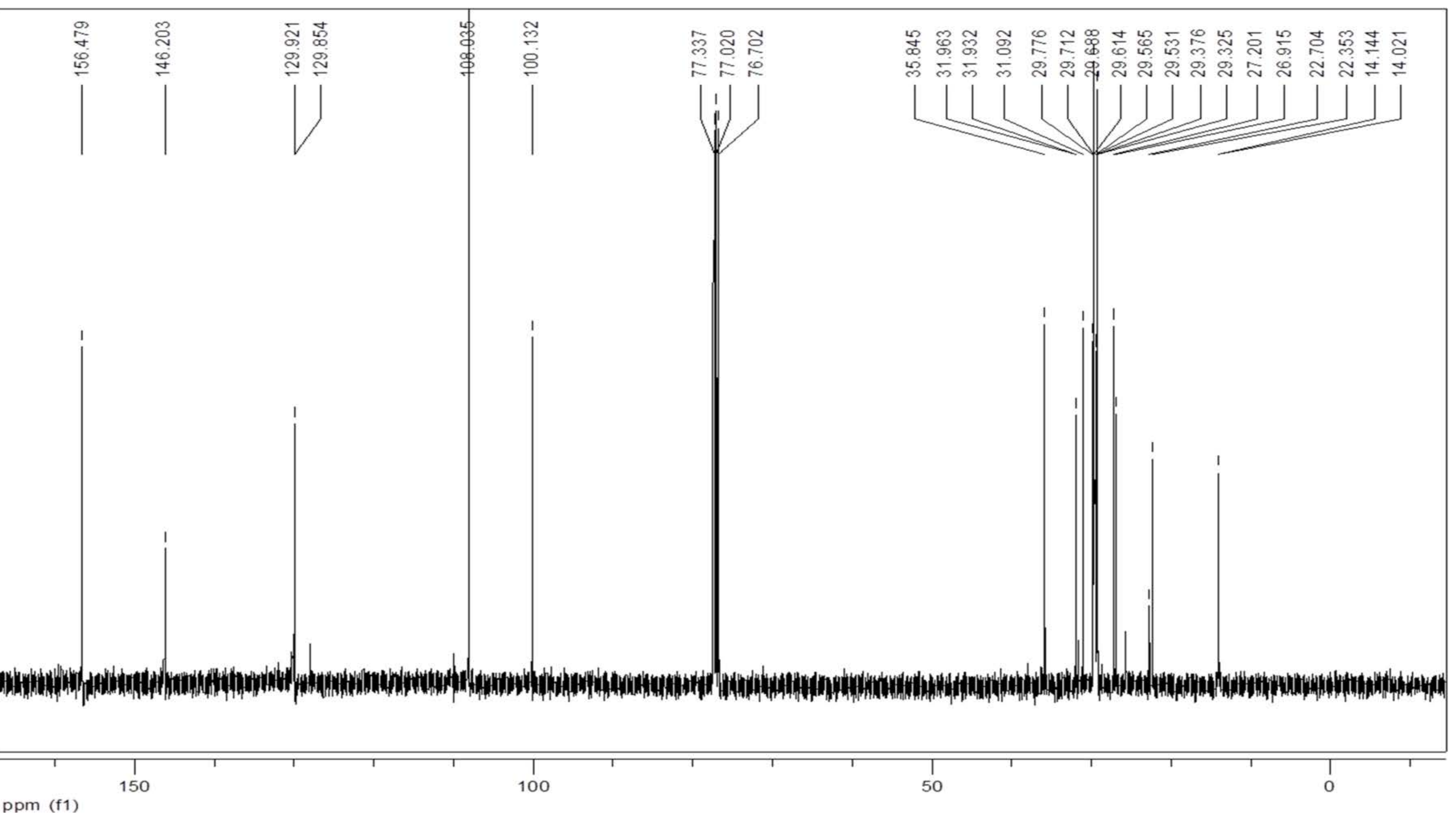
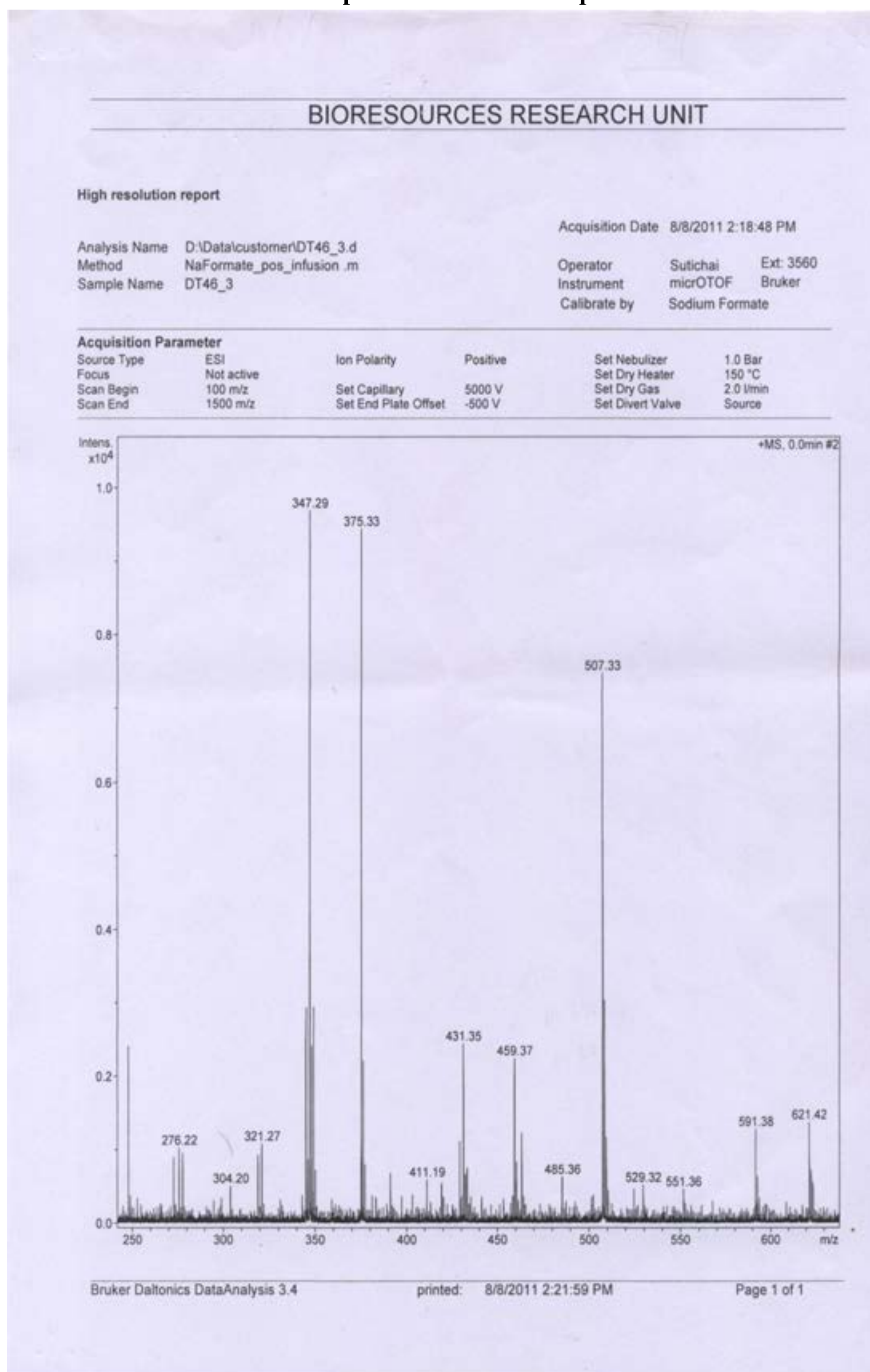
The proton NMR spectrum data of compound 2



The carbon NMR spectrum data of compound 2



The mass spectrum data of compound 2



APPENDIX F

20 mM of phosphate buffer, pH 7.0

At the beginning, 100 mM phosphate buffer is prepared.

Buffer I, dissolve 1.56 g of monobasic $NaH_2PO_4.2H_2O$ in distilled water until the final volume is 100 ml.

Buffer II, dissolve 1.42 g of dibasic Na_2HPO_4 in distilled water until the final volume is 100 ml.

For 100 ml of 100 mM phosphate buffer: 100 mM buffer I (39 ml) was mixed with 100 mM buffer II (61 ml).

For 100 ml of 20 mM phosphate buffer: 40 ml of 100 mM phosphate buffer is mixed with 160 ml distilled water.

Agarose gel electrophoresis

1) 1.8% (w/v) agarose gel

- agarose 0.72 g
- 1x TBE buffer 40 ml

2) 1x Tris Boric EDTA buffer (TBE buffer), pH 8.0

- Tris aminomethane (50 mM) 108 g

- EDTA (0.65 mM) 7.44 g

- Boric acid (50 mM) 50.4 g

Adjust pH to be 8.0 and quantitate the volume to be 1,000 ml.

Phosphate buffer saline (PBS)

1. NaCl	0.138 M
2. KCl	3 mM
3. $Na_2HPO_4.2H_2O$	8 mM
4. KH_2PO_4	2 mM

Adjust pH to be 7.4 and quantitate the volume to be 1,000 ml.

BIOGRAPHY

Miss Dungporn Teerasripreecha was born on November 17th, 1986 in Bangkok, Thailand. She finished her high school from Donmuang Taharnargardbumrung School, Bangkok, in 2004. After that, she got a Bachelor's Degree in Biology from Department of Biology, Faculty of Science, Chulalongkorn University in 2008. At present, she is a graduate candidate in Master's Degree in Zoology, Department of Biology, Faculty of Science, Chulalongkorn University.

Presentation:

1. **Teerasripreecha, D**., Phuwapraisirisan, P., and Chanchao, C. 2011. Antiproliferative activity of *Apis mellifera* propolis from Nan Province, Thailand. The 4th Suranaree University of Technology Graduate Conference, Nakhon Ratchaseema, Thailand. Number 1SC105, p. 455-466.
2. **Teerasripreecha, D**., Phuwapraisirisan, P., and Chanchao, C. 2010. Antiproliferative activity of *Apis mellifera* propolis from Nan province, Thailand. Abstract. The 15th Biological Sciences Graduate Congress, Kuala Lumpur, Malaysia. 108. (December 15-17, 2010)