ผลของคาร์ดานอลจากพรอพอลิสของผึ้งพันธุ์ *Apis mellifera* ต่อเซลล์มะเร็งเต้านม BT-474

นางสาวสุรีย์รัตน์ บัวหอม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

EFFECTS OF CARDANOL FROM *Apis mellifera* PROPOLIS ON BT-474 BREAST CANCER CELL LINE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2014

Thesis Title	EFFECTS OF CARDANOL FROM *Apis mellifera* PROPOLIS ON BT-474 BREAST CANCER CELL LINE
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สุรีย์รัตน์ บัวหอม : ผลของคาร์ดานอลจากพรอพอลิสของผึ้งพันธุ์ *Apis mellifera* ต่อเซลล์มะเร็งเต้านม BT-474 (EFFECTS OF CARDANOL FROM *Apis mellifera* PROPOLIS ON BT-474 BREAST CANCER CELL LINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.ปรีชา ภูวไพรศิริศาล, ดร.เกรียงศักดิ์ เลิศประภามงคล, 111 หน้า.

พรอพอลิส เป็นผลิตภัณฑ์อย่างหนึ่งจากผึ้ง มีลักษณะเป็นสารเหนียวคล้ายยางไม้ มีฤทธิ์ทางชีวภาพมากมาย ดังนั้นในงานวิจัยนี้ได้สกัดสารออกฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งเต้านม BT-474 ที่ชื่อว่า คาร์ดานอล จากผึ้งพันธุ์ (*Apis mellifera*) ที่จังหวัดน่าน ประเทศไทย โดยใช้การสกัดอย่างหยาบด้วยตัวทำละลายอินทรีย์ 3 ชนิด คือ เมทานอล ไดคลอโรมีเทน และเฮกเซน แล้วทำการสกัดบริสุทธิ์โดยใช้ Quick column chromatography และ Adsorption chromatography ยืนยันผลว่าเป็นคาร์ดานอลโดยใช้ Thin layer chromatography และ Mass spectrometry จากวิธี 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay และการคำนวณหาค่าความเข้มข้นที่ทำให้เซลล์ตาย 50% (IC_{50}) พบว่าคาร์ดานอลที่ได้มีฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งเต้านม BT-474จริงและมีค่า IC_{50} เท่ากับ 15.57 ± 1.73 ไมโครกรัม/มิลลิลิตร คาร์ดานอลส่งผลให้เกิดการเปลี่ยนแปลงทางรูปร่างของ BT474 กล่าวคือ เซลล์เกิดการหดตัว แล้วหลุดลอยขึ้นมาจากพื้นผิว และส่งผลให้เกิดการตายของเซลล์ จากการศึกษาหารูปแบบการตายของเซลล์โดยการย้อมสีด้วย Annexin V และ Propidium iodode (PI) พบว่าเกิดการตายของเซลล์ช่วง Late apoptosis เกิดทั้งการตายแบบ Apoptosis และการตายแบบ Necrosis ต่อมาได้ศึกษาระยะพักในวัฏจักรของเซลล์มะเร็งโดยการย้อมสี PI พบว่าระยะที่ถูกยับยั้งคือ ระยะย่อย G_1

นอกจากนี้ยังศึกษาการเปลี่ยนแปลงระดับการแสดงออกของยีนในกลุ่มที่เกี่ยวข้องกับการแบ่งเซลล์ทั้งในระดับของการถอดรหัสข้อมูลทางพันธุกรรมและการแปลรหัสข้อมูลทางพันธุกรรม ในส่วนแรกใช้วิธี Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR*)* ส่วนหลังใช้วิธี Western blot เพื่อบ่งบอกถึงกลไกการทำงานของคาร์ดานอลในการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง พบว่า คาร์ดานอลส่งผลให้เซลล์เกิดการหยุดของวัฏจักรการแบ่งเซลล์ในระยะย่อย G_1 ซึ่งสอดคล้องกับผลของการย้อมเซลล์ด้วย PI โดยเข้าไปเพิ่มระดับการแสดงออกของยีน *ERK, JNK* และ *p38* รวมทั้งกระตุ้นให้มีการแสดงออกของ *p21* เพิ่มสูงขึ้น แต่ไปยับยั้งการแสดงออกของ *Cyclin D1* และ *CDK4* ในระยะย่อย G_1 ของวัฏจักรเซลล์ และยั้งยั้งการแสดงออกของ *Cyclin E* และ *CDK2* ซึ่งเป็นยีนที่มีความสำคัญต่อวัฏจักรของเซลล์ในการเข้าสู่ระยะย่อย S ดังนั้นเซลล์จึงไม่สามารถเกิดการจำลองสารพันธุกรรมและการแบ่งเซลล์ ส่งผลให้เกิดการตายของเซลล์มะเร็งเต้านม BT-474

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5572161023 : MAJOR BIOTECHNOLOGY
KEYWORDS:

SUREERAT BUAHORM: EFFECTS OF CARDANOL FROM *Apis mellifera* PROPOLIS ON BT-474 BREAST CANCER CELL LINE. ADVISOR: . ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., KRIENGSAK LIRDPRAPAMONGKOL, Ph.D., 111 pp.

Propolis is one of products from honeybee. It is sticky resin like plant resin and has many bioactivities. Thus, in research, cardanol, an anti-proliferative compound against breast cancer BT-474 cells was isolated from *Apis mellifera* propolis in Nan province, Thailand. Crude propolis was extracted by three organic solvents which were methanol, dichloromethane, and hexane. Later, it was purified by quick column and adsorption chromatography. The obtained cardanol was proved by thin layer chromatography and mass spectrometry. Due to 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay and the calculated inhibition concentration at 50% (IC_{50}), the expected cardanol could be really active against BT-474 cells with the IC_{50} value of 15.57 ± 1.73 µg/ml. Cardanol led to the morphology change of treated BT-474 cells such as cell shrinkage, cell detachment from substratum which, later, caused the cell death. In order to investigate the program cell death, cells were stained with annexin V and propidium iodide (PI). It was found that cells were dead via late apoptosis, including both apoptosis and necrosis. Next, cells were stained by PI in order to determine cell cycle arrest. It was shown that cell cycle was arrested at G_1 subphase.

Moreover, the change in expression of genes involving in cell division was observed at both transcriptional and translational levels in order to know a mechanism on how cardanol could inhibit the proliferation. For the former, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used. For the latter, western blot was used. It was found that cardanol induced the arrest in cell cycle at G_1 subphase. The obtained data from qRT-PCR and western blot was coincided to the data of qRT-PCR in term that cardanol increased the expression level of *ERK*, *JNK*, and *p38.* Also, it up-regulated the *p21* expression. In contrast, it could decrease the expression of *cyclin D1* and *CDK4* at G_1 subphase. Also, it decreased the expression of *cyclin E* and *CDK2* which were important for the cell cycle to continue to the S subphase. Hence, within cells, no DNA replication and cell division occurred. That led to the death of BT-474 cells.

Field of Study: Biotechnology

Academic Year: 2014

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ACKNOWLEDGEMENTS

I would like to thank to my advisor, Assoc. Prof. Dr. Chanpen Chanchao, who has good advices and guidance to this thesis. Also, I would like to thank my co-advisors, Asst. Prof. Dr. Preecha Phuwapraisirisan and Dr. Kriengsak Lirdprapamongkol for their encouragements and helpful guidance throughout this research.

I would like to express my deep gratitude to Dr. Noppadon Kitana, Dr. Yupyn Chintapakorn, and Asst. Prof. Dr. Dumrongkiet Arthan for serving as my thesis committee.

Specially, I am thankful to Mrs. Songchan Puthong, a researcher at Institute of Biotechnology and Genetic Engineering, Chulalongkorn University for all of her help and kindness through cell culture.

In addition, I would like to thank all members at Laboratory of Biochemistry, Chulabhorn Research Institute and all members at Molecular Biology Research Lab in Department of Biology, Faculty of Science, Chulalongkorn University for all of their help and friendship.

I am truly appreciated to the financial supports from the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Ratchadapisek Sompoch Endowment Fund of Chulalongkorn University (RES560530041-FW).

Finally, I would like to thank my family members for their understanding and support without any condition.

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CHAPTER I
INTRODUCTION

Cancer is considered to be one of the most life-threatening diseases in the world. According to the international agency for research on cancer (IARC) of the world health organization (WHO), cancers affect millions of human and the number of the disease incidence also keeps increasing. Due to Globocan (2012), it was estimated that new cancer cases have been raised from 12.7 million in 2008 to 14.1 million in 2012. Furthermore, the number of cancer causing dead patients was increased from 7.6 million in 2008 to 8.2 million in 2012 (Ferlay et al., 2012)[12]. The significant increment of the cancer cases and deaths strongly suggests that cancers will be more and more serious and challenging issue for the world to cope with. The increment could be due to many factors like chemical, U.V., food, life style, etc. In high-income countries, the factors are smoking, overweight and obesity, and alcohol use, however in low-income countries, the infection of papilloma viruses was reported to be the main factor (Danaei et al., 2005)[15].

There are more than 200 types of cancers. Among them, the breast cancer is the most typical cause of cancer death in women. In 2012, there were about 522,000 dead women caused by breast cancer. Noteworthy, this number was increased about 14% from the number recorded in 2008. Breast cancer is often the cause of cancer lethality in developing countries because the shifting of lifestyle to what common in industrialized countries leading to be prone to reproductive, dietary, and hormonal risk factors. Together with the difficulty of reaching for the advanced medical treatment, the breast cancer rate in the developing countries is the highest (American Cancer Society, 2013).[51]

At present, there are many ways for cancer treatment such as surgery, chemotherapy, and radiotherapy. However, some treatment has a dissatisfying side effect such as nausea, dizziness, drug resistance. Thus, finding a new anti–cancer agent from natural product is still challenging.

Propolis is one of nature products from bees. Usually, it is sticky and dark brown. It was comprised of plant resin (50%), wax (30%), oil (10%), pollen (5%), and others (5%) (Burdock, 1998)[4]. Since bees harvested food from plants, many chemical compounds had the same chemical structure as those found in plants. However, many chemical compounds were modified by bee's enzymes secreting from their saliva glands. Bees used propolis to repair a hive. Furthermore, they used propolis to wrap dead bees or intruders in order that bacteria would not be spread within a hive. As known, bees are social insects. Within a hive, there were many thousands of bees living very close together. Thus, the pathogen infection could be spread very fast and easily. This fact could draw our interest to focus on antibacterial undoubtedly. Until now, there are many reports presenting bioactivities of propolis such as antitumor activity (Kimoto et al., 1998)[26], anti-microbial activity (Kujumgiev et al., 1999)[28], anti-inflammatory activity (Park and Kahng, 1999)[9].

Both crude and purified forms of propolis were reported to be useful in traditional medicine. For example, cardanol, a phenolic compound found in Anacardiaceae family, could be purified from *Apis mellifera* propolis in Nan province (Teerasripreecha et al., 2012)[54]. It was in the same group as anacardiacid, catechol, resorcinol, and ginkgolic acid. In addition, cardanol was reported to show many bioactivities such as antimicrobial activity (Gopalakrishnas et al., 2011)[16], antioxidant activity (Trevisan et al., 2005)[56], and anticancer activity (Kawati et al., 2011)[23]. According to Teerasripreecha et al. (2012)[54], purified cardanol could inhibit the proliferation of breast cancer BT-474 cell line without damaging normal fibroblast

cells. The IC_{50} value was revealed. Nonetheless, how cardanol could inhibit the proliferation of BT-474 was not reported yet. Thus, in this research, it was continued from what Teerasripreecha et al. (2012)[54] reported in order to reveal the mechanism on how cardanol worked on BT-474 cancer cell line.

The objectives of this work were as below.

1. To determine how cardanol could inhibit the proliferation of BT-474 cancer cell line by observing the morphology and growth curve

2. To investigate the program cell death and cell cycle arrest in cardanol-treated BT-474 cancer cell line

3. To reveal a mechanism on how cardanol inhibits the proliferation of the treated cancer cells by the change in expression of cancer relating genes at both transcriptional and translation levels

CHAPTER II
LITERATURE REVIEW

2.1 Biology of Apis mellifera

Honeybee (*A. mellifera*) is social insect. It is native to Europe, the Middle East, and Africa. It is widely spread. It has been known as an economically valuable pollinator for crop monoculture worldwide (McGregor, 1976)[35]. It produces many useful products which are honey, bee pollen, beeswax, royal jelly, and propolis. The taxonomy of *A. mellifera* is presented as below:

Taxonomy identification of *A. mellifera* (Wongsiri, 1989)[62]

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Family Apidae

Genus *Apis*

Species *Apis mellifera*

In the United States, *A. mellifera* had many subspecies like *A. mellifera ligustica* Spinola, *A. mellifera carnica* Pollmann, *A. mellifera mellifera* Linnaeus, *A. mellifera causcasia* Pollmann, and *A. mellifera iberiensis* Engel (Ellis et al., 2013).

Within a hive, there are 3 castes of honeybees which are a queen, workers, and drones. A developmental period from egg to adult is varied depending on a caste. For drones, the life cycle takes about 21 days. They are haploid male since

they are developed from unfertilized eggs. Their body size is larger than workers' body size. For workers, the life cycle also takes about 21 days. They are diploid female since they are developed from fertilized eggs. However, they are infertile. Although, their body size is the smallest among bees in these three castes, they are in charge of almost tasks in a hive such as hive construction, nursing, hive defensing, hive cleaning, guarding, food foraging. For a queen, its development takes about 15-16 days. Its body size is the largest. Comparing to workers, a queen is fertile. Also, it can release pheromones to control bees in the colony. Many queen pheromones can regulate colony homeostasis, growth, and reproduction (Winston, 1989)[61].

2.2 Propolis

Honeybees can produce many products which are honey, royal jelly, bee pollen, beeswax, bee venom, and propolis. Among these bee products, propolis has long been used in traditional medicine. It is derived from plant resin. Thus, a component of propolis is depended on geographic locations. Propolis is dark brown and sticky (Figure 2.1). Its composition was plant resin (50%), wax (30%), oil (10%), pollen (5%) and others (5%) (Burdock, 1998)[4]. To produce propolis, honeybees collected plant materials and mixed it with wax. Honeybees used propolis to seal a crack in a hive.

Figure 2. 1Propolis of *Apis mellifera*. A bee hive (Left) and propolis (Right)

Chemical compositions in propolis have been reported to have beneficial bioactivities as mentioned in 2.3.

2.3 Bioactivity of propolis

2.3.1 Antibacterial activity

In 1999, Kujumgiev et al.[28] reported the antibacterial activity of propolis which was collected from different geographic origins. They were extracted by 70% ethanol and tested with *Staphylococcus aureus* 209 and *Escherichia coli.* The antibacterial activity was determined by the diameter of inhibition zone. The result showed that all propolis extracts were active against *S. aureus* which was Gram-positive bacteria. However, no propolis extract was active against *E. coli* which was Gram-negative bacteria. In addition, in 2013, Popova et al.[45] reported that crude alcohol extracts of Omani propolis could inhibit the growth of both *S. aureus* and *E. coli*. Nonetheless, the minimum inhibition concentration (MIC) from all crude extracts could be more sensitive to *S. aureus* than *E. coli*.

In 2000, Koo et al. reported the antimicrobial activity of propolis. The propolis was tested on fifteen microorganisms which were as follow: *Candida albicans* - NTCC 3736, F32; *S. aureus* - ATCC 25923; *Enterococcus faecalis* - ATCC 29212; *Streptococcus sobrinus* 6715; *Streptococcus sanguis* - ATCC 10556; *Streptococcus cricetus* – HS - 6; *Streptococcus mutans* - Ingbritt 1600; *Streptococcus mutans* - OMZ 175; *Actinomyces naeslundii* - ATCC 12104, W1053; *A. viscosus* OMZ 105; *Porphyromonas gingivalis*; *Porphyromonas endodontalis*, and *Prevotella denticola*. By determining by an agar diffusion method, the propolis extract could inhibit all those mentioned microorganisms significantly ($p < 0.05$). The largest inhibitory zone was observed from *Actinomyces* spp. Moreover, cell adherence and water-insoluble glucan formation could be inhibited from propolis extracts at a final concentration of 400 and 500 μg/ml, respectively. Thus, it could be said that the propolis extracts showed in vitro antibacterial activity as well as inhibition of cell adherence and water-insoluble glucan formation.

In 2004, Yildirim et al.[64] reported the effect of water extract of propolis (WEP) from *A. mellifera* in Turkey. The data showed the inhibition against *Mycobacterium tuberculosis* ($H_{37}R_v$) in vivo. WEP (0.01 g) was injected to a guinea pig infected by *M. tuberculosis*. After 30 days, all tissue were embedded in paraffin for histopathological examination. It showed that *M. tuberculous* did not spread to lung tissue. Thus, it could be concluded that the WEP could prevent the spread of *M. tuberculosis* since it helped not to develop the tuberculous disease.

2.3.2 Antifungal activity

In 1999, Kujumgiev et al.[28] reported the antifungal activity of propolis against *Candida albicans* 562 by using an agar cup method. The antifungal activity was determined from the diameter measurement of inhibitory zone. The diameter at 14.3 ± 0.06 mm could be obtained from propolis collected from an area near Rio Claro City, Sao Paulo State, Brazil. In addition, Ota et al. (2001) [42]reported fungal activity of propolis by sensitivity test on 80 stains of *Candida* yeasts. Twenty strains belonged to *C. albicans*. Twenty strains belonged to *C. tropicalis*. Twenty strains belonged to *C. krusei* while 15 strains belonged to *C. guilliermondii*. The hydroalcoholic propolis extract showed anti-fungal activity to all used strains.

2.3.3 Anti-inflammatory activity

In 1999, Park and Kahng [9]reported the ethanolic extract of propolis (EEP) on chronic inflammation which was evaluated by using rat adjuvant arthritis. The arthritis index was suppressed by EEP treatment (50 mg/kg/day and 100 mg/kg/day, P.O.) Its analgesic effect using the tail-fick test was compared to prednisolone at 2.5 mg/kg/day, P.O. and acetyl salicylic acid at 100 mg/kg/day, P.O. The result showed inhibitory effect on the paw edema whereas EEP at 200 mg/kg/day, P.O. could show the inhibitory effect after carrageenan injection at 3 and 4 h. Thus, the ethanolic extract showed anti- inflammatory effect on both chronic and acute inflammation.

In 2005, Hu et al.[19] reported anti-inflammatory effect of EEP and water - soluble derivatives (WSD) in male animal models which were imprinting control region (ICR) mice and Wistar rats. After those animals were treated with EEP and WSD, outstanding symptoms like thoracic capillary vessel leakage in mice, carrageenan-induced oedema, carrageenan-induced pleurisy, and acute lung damage in rats could

be observed. Since the level of prostaglandin-E (2) and nitric oxide, and interleukin-6 (IL-6) was decreased in the treated animals, it could be implied that EEP and WSD might inhibit the activation and differentiation of macrophage cells which were directly involved in causing inflammation.

2.3.4 Antiviral activity

In 1999, Kujumgiev et al.[28] reported the antiviral activity of propolis against avian influenza virus (H7N7). By determining the cytopathogenic effect (CPE), the selectivity index (SI) was determined by the ratio of TC_{50} (the concentration causing visible morphological changes in 50% of cells) to EC_{50} (the concentration reducing CPE in 50% of control). The propolis in this research could show very good effectiveness in CPE because the SI was greater than 4. This value was interpreted to be significant.

In 2010, Diaz-Carballo et al.[7] reported two anti-retroviral (lentiviral) compounds, 7-epi-nemorosone and plukenetione A, from Caribbean propolis. Both of them belonged to polyisoprenylated acylphloroglucinols. Considering the inhibition of viral infection, the former compound (< 1 μM) was more active than the latter compound (2 μM). They found that 7-epi-nemorosone could inhibit the replication of virus by interrupting the Akt/PKB signaling cascade. Considering the inhibition of reverse transcriptase, only plukenetione A could inhibit this enzyme with the IC50 vlaue of 1.75 μM. It was additionally reported that the role of plukenetione A in inhibition was by interfering a catalytic site of this enzyme.

2.3.5 Antioxidant activity

In 2011, Sulaiman et al.[52] reported the antioxidant activity of *A. mellifera* propolis collected from four geographical regions in Iraq (Baghdad, Dunuk, Mosul, and Salahah-Din). HPLC-ESIMS was used for analysis and identification of bioactive compounds in those propolis extracts. Major chemical components were shown to be phenolic acids including flavonoids, aromatic acids, and their esters. It could be an evident that these samples exhibited interesting anti-oxidation which might be justified as a source of natural antioxidants. It also supported that propolis harvested from different geographical origins had different physical properties.

In 2007, Mohammadzadeh et al.[38] reported antioxidant activity of propolis harvested from three different regions in Iran where were Tehran, Islahan, and Khorasan. The antioxidant power was measured by ferric reducing ability of plasma (FRAP) assay and was compared with Trolox at concentration of 100, 1000, and 2000 µg/ml. FRAP values of CEE were ranged from 31.5 ± 14.6 to 1650 ± 72 µM when CEE at the concentration of 100, 1000, and 2000 µg/ml were used. Comparing to positive control, FRAP values of Trolox were ranged from 125.25 ± 9.95 to 3,381.64 ± 113.83 µM when Trolox at the concentration of 100, 1000, and 2000 µg/ml were used. The flavonoid and polyphenol content in CEE were measured by aluminum nitrate and Folin-Ciocalteu colorimetric methods. The results showed that there was flavonoid at the amount of 1.22 ± 0.33 – 7.79 ± 0.39 g/ 100 g CEE and polyphenol at the amount of 3.08 ± 0.02 – 8.46 ± 0.03 g/ 100 g CEE. They concluded that CEE from Iran could prevent free radical-relating diseases.

2.3.6 Antiproliferative activity

In 1998, Kimoto et al.[26] reported artepillin C (3,5–diprenyl–4–hydroxycinnamic acid) extracted from Brazilian propolis could be cytotoxic to human and murine malignant tumor cells both in vitro and in vivo. Also, artepillin C could damage solid tumor and leukemic cells. For in vitro level, MTT assay, DNA synthesis assay, and morphological observation were done. However, for in vivo level, human tumor cells were transplanted into nude mice. Later, 500 g of artepillin C was injected. Identifying by histology, cytotoxic effect to carcinoma and malignant melanoma could be seen. Furthermore, apoptosis, abortive mitosis, and massive necrosis were revealed. In addition, artepillin C could activate the immune system by increasing the ratio of CD4/CD8 T cells and total number of helper T-cells.

In 2005, Mishima et al.[37] reported baccharin and drupanin, two related cinnamic acid derivatives, from Brazilian honeybee propolis. Both compounds could give in vivo antitumor activity in mice bearing sarcoma S-180 cells. They could induce tumor cell death and were less genotoxic to normal hematopoietic cells than present anti-cancer drugs. Moreover, Orsolic and Basic (2003) [41] reported the antitumor effect of water-soluble derivative of propolis (WSDP) from Croatia and Brazil on mammary carcinoma cells (MCA), human epithelial carcinoma cell line (HeLa), and Chinese hamster lung fibroblast cells (V79). The result showed that the percentage of apoptosis of MCA cells was increased from 20% to 24% and 26% after MCA cells were incubated in 50 μg/ml of WSDP from Brazil and Croatia, respectively. In addition, the percentage of apoptosis of HeLa cells was increased from 2% to 10% by Croatian propolis and 9.5% by Brazilian propolis. However, the percentage of apoptosis of V79 was decreased from 18% to 8% by Croatian propolis and 12% by Brazilian propolis.

2.3.7 Immunomodulatory activity

In 2010, Orsatti et al.[41] reported Brazillian green propolis had immunomodulatory action. Male BALB/c mice were divided into 2 groups. Ten mice were in each control and treated group. Control mice were treated 0.9% NaCl (0.1 ml) while the latter, mice were treated with CEE (200 mg/kg, 0.1 ml) for 3 days. The result showed that CEE could improve innate immunity at the initial step by up-regulating *toll-like receptor (TLR)-2* and *TLR-4* expression and pro-inflammatory cytokine or interleukin (IL)-1 and IL-6 production in macrophages and splenocyte cells.

Recently, how active compounds isolated from natural products could give immunomodulatory action at molecular mechanisms has been focused. For example, Bufalo et al. (2014)[3] reported this activity by evaluating cell markers and cytokine production of human monocytes. Propolis could up-regulate the expression of TLR-4 and cluster of differentiation (CD) 80. Also, it could affect the tumor necrosis factor (TNF) - α and IL-10 with dose-dependent manner. If TLR-4 was blocked, the cytokine production would be decreased.

2.3.8 Anti-allergy, rhinitis, and asthma

In 2003, Khayyal et al.[25] reported properties of propolis in order to develop to be new drugs. The crude aqueous extract (CAE) of propolis was used as an adjuvant form to treat asthma patients. They had been treated daily for 2 months. It was found that treated patients showed a reduction in incidence and severity of nocturnal attacks but an improvement of ventilator functions. The CAE had flavonoid in its composition which was later proved to decrease prostaglandin, leukotrienes,

pro-Inflammatory cytokines, TNF-α, intercellular adhesion molecule (ICAM)-1, IL-6, IL-8 but increased IL-10.

In 2009, Shinmei et al.[50] reported effect of Brazilian propolis on sneezing and nasal rubbing by using male BALB/c mice induced to have allergic rhinitis by antigen. Granular propolis dissolved in 5% gum arabic and CEE dissolved in 1% MeOH were used. The data showed that, for a single administration, propolis at a dose of 1,000 mg/kg caused no significant inhibition on sneezing and nasal rubbing which was similar to tranilast (a drug) at a dose of 300 mg/kg. However, for repeated administration, propolis at the same concentration could cause a significant inhibition while the drug could still not cause a significant inhibition. A concentration-relating inhibition to histamine release from rat peritoneal mast cells induced by antigen implied that propolis could relief the symptoms of allergic rhinitis through the inhibition of histamine release.

2.4 Cardanol

Cardanol was a phenolic compound found in Anacardiaceae family. It was in the same group as anacardiacid, catechol, resorcinol, and ginkgolic acid. The compound contained aromatic, hydrophilic, and hydroxyl groups. The chemical structure of cardanol was shown in Figure 2.2.

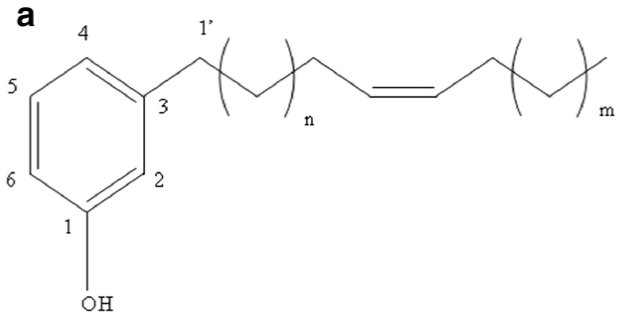


Figure 2. 2Chemical structure of cardanol (Teerasripreecha et al.,2012)[54].

The compound has been reported to have many biological effects as mentioned below.

2.4.1 Antimicrobial activity

In 2011, Gopalakrishnas et al.[16] reported cardanol which was extracted from cashew nut shell liquid (CNSL), by product from cashew nut processing industry of M/S Sathya Cashew Chemical in India. Two more compounds, p-sulphanilic acid and p-anisidine, were also isolated from CNSL. In order to test for antimicrobial activity, *Klebsiella pneumoniae* (NCIM 5082), *Pseudomonas aeruginosa* (NCIM 2026), *Solmonella typhi* (NCIM 2501), *Bacillus cereus* (NCIM 2703), and *Streptococcus pyogenes* (NCIM 2608) were used. The p-sulphanilic acid showed antibacterial activity with the inhibition zone of 22 mm in diameter against *K. pneumonia* at the concentration of 80 μg/ml and with the inhibition zone of 17 mm in diameter against *B. cereus* at the same concentration. In contrast, p-anisidine presented lower antibacterial activity than p-sulphanilic acid.

2.4.2 Antioxidant activity

In 2005, Trevisan et al.[56] reported antioxidant activity of anacardic acid, cardanol, and cardol collected from cashew apple, nut (raw and roasted forms), and cashew nut shell liquid (CNSL) in Brazil. Compounds from CNSL were extracted and separated by semi-preparative HPLC and were identified by electrospray ionization mass spectrometry (ESI-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance spectroscopy (NMR). The antioxidant capacity was found to be correlated significantly ($P<0.05$) with the concentration of alky phenols in the sample extracts. A mixture of anacardic acid (10 mg/ml) showed the highest

antioxidant capacity with the IC_{50} value of 0.60 mM, comparing to cardanol and cardol with the IC_{50} value of > 4.0 mM.

2.4.3 Anticancer activity

In 2008, Ola et al.[40] reported anacardic acid and cardanol extracted from CNSL in Timor Island, Indonesia. Anacardic acid was isolated as calcium anacardate and was mixed with ammonia. Cardanol could be isolated from the acid-free CNSL after it was extracted by hexane / ethyl acetate (98: 2). Also, cardol could be isolated by ethyl acetate / hexane (80: 20). Anacardic acid and cardanol were analysed by using Infrared Spectroscopy (IR) and Gas chromatography - Mass spectrometry (GC - MS) After that, all compounds were tested against Hela cancer cell lines using MTT assay. The result showed anacadic acid and cardanol were the anti-cancer agents.

2.5 Breast cancer

Breast cancer was reported to be the most common cancer worldwide as well as the leading cause of Thai women death (Suwisith et al., 2008)[53]. Food, chemicals, and life style may cause a risk factor for this disease.

According to Kotepui and Chupeerach and all medical records of breast cancer patients from National Cancer Registry of Thailand,[34] the general population statistics on age, gender, and other factors were revealed. The data showed that during the period of 10 years, there were 7,711 breast cancer cases. The disease incidence of $4.13/10^5$ which was found in people under 40 years old was lower than the disease incidence of $39.2/10^5$ which was found in people older than 40 years old. Thus, more breast cancer patients were found in older people.

2.6 MTT assay

The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromine (MTT) assay is used to measure viable cells and proliferation of cells. It is a colorimetric method. MTT will be reduced to be a purple formazan by succinate dehydrogenase enzyme from mitochondria in living cells (Figure 2.3). After that, formazan crystal will be dissolved in dimethyl sulfoxide (DMSO) and the measurement will be at the absorbance of 540 nm.

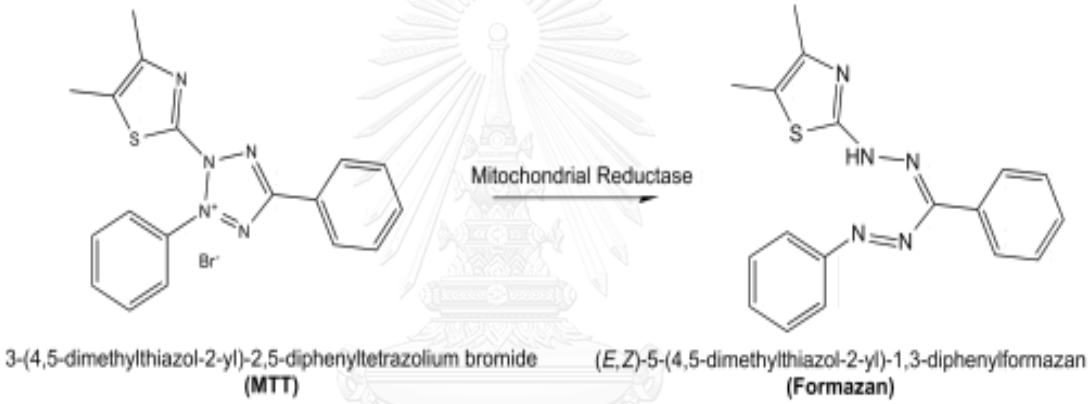


Figure 2. 3It presents how MTT works. The color will change from yellow of MTT to be purple of formazan.

In 2009, Han et al.[22] reported two main phenolic compounds which were oleuropein and hydroxytyrosol in olive oil. They could affect the growth of human breast cancer cells (MCF-7). By using MTT assay, it showed that, after 48 h treatment with olive leaf extract at the dose of 0.1%, cancer cell proliferation were inhibited to 60%, comparing to the vehicle treated cancer cells (negative control). More cytotoxicity could be found in oleuropein (200 μg/ml) and hydroxytyrosol (50 μg/ml).

2.7 Programmed cell death

It is an important process in the maintenance of biological cells and system. It can be divided into two types which are apoptosis and necrosis. Apoptosis occurs in normal physiological conditions and does not damage neighbor cells. In contrast, necrosis occurs in severely physiological condition resulting in damaging other cells (Elmore, 2007)[8].

2.7.1 Apoptosis

It can make a cellular shrinkage, chromatin condensation, DNA fragmentation, and loss of mitochondria. Apoptosis has both intrinsic and extrinsic pathways. For intrinsic pathway, caspase-9 will be stimulated. In mitochondria, many proteins will be involved like proteins in bcl-2 family, small mitochondrial-derived activator of caspases (SMC), and inhibitor of apoptosis proteins (IAPs). For extrinsic pathway, it begins from outside of the cell. Pro-apoptotic receptor on cell membrane will be activated by pro-apoptotic ligands. Then, this ligand - receptor binding will activate the death - inducing signal complex. After that, the same process will occur in the extrinsic pathway as in the intrinsic pathway (Figure 2.4).

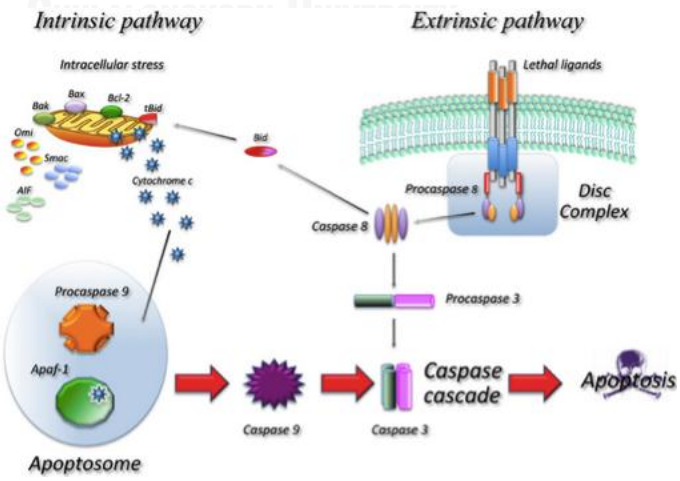


Figure 2. 4Apoptosis presenting both intrinsic and extrinsic pathways (Favaloro et al., 2012)[11].

2.7.2 Necrosis

Necrosis is an accidental cell death. This process can occur after cells are exposed to physical or chemical substances such as UV radiation, higher temperature, anti - cancer drug, ionizing radiation, etc. The obvious response of necrotic cell is the change in morphology including nuclear swelling, chromatin flocculation, breakdown of cytoplasmic structure and organelle function, and cytolysis by swelling. This situation can induce inflammatory response (Wei and Thompson, 2014)[59].

2.7.3 Morphology of apoptotic and necrotic cells

For apoptosis, plasma membrane remains the same without integrity loss. However, chromatin aggregation, nuclear and cytoplasmic condensation, cell shrinkage, and formation of cytoplasm and nucleus into membrane vesicle (apoptosis bodies) will be obviously observed. Apoptotic cell does not damage other cells. In contrast, necrotic cell damages plasma membrane including membrane blebbing, loss of integrity, and cell swelling. Normally, necrotic cells can induce inflammatory response (Figure 2.5).

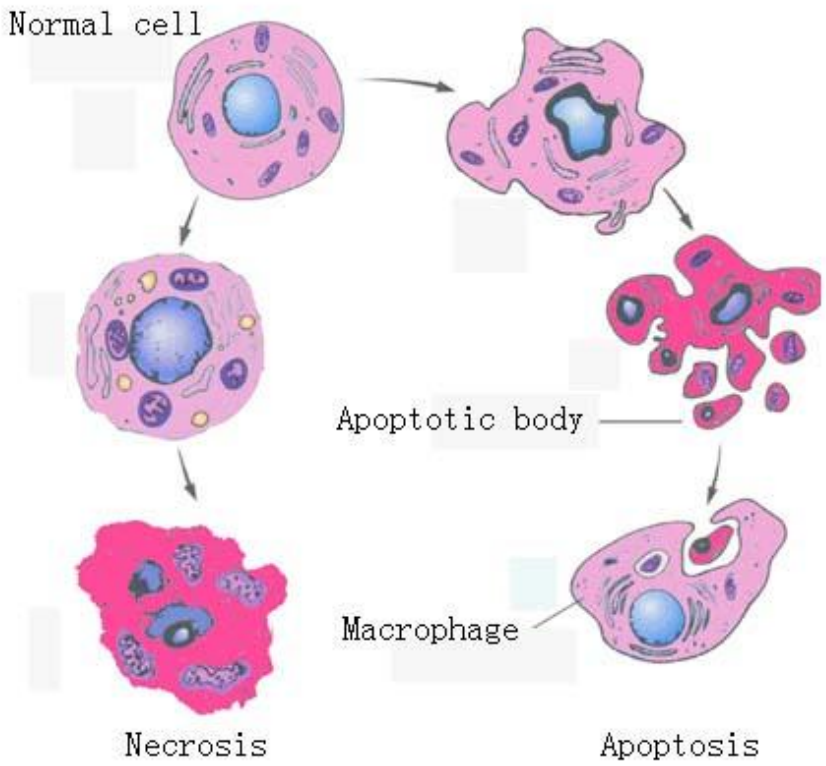


Figure 2.5 Different morphology between apoptotic and necrotic cells (Baba, 2009).**[1]**

2.7.4 Determination of apoptosis and necrosis

Program cell death is analysed by staining cells with Annexin V and propidium iodide (PI). Also, it can be used to determine cell cycle arrest by staining cells with (PI). Result of staining can be detected by using flow cytometry.

For the basic background, apoptosis will cause a loss of membrane phospholipid asymmetry (Koopman et al., 1994).[27] Fluorescein isothiocyanate (FITC) labeling annexin V will bind to phosphatidyl serine. Thus, this FITC labeling annexin V can bind to dead cells only. For live cells, since FITC labeling annexin V cannot bind, a negative result (no fluorescence) will be observed while, for apoptotic

cells, a positive result (green fluorescence) will be observed instead. For necrotic cells, both FITC labeling annexin V and PI can double label so the combination of red and green fluorescence can be noticed (Schutte et al., 1998).[48]

Not only flow cytometry can be used to analyse apoptosis but it is also used to analyse cell cycle arrest. The cell cycle position (subphase G_1, S, and G_2/M) is identified by DNA content which is determined by PI staining.

In 2007, Hsu et al.[18] reported caffeic acid phenyl ester (CAPE) was an anti-cancer component from propolis. The percentage of apoptotic cells were analyzed by using flow cytometry after the cells were stained with annexin V and PI. HeLa cervical cancer cell lines (ME 180) were incubated with 25 μM of CAPE at 0, 8, 16, 24, and 48 h. The result showed that CAPE induced S and G_2/M phase cell cycle arrest and initiated apoptosis in human cervical cancer cell lines. CAPE up-regulated the expression of E2F-1 target gene, cyclin A, cyclin E, and apoptosis protease activating of factor-1 (Apaf-1) but down-regulated cyclin B. Also, it could induce myeloid leukemia cell differentiation protein (*Mcl-1*).

Furthermore, et al. (2012) [5]reported an effect of chrysophanol, an anthraquinone compound, by using flow cytometry. The compound involved in necrosis of human live cancer cell line (J5) in a dose and time dependent manner. After the cancer cells were incubated with chrysophanol (50 μM) at 0, 6, 12, and 24 h, more percentage of necrotic cells could be observed (2.58%, 8.58%, 17.84, and 38.08%, respectively). The compound could additionally stimulate reactive oxygen species (ROS) production, DNA damage, mitrochondrial dysfunction, loss of ATP, and promotion of LDH activity, which resulted in cell necrosis.

2.8 Biology of cell cycle

It is complex series of signal pathways. The division of cell cycle consists of two phases of interphase and mitosis (Figure 2.6). For interphase, it contains three subphases. The first one is G_1 subphase (first growth subphase). It is for cell growth. Volume of cytoplasm and numbers of organelles and materials in cytoplasm will be double. The second one is S subphase (DNA synthesis subphase). During this period, DNA replication will occur. The third one is G_2 subphase (second growth subphase). The situation of cell at this subphase is similar to what occurs in G_1 subphase. After interphase is complete, cell will continue to mitotic phase which is consisted of prophase, metaphase, anaphase, and telophase (Israels, 2014).[20]

Abnormality in division of cell cycle can cause cancer cell. That may come from genetic mutations and process malfunctions which leads to uncontrolled cell proliferation.

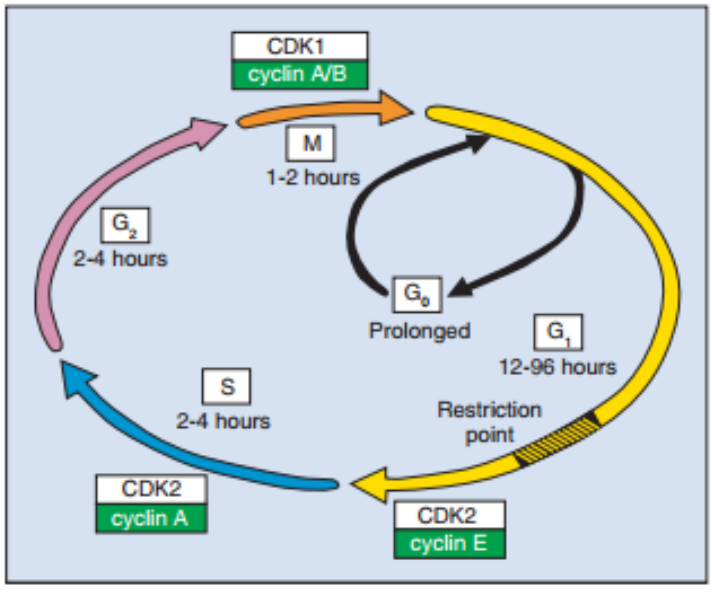


Figure 2.6 Cell cycle (Israels, 2014)[20].

2.9 Cell cycle arrest

Within a cell cycle, there are many checkpoints to determine whether the division will proceed or stop. In 2001, Choi *et al*.[21] reported quercetin could induce cell cycle arrest and apoptosis in human breast cancer cell (MCF-7). Quercetin had the mechanism of growth inhibition for MCF-7. Mitotic index determined by MPM2 staining showed that cells in M phase could increase the amount of cyclin b1 and Cdc2 kinase activity. However, longer treatment could cause more cells accumulate in G_2 subphase. The level of cyclin b1 and cyclin b1 associated cdc2 kinase activity would be decreased. Furthermore, after treatment of 48 h, induced $p21^{CIP1/WAF1}$ could increase cdc2-cyclin b1 complex. In overall, the result showed two different mechanisms which the first one could arrest the cell cycle at G_2 subphase and could induce apoptosis.

In G_1 subphase, there are two major types of cyclins which are cyclin D and cyclin E (Figures 2.7 and 2.8). During cell cycle arrest, it will response to mitogen deprivation or CDK inhibitors of the CIP/KIP family including $p21^{clp1}$, $P27^{kip1}$, and $p57^{kip2}$ (Morgan, 1995)[39]. They will bind to CDK2 and CDK4 complexes. Both can phosphorylate tumor suppressor proteins (pRB, p107, and p130) in the retinoblastoma family and finally stop the growth (Lipinski and jacks, 1999). Cyclin D activates CDK4 and CDK6. In addition, cyclin E activates CDK2. Therefore, G_1 phase depends on cyclin D and CDK4/6 protein complex. Also, it depends on cyclin E and CDK2 (Sherr, 1994).[49]

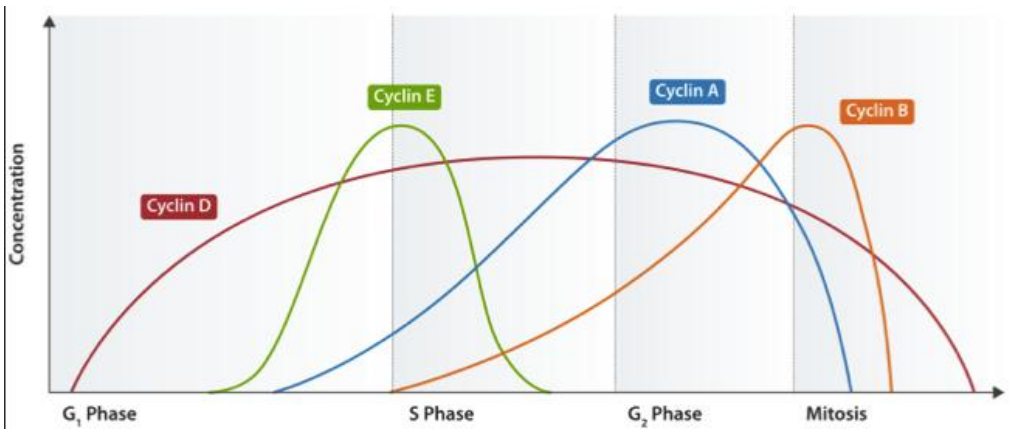


Figure 2.7 Differential expression of cyclins during the cell cycle. (http://www.labome.com/method/Cell-Based-Assays-the-Cell-Cycle-Cell-Proliferation-and-Cell-Death.html)

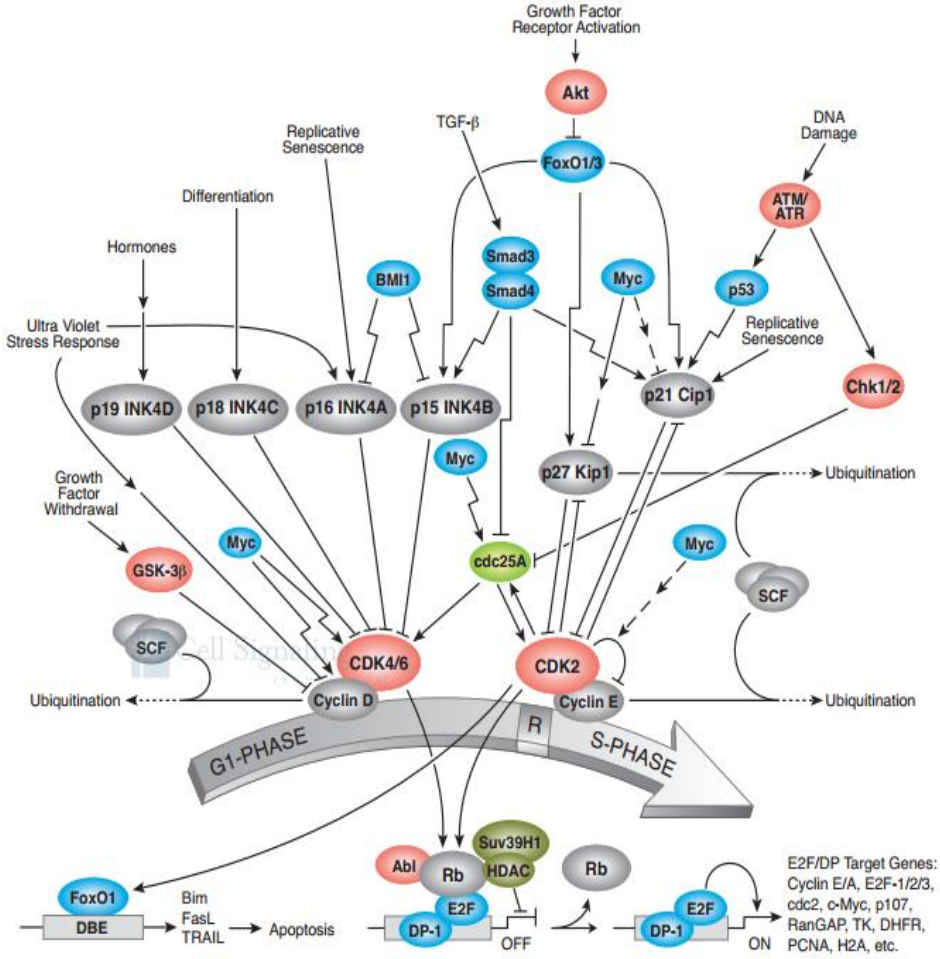


Figure 2.8 The G_1 checkpoint for cell arrest. (http://media.cellsignal.com/www/pdfs/science/pathways/Cell_Cycle_G1S)

2.10 Gene expression

Gene expression is the processes of generating gene products from genes. Gene as a DNA sequence locating on chromatin can be transcribed into messenger RNA (mRNA) as an intermediate form of genetic materials. This process is called transcription. Later, with the function of ribosome and transfer RNA (tRNA), mRNA will be further translated into protein. This process is called translation. Thus, for detecting an expression of any interesting gene, it can be assayed in 2 steps. The first one is at the transcriptional level so reverse transcriptase polymerase chain reaction (RT PCR) is used. The second one is at the translational level so western blot is used.

2.10.1 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

This technic is used to determine the expression change of interesting genes at the transcriptional level. Therefore, total RNA (messenger RNA) from target cells must be required (Willard et al., 1999)[13]. An expression level of interesting gene should be normalized to control gene. A crossing point (Cp) will be used to calculated for the relative gene expression level with the below formular.

The relative gene expression level = $2^{(\text{Cp control - Cp target})}$

The Cp value correlates to the amount of the initial templates indicating the expression of the target mRNA (Livak and Schmittgen, 2001).[32]

In 2012, Lirdprapamongkol et al.[31] reported a mechanism of chrysin extracted from *Apis mellifera* propolis from Chiangmai province on inhibiting the growth of human lung and cervical cancer cells lines. For cancer cells treated 60 μM chrysin for 3 h, the expression of *mcl-1* was decreased by 52%. The result could lead to an implication that modulation of tumor necrosis factor-related apoptosis-

inducing ligand (TRAIL) of cancer cells could be sensitized by down-regulation of mcl-1.

2.10.2 Western blot

It is a technic used to identify the expression of target gene at the translational level. A mixture of protein is separated based on its molecular size by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). For SDS PAGE gel, it contains stacking and separating gels. After electrophoresis, separated protein will be transferred onto nylon membrane or PVDF membrane. Then, the membrane will be labeled with both primary and secondary antibody which was specific to protein of interest. Positive control will be used to confirm the identity of the protein and activity of antibody while negative control will be used to confirm non-specific binding (Mahmood and Yang, 2012)[33].

In 2008, Kim et al. reported naringin, a flavonoid compound found in citrus fruit extracts, could inhibit urinary bladder carcinoma (5637) proliferation. By western blot, it could reveal that the cell cycle of treated cancer cells with 100 μM naringin was arrested at G_1 subphase. It could induce the expression of p21WAF1, regulate the progression of cell cycle at G_1-S subphase transition checkpoint which was independent from the p53 pathway. It could down-regulate the expression of cyclins and cyclin dependent kinases (CDKs). In addition, naringin could induce phosphorylation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase.

CHAPTER III

METERIALS AND METHODS

3.1 Equipments

- Rotary evaporator, Buchi Rotavapor R-114, Switzerland
- Microwave oven, Sharp Carousel R7456, Thailand
- Centrifuge, Hettich, Germany
- Column (250 ml in size), Schott Duran, Germany
- Flask (50, 250, 500, 1,000 ml in size), Schott Duran, Germany
- Microtube (1.5 ml in size), Sarstedt, Germany
- Centrifuge tube (15 and 50 ml in size), Sarstedt, Germany
- Tissue culture plate 96 well, DSI, Thermo Fisher Scientific, Denmark
- Tissue culture flask, Nunclon DSI, Thermo Fisher Scientific, Denmark
- Pipette tips (200 and 1,000 µl in size), BioScience, Inc., USA
- Pipette tips (10 µl in size), BioScience, Inc., USA
- Autoclave, Conbraco Industry, Inc., USA
- Electrophoresis chamber set, model: Mupid, Advance Co., Ltd., Japan
- Flow cytometry (an FC 500 MPL cytometer), Beckman Coulter
- Image Quant LAS 4000 mini, GE Healthcare Life Sciences

3.2 Chemicals

- Methanol (CH_3OH), TSL Chemical, Thailand
- Methylene chloride (CH_2Cl_2), TSL Chemical, Thailand
- Hexane ($C_6H_{14}C_6H_{12}$), TSL Chemical, Thailand
- Fetal Bovine Serum, PAA Laboratories GmbH, USA
- RPMI 1640 medium, Biochrome, Germany
- Trypsin, Sigma, USA
- Glycine, Biochemical, England
- Silica gel 60 for column chromatography, 0.063-0.200 mm in Ø
- Silica gel 60 G for thin layer chromatography
- . TLC silica gel 60 F254, Merck KGaA, Germany
- Agarose, low EEO, Research Organics Inc., USA
- Annexin V, Alexa Fluor, Life Technologies
- Propidium iodide solution, Sigma, USA
- Immobilon-P nylon membrane, Millipore, Beaford, MA, USA
- Anti-rabbit IgG HRP-linked antibody, Cell Signaling Technology
- WesternBright ECL reagent, Advansta, Menlo Park, CA, USA

3.3. Propolis collection

Propolis of *Apis mellifera* was collected from bee hives in a bee farm in Pua district, Nan province, Thailand in January, 2012. They were located in a mango tree garden. Propolis was wrapped by aluminum foil and kept in the dark at -20 $^{\circ}$C until used.

3.4 Preparation of crude extract

It was perform using the methodology described by Teerasripreecha et al. (2012)[54]. Propolis (90 g) was dissolved in 400 ml of 80% (v/v) methanol (MeOH) and was shaken at 100 rpm, 15 $^{\circ}$C, for 18 h. Then, it was spun at 4930 g, 20 $^{\circ}$C for 15 min. The supernatant was evaporated by rotary evaporator (Buchi rotavapor R-114) until dryness to obtain crude MeOH extract (CME). The pellet was further mixed with 400 ml of dichloromethane (CH_2Cl_2) and later was shaken at 0.0028, 15 $^{\circ}$C, for 18 h. After that, it was spun at 4930 g, 20 $^{\circ}$C for 15 min. The supernatant was collected and evaporated by the rotary evaporator to leave crude CH_2Cl_2 extract (CDE). Next, the pellet was dissolved in 400 ml of hexane and was shaken at 0.0028 g, 15 $^{\circ}$C, for 18 h. After spun, the supernatant was evaporated by the rotary evaporator to afford crude hexane extract (CHE). All obtained crude extracts (CME, CDE, and CHE) were weighed and its character was recorded. All three crude extracts were tested for antiproliferative activity and kept in the dark at -20 $^{\circ}$C until used.

3.5 Isolation of cardanol by chromatography

This step was done by the following protocol reported by Teerasripreecha et al. (2012) [54]with slight modifiiation.

3.5.1 Quick column chromatography

The sintered glass (250 ml in volume) was packed with silica gel 60 G (0.063-0.2 mm). A vacumn pump was used to pack silica gel tighter. An active crude extract was mixed with silica gel 60 until it was homogeneous and not sticky. Then, the mixture was put on top of the packed silica gel. A piece of filter paper (Whatman, 110 mm in Ø) was put on top of the transferred sample. After that, the cotton was put to cover the gel. The final volume of 1.5 l of solvent (500 ml/ time, 3x) of hexane 25% (v/v) , CH_2Cl_2 -hexane 50% (v/v), CH_2Cl_2 - hexane 75% (v/v), CH_2Cl_2 100% (v/v), MeOH - CH_2Cl_2 4% (v/v), and MeOH - CH_2Cl_2 30% (v/v) was added to the column, respectively. Fractions were collected. The pattern of chemical compounds in all fractions was observed by thin layer chromatography (TLC). Fractions having similar TLC profile would be pooled together. All pooled fractions would be evaporated and weighed. A character of each pooled fraction would be recorded. All pooled fractions were assayed by MTT assay for antiproliferative activity.

3.5.2 Adsorption chromatography

Silica gel 60 (90 g) mixed with hexane (250 ml) was packed into a column. Next, the active fraction was mixed with silica gel 60 (5-7g) until it was not sticky. The mixture was left at RT until dry. After that, it was put on top of the column and was covered on top with cotton. Then, it was eluted with 500 ml of 100% (v/v) hexane, 50% (v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, and 100% (v/v) MeOH, respectively. Fractions (2.5 ml each) were collected and screened for composition pattern by TLC. Fraction having cardanol on the TLC plate would be pooled together and evaporated. After that, it was weighed.

3.5.3 Thin layer chromatography (TLC)

It was used to determine the purity of sample. A TLC plate (a silica coated plate, Merck) was cut into the size of 5 ×5 cm^2. At the beginning, the line was drawn from the bottom of the plate at 0.5 cm by a pencil. Then, a sample was loaded on the drawn line by a capillary tube. If the sample was very sticky, it could be diluted with organic solvent. After that, the TLC plate was placed in a solvent chamber. The solvent of 100% (v/v) CH_2Cl_2, was used for the mobile phase. After, a compound reached almost the top of the TLC plate, it would be pulled out of the chamber. After it was dry at RT, the pattern of compounds on the TLC plate could be observed under ultraviolet light.

3.5.4 Mass spectrometry (MS)

A pure and active fraction was analyzed for chemical structure by MS. It could work by ionizing a sample. A spectrum was produced according to mass atom of the sample.

3.6 Antiproliferative activity

3.6.1 Cell culture

Breast cancer BT-474 cell line (ATCC no. HTB 20) was obtained from Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. It was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% (v/v) fetal calf serum (FCS). For seeding cells, 1 x 10^5 cells were transferred to 5 ml medium in a 25-cm^2 flask and were incubated at 37 $^{\circ}C$ with 5% (v/v) CO_2. It would be re-passaged when it reached the 70-80% confluency.

3.6.2 Cytotoxicity test

It was done by using 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay. BT-474 cell line ($5x10^3$ cells in 200 µl) was placed in a well of 96 well plate. It was incubated at 37 ^{o}C with 5% (v/v) CO_2 for 24 h. Cardanol was dissolved in dimethylsulfoxide (DMSO). After that, 2 µl of cardanol was transferred to each well in order to make the final concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 µg/ml, respectively. DMSO (2 µl/well) was used as control. Later, cells were incubated for 72 h. Ten µl of 5 mg/ml of MTT solution was added to each well and was incubated for another 4 h. After that, the solution was removed. The solution containing 150 µl of DMSO and 25 µl of 0.1 M glycine was added to dissolve formazan crystal. The absorbance was measured at 540 nm by a microplate reader. The percentage of cell survival was calculated according to the following formula.

$$\% \text{ cell survival (PS)} = \frac{\textbf{Absorbance of sample}}{\textbf{Absorbance of control}} \times 100$$

The PS of control was set to be 100%. A graph was drawn where a Y axis represented % PS and an X axis represented concentrations of cardanol. A line was drawn horizontally from the 50% PS point until it reached the graph line. Then, it was drawn vertically until it reached the X axis. The touched point represented the IC_{50} value of cardanol.

3.6.3 Growth curve

Untreated and treated BT-474 cells (with IC_{50} value) were prepared. By MTT assay, the absorbance at 540 nm was measured at every 1, 2, 3, 5, and 7 days. The graph presenting % PS (Y-axis) and time (X-axis) was drawn. The trend line was compared to untreated cell line. Triplication of experiment was done.

3.6.4 Cell morphology

Three groups of BT-474 cells (2 x 10^5 cells/ml) were prepared: 1) untreated cells (control); 2) treated cells with 30 µg/ml of cardanol; and 3) treated cells with 0.5 µg/ml of doxorubicin (positive control). The morphology of all groups of cells was observed after 0, 24, 48, and 72 h incubation by an inverted microscope (Ziess) connected to a digital camera (Cannon EOS 7D).

3.7 Program cell death

It was done by following Hsu et al. (2013).[18] Three groups of BT-474 cells (3-5x10^6 cells/ml) were prepared: 1) stained cells (control); 2) stained and cardanol treated cells (30 µg/ml); and 3) stained and doxorubicin treated cells (0.5 µg/ml) as positive control. Later, cells were spun at 3,000x g, 4 $^{\circ}$C for 10 min. Then, it was washed by 1 ml of cold 1x phosphate buffer saline (PBS) and was spun at 3,000x g, 4 $^{\circ}$C for 10 min. After that, the supernatant was removed. Fifty µl of 1 x binding buffer, 1 µl of annexin V-FITC, and 5 µl of propidium iodide (PI) was added to stain cells in the dark, RT for 10 min. Fluorescence wavelength at 488 nm was used. The data was analysed by flow cytometry (an FC 500 MPL cytometer, Backman Coulter). Duplication of experiments was performed.

3.8 Cell cycle arrest

Three groups of cells (1x10^6 -1x10^8 cells/ml / each group) were prepared: 1) untreated BT-474 cells (control); 2) cardanol treated cells at 30 µg/ml, and 3) doxorubicin treated cells at 0.5 µg/ml (positive control). Cells were incubated and harvested at 24, 48, and 72 h. After treatment, cells were washed with cold 1x PBS and were spun at 2,000x g, RT for 5 min. After drained, the pellet was fixed with 500

µl of cold 1x PBS and 200 µl of 70% EtOH at -20 °C for overnight or on ice for 4 h. Then, it was spun at 2,000x g, RT for 5 min and was washed with cold 1x PBS. Later, the pellet was resuspended in 250 µl of cold 1x PBS. RNase at the volume of 2.5 µl (or final concentration of 0.1 mg/ml) was added and mixed. The suspension was incubated at 37 °C for 30 min. Then, it was spun at 2,000x g, RT for 5 min. The pellet was resuspended in 12.5 µl of 1 mg/ml propidium iodide (PI). The suspension was incubated at RT in the dark for 30 min. The data was analysed by flow cytometry (an FC 500 MPL cytometer, Backman Coulter). The result was interpreted from 4 phases which were 1) sub G_1 phase (apoptotic cells), 2) G_1 phase (diploid chromosome content), 3) S phase (DNA synthesis), and 4) G_2/M subphase (double diploid) as in Figure 3.1.

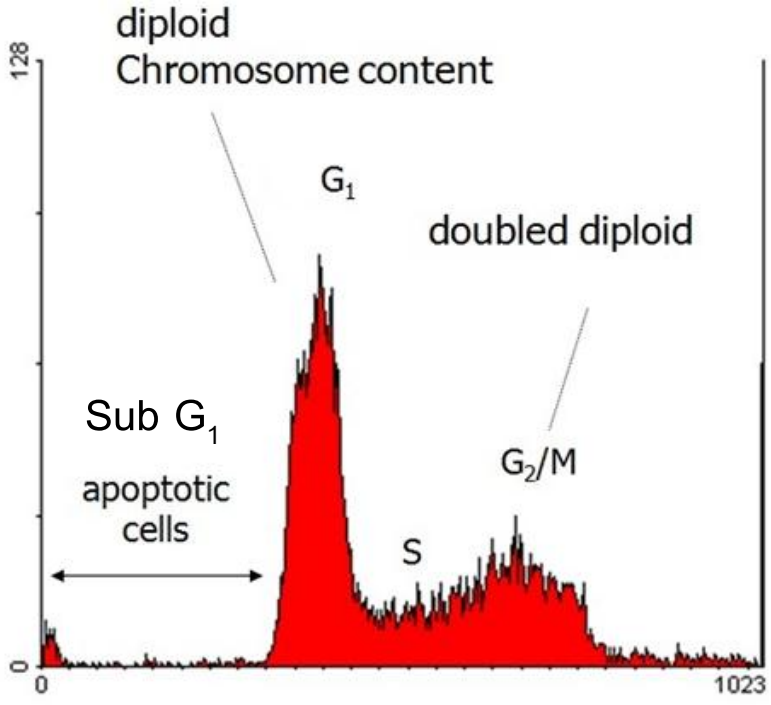


Figure 3.1 How to interpret cell cycle arrest using PI staining and flow cytometry.

3.9 Change in gene expression

3.9.1 Transcriptional level

Total RNA extraction

Total RNA was extracted from 1) untreated BT-474 cells (control), 2) cells treated with cardanol at 30 µg/ml, and 3) cells treated with doxorubicin at 0.5 µg/ml by using RNeasy Plus Mini Kit (Cat. # 74134, Qiagen). After harvesting cells by trypsinization, the cell suspension was spun at 8,451 x g, RT for 5 min and drained. The cell lysate was added with 350 µl of buffer RLT Plus and homogenized. The homogenized lysate was transferred to a gDNA eliminator and was spun at 8,451 x g, RT for 30 sec. The flow through (FT) was saved. The volume of 350 µl of 70% EtOH was added to FT and was mixed well by pipetting. The volume of 700 µl of the mixture was transferred to an RNeasy spin column which was already placed in a 2 ml collection tube. It was spun at 8,451 x g, RT for 15 sec. FT was removed. The volume of 700 µl buffer RW1 was added to the RNeasy spin column. It was spun at 8,451 x g, RT for 15 sec. After the removal of FT, 500 µl of buffer RPE was added to the RNeasy spin column. It was spun at 8,451 x g, RT for 15 sec. Againand wash , the spin column was washed by 500 µl of buffer RPE. It was spun at 8,451 x g, RT for 2 min. Extracted RNA was eluted from the column by using 20 µl of RNase - free H_2O. The absorbance at 260 nm of extracted RNA was measured by using a spectrophotometry (Genesys 10 series, Thermo Scientific). The concentration of RNA was calculated according to a formula below.

Concentration of RNA (µg/ml) = (Abs260) x Dilution factor x (40)

The purity of extracted RNA was estimated from the Abs 260/280 ratio which should be about 1.8-2.2. The RNA samples were stored at -20 $^{\circ}$C until use.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Two groups of genes were selected. The first group belonged to death receptor group containing apoptosis regulated genes like *b-cell lymphoma-2* (*Bcl-2*), *myeloid cell leukemia-1 (Mcl-1*), *mitogen activating protein-kinase activating death domain* (*MADD*), *cellular FLICE-like inhibitory protein* (*c-FLIP*), and *human death receptor 5* (*DR5*). The second group belonged to cell cycle regulating gene. It contained *p21*, *cyclin D1*, *cyclin E*, *cyclin A*, *cyclin-dependent kinase 4* (*CDK4*), *cyclin-dependent kinase 6* (*CDK6*), and *cyclin-dependent kinase 2* (*CDK2*). The used conditions were done by following Lirdprapamongkol et al. (2013)[31]. The reaction mixture was prepared by using One Step SYBR PrimeScript RT-PCR Kit II (cat. # TKR-R-RR064A, Takara). A PCR reaction mixture (20 µl final volume) contained RNA (10 ng), 10 µl of 2x one step SYBR RT-PCR buffer, 1 µl of Prime Script 1 step enzyme mix, 0.5 µl of each forward and reverse PCR primers (20 µM stock), and RNase-free d-H_2O. Forward and reverse primers were listed in Table 3.1

Table 3.**1Forward and reverse primers used for qRT-PCR.**

Nucleotide sequence of primers (5' → 3')	**Annealing temperature (°C)**
Reference gene: *ß-actin* (Lirdprapamongkol et al., 2013)[31] F: GACCTGACTGACTACCTCATGA R: AGCATTTGCGGTGGACGATGGAG	55
MADD (Li et al., 2011)[30] F: TCAACCCACTCATCTATGGCAATG R: GCGGAATTGAAGAACCGTACCA	60
c-FLIP (Li et al., 2011)**[30]** F: CCAGAGTGTGTATGGTGTGGAT R: TCTCCCATGAACATCCTCCTGAT	60
Bcl2 (Li et al., 2011)[30] F: TGGGATGCGGGAGATGTG R: CGGGATGCGGCTGGAT	60
Mcl1 (Lirdprapamongkol et al., 2013)**[31]** F: AGCAGAGGAGGAGGAGGAC R: GCCTGCTCCCGAAGGTA	55
DR5 (Pilai et al., 2011)[44] F: TGCTGCTCAAGTGGCGC R: GGCATCCAGCAGATGGTTG	60
P21 (Weglarz et al., 2006)**[58]** F: CACTCCAAACGCCGGCTGATCTTC R: TGTAGAGCGGGCCTTTGAGGCCCTC	55

Nucleotide sequence of primers (5' → 3')	Annealing temperature (°C)
E2F1 (Gil et al., 2008)[14] F: GCCACTGACTCTGCCACCA R: GGACAACAGCGGTTCTTGCT	60
Cyclin A (Gil et al., 2008)[14] F: GAAGACGAGACGGGTTGCA R: AGGAGGAACGGTGACATGCT	60
Cyclin D1 (Ullmannova et al., 2003)**[57]** F: AATGACCCCGCACGATTTC R: TCAGGTTCAGGCCTTGCAC	60
Cyclin E (Potemski et al., 2006)[46] F: TTCTTGAGCAACACCCTCTTCTGCAGCC R: TCGCCATATACCGGTCAAAGAAATCTTGTGCC	58
CDK2 (Chiang et al., 2010)[6] F: TTTGGAGTCCCTGTTCGTAC R: TGCGATAACAAGCTCCGTCC	58
CDK4 (Chiang et al., 2010)[6] F: CTTTGACCTGATTGGGCTGC R: GGAGAGGTGGGAGGGGAATG	58
CDK6 (Ullmannova et al., 2003)**[57]** F: TCTTGCTCCAGTCCAGCTAC R: AGCAATCCTCCACAGCTCTG	60

The relative expression level of gene was normalized to the expression of ß-actin gene as control. It was calculated by using crossing point (Cp) value according to a formula below.

The relative level = $2^{(Cp\ actin-Cp\ target)}$

The gene expression level was compared between untreated sample and treated sample as the fold change which was calculated according to $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001).[32]

3.9.2 Translational level

Western blot

It was performed by following Lirdprapamongkol et al. (2013).[31] BT-474 cancer cells ($2x10^5$ cells/ml) were treated with cardanol (conc. of IC_{20} value) for 24 h. The samples were prepared in 2 groups which were untreated and treated groups. Cells were lysed in 150 µl of radioimmunoprecipitation assay buffer (RIPA) which contained half protease phosphatase and phosphatase inhibitor cocktail with EDTA solution (catalog# 78440, Thermo Scientific, USA) on ice. Then, the concentration of protein was measured by Bradford assay. Briefly, 20 µg of protein were loaded onto a well of 10% Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE) as 10% separating gel and 10% stacking gel. After electrophoresis at 15 mA for 105 min, the protein was transferred to immobilon-P nylon membrane (Millipore, Beaford, MA, USA) by electroblotting at 100 V for 90 min. The membrane was later blocked with 3% (w/v) Bovine Serum Albumin (BSA) for 1 h, gently shaking at RT. After that, the membrane was cut and probed with the diluted primary antibodies with 3% (w/v) Bovine Serum Albumin (BSA) (1: 1000 ERK, 1: 5000 pERK, 1: 1000 p-38, 1: 1000 p-p38, 1: 1000 JNK, 1: 1000 pJNK, 1: 1000 cyclin D1, and 1: 1000 p21) for overnight, 4 ^{o}C, in the dark. Then, the membrane was washed by 1x TBS/T. Later, a probe with the

diluted secondary antibody in TBS/T containing 5% skim milk (1: 10000 mouse, 1: 5000 rabbit) was added with gently shaking at RT for 1 h. The result was visualized by using western bright ECL reagents (Advansta, Menlo Park, CA, USA) and image was captured by using Image Quant LAS 4,000 mini (GE Healthcare Life Sciences). The detail of all used reagents was shown in appendix E.

3.10 Statistical analysis

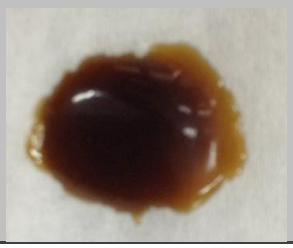
Data was expressed as mean ± standard deviation (S.D.) from triplication in each experiment. The data was analyzed by one way analysis of variance (ANOVA) followed by Tukey test of multiple comparisons. A p-value at < 0.05 was considered to be significant. All analyses were done by using SPSS program version 19.0.

CHAPTER IV
RESULTS

4.1 Crude solvent extract of propolis from *Apis mellifera*

After extraction of *A. mellifera* propolis (90 g) with three organic solvents (MeOH, CH_2Cl_2, and hexane), the result was shown in Table 4.1. The highest yield was obtained from CHE (21,600 mg). In addition, the color of pigments could be most removed from CHE. Thus, it looked hazel while the rest still looked very brown. Although, all crude extracts showed the antiproliferative activity to BT-474, CDE (medium polar solvent) was the most active (IC_{50} = 29.97 ± 6.17 μg/ml. Thus, CDE was further purified by chromatography.

Table 4.1 **Character and IC_{50} value of crude extracts (CME, CDE, and CHE).**

Crude extract	Weight (mg)	% yield of propolis	Character	IC_{50} (µg/ml)
1) Crude MeOH extract (CME)	600	0.33	Sticky and dark brown 	38.26 ± 4.28
2) Crude CH_2Cl_2 extract (CDE)	1,540	1.71	Sticky and yellow brown 	29.97 ± 6.17
3) Crude hexane extract (CHE)	21,600	24	Hazel 	39.18 ± 6.27

4.2 Effect of cardanol

Since CDE (1,540 mg) was the most active. It was further purified by quick column and adsorption chromatography. The obtained cardanol was confirmed by Rf value on a plate of thin layer chromatography and spectrum of mass spectrometry (Appendix A). Cardanol at the weight of 0.52 mg was obtained. The IC_{50} value was calculated to be 15.57 ± 1.73 µg/ml (Figure 4.1). That was similar to Teerasripreecha et al. (2012)[54] which showed the IC_{50} value of cardanol on inhibiting BT-474 cell lines at 13.95±0.9 µg/ml.

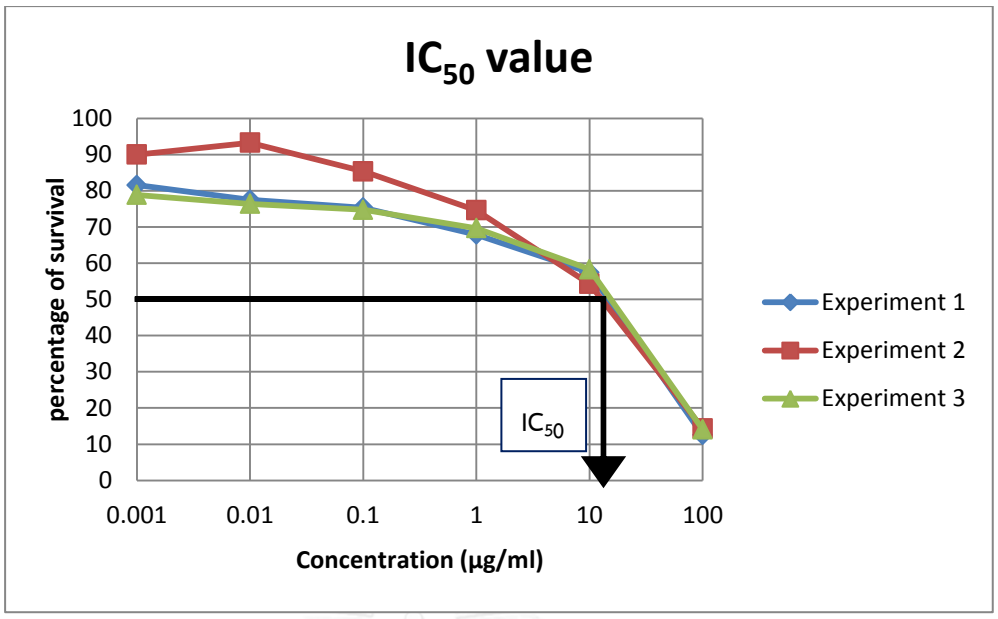


Figure 4.1 The IC_{50} value of cardanol from triplicate experiments.

4.2.1 Growth curve

The growth of untreated and treated BT-474 cells with various concentrations of cardanol (0.001-100 µg/ml) was observed (Figure 4.1). Among 4 phases of usual growth curve, the percentage of cell survival (PS) was recorded starting from lag phase to log or exponential growth phase because all treated cells at any concentration were dead at almost the end of log phase. Cardanol at the concentration of 100 µg/ml was the most effective in inhibition. In overall, the inhition by cardanol was time- and dose-dependent manner.

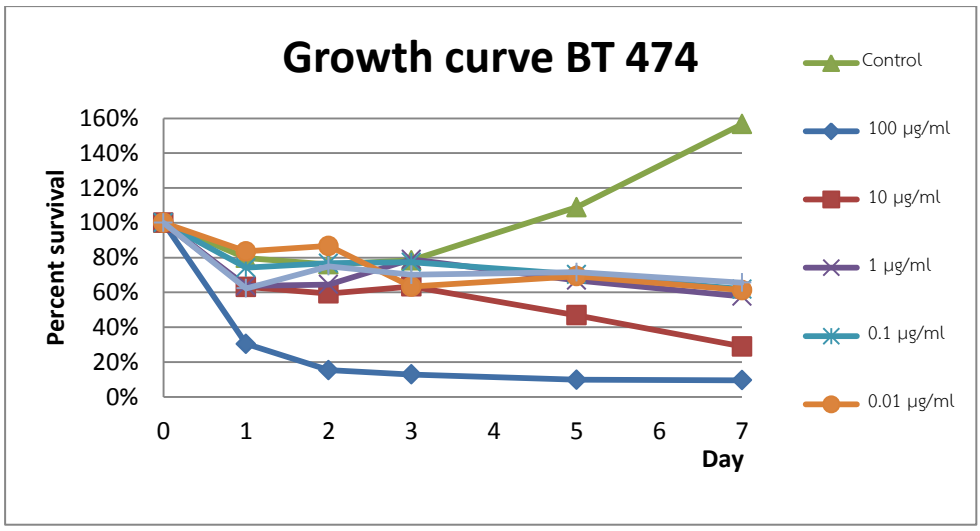


Figure 4.2 Growth curve of untreated and treated BT-474 breast cancer cell line.

4.2.2 Change in morphology of BT-474 cells

Both untreated and cardanol treated BT-474 cells (IC_{50} value conc.) had been incubated for 96 h. Every 24 h, cells were recorded for morphology change. The morphology of untreated BT-474 cells was shown in Figure 4.3. In overall, cells were alive. It looked flat and was attached to the substratum. However, the morphology of cardanol treated cells was differently changed from control. Floating cells could be observed after 24 h incubation. Cells started to shrink. Large clump cells could be observed at 48 h. Later, much less cell number could be seen at 72 h.

For this experiment, doxorubicin (0.5 µg/ml), a present chemotherapeutic drug, was used as positive control. The morphology of doxorubicin treated BT-474 cells was also differently changed from control but was similar changed as cardanol treated BT-474 cells. However, smaller clump of custard apple shaped cells and less number of livable cells.

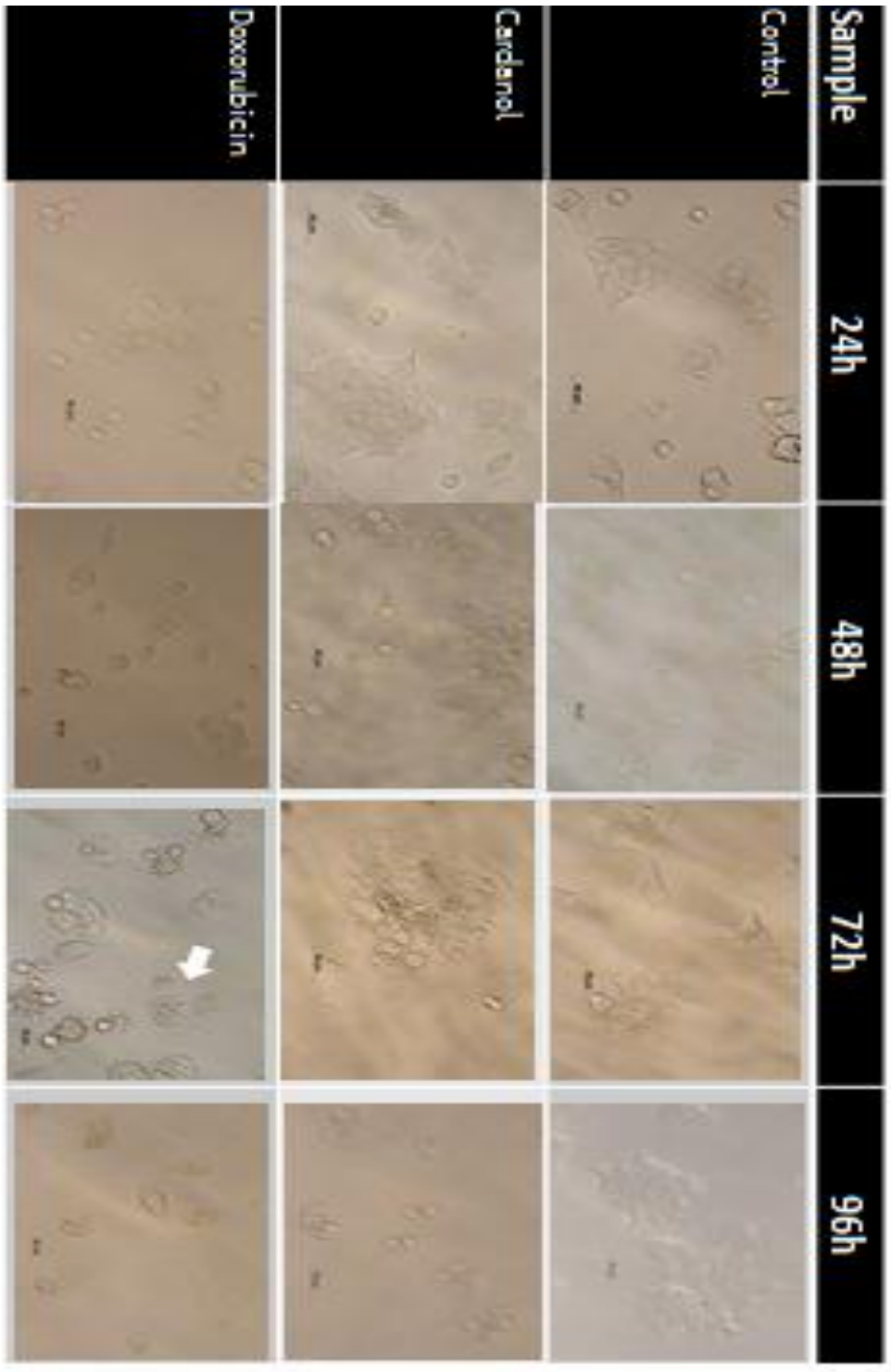


Figure 4.3 The morphology of cardanol treated BT-474 cells at 24, 48, 72, and 96 h. They were shown in 4.3. All images were magnified at 200x. Triplication of experiments was done.

4.3 Program cell death

4.3.1 Apoptosis or necrosis

Program cell death was determined by the distribution of annexin V and PI stained cells using flow cytometry. Cells were from 3 groups which were 1) stained untreated cells, 2) stained and cardanol treated cells, and 3) stained and doxorubicin treated cells. In each data set, four boxes would be reported. Box 1 represented necrotic cells. Box 2 represented late apoptosis and necrosis. Box 3 represented livable cells and box 4 represented early apoptosis.

For 24 h treatment, it was shown as in Figure 4.4 (A-C), Table 4.2, and Figure 4.4.

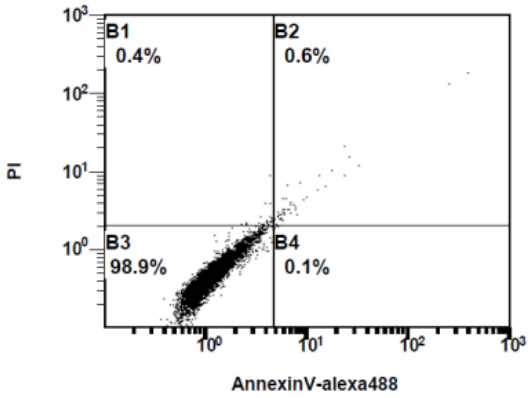
For 48 h treatment, it was shown as in Figure 4.6 (A-C), Table 4.3, and Figure 4.5.

For 72 h treatment, it was shown as in Figure 4.8 (A-C), Table 4.4, and Figure 4.6.

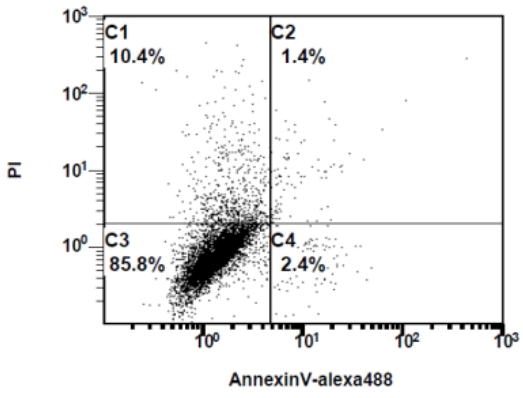
And all data analysis were shown in appendix B

By using flow cytometry, the scattering of stained cells at 24 h of treatment was shown as followed.

(4.4A) **Untreated**



(4.4B) **Cardanol treated, 30 µg/ml**



(4.4C) **Doxorubicin treated, 0.5 µg/ml**

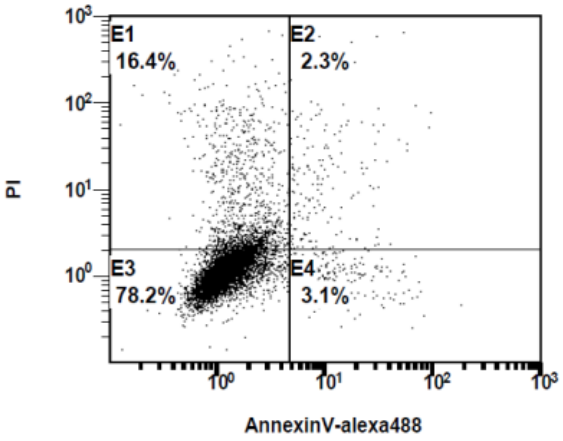


Figure 4.4 Program cell death. After 24 h incubation, the result of untreated cells, cardanol treated cells, and doxorubicin treated cells were shown in 4.4A, 4.4B, and 4.4C, respectively. Duplication of experiments was done.

According to Figure 4.4, the percentage of cell death whether by early apoptosis, late apoptosis, or necrosis was shown in Table 4.2 and Figure 4.5. In overall, it looked that both cardanol treated (9.05%) and doxorubicin treated (13.2%) cells were mainly dead by necrosis. Within the same treatment, no significant difference was observed.

Table 4.2 **The percentage of dead cells (24 h) which was analysed from the data of flow cytometry.**

BT-474 cells	Livable cells (V^-/PI^-)	Early apoptosis (V^+/PI^-)	Late apoptosis (V^+/PI^+)	Necrosis (V^-/PI^+)
Control	95.80±4.38	0.45±0.49	0.55±0.07	3.20±3.96
Cardanol treated	88.60±3.96	1.40±1.41	0.95±0.64	9.05±1.91
Doxorubicin treated	81.25±4.31	1.75±1.91	3.80±2.12	13.2±4.53

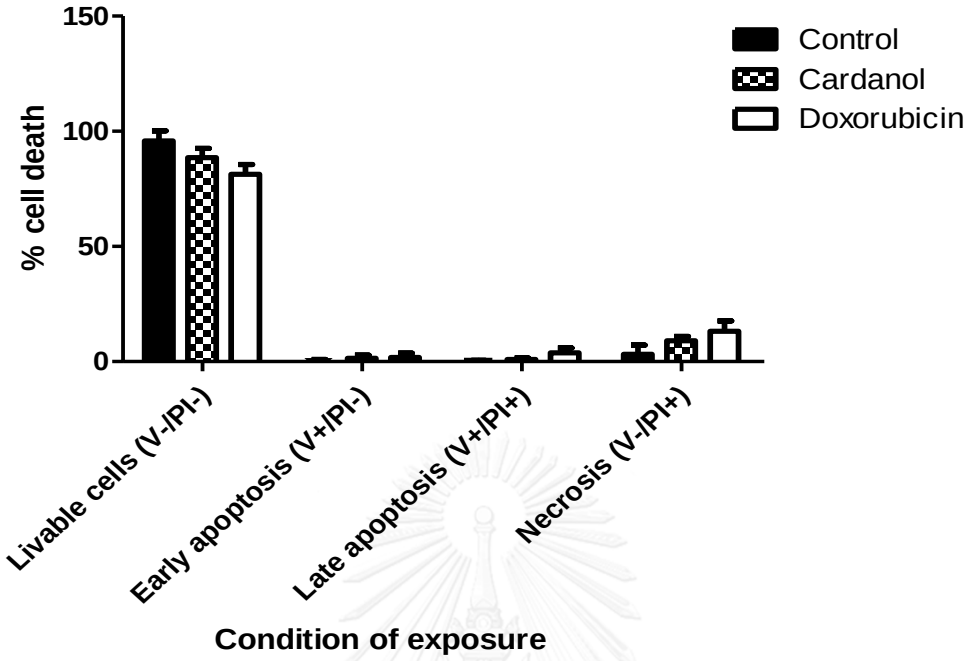


Figure 4.5 The percentage of cell death after 24 h treatment. Three groups of cells which were stained untreated cells, cardanol treated cells, and doxorubicin treated cells were used. The percentage of livable cells, early apoptosis, late apoptosis, and necrosis was shown.

By using flow cytometry, the scattering of stained cells at 48 h of treatment was shown as followed.

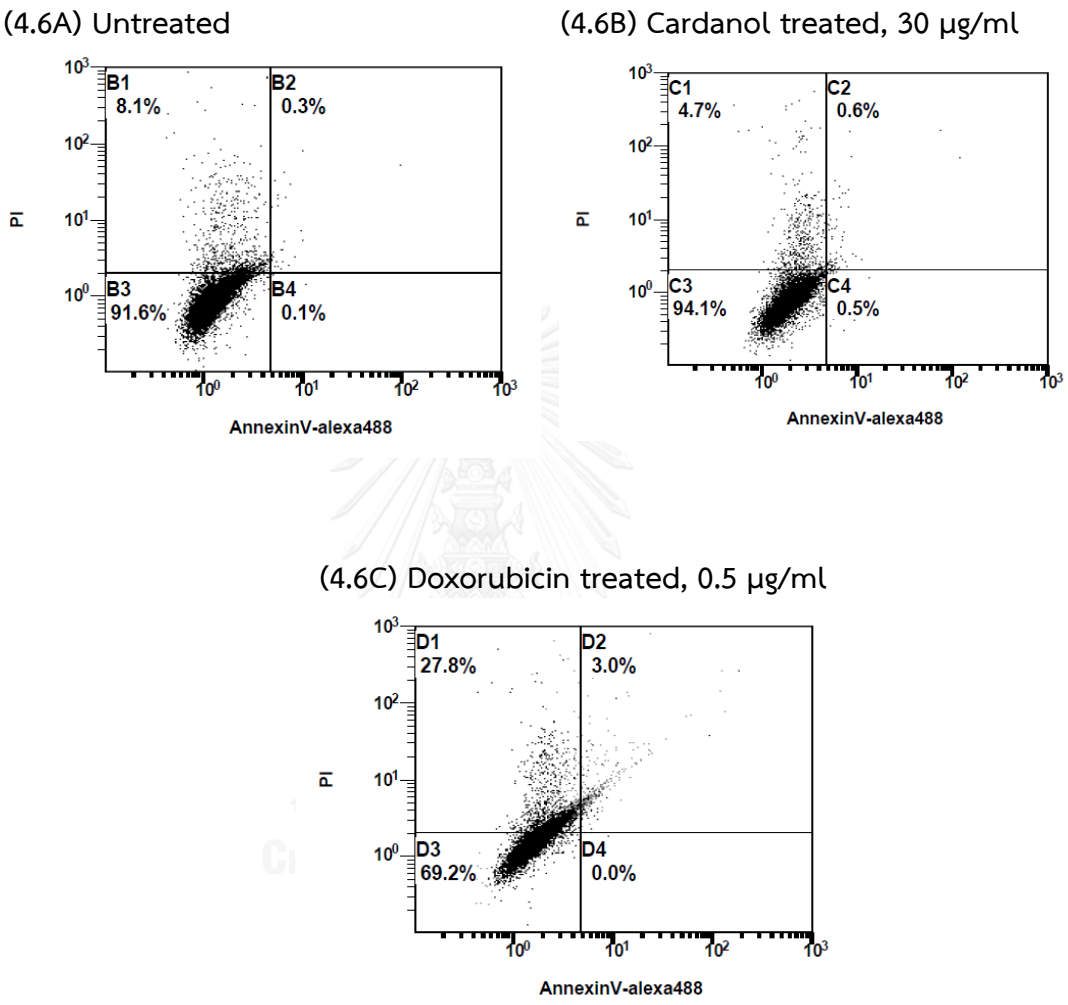


Figure 4.6. Program cell death. After 48 h incubation, the result of untreated cells, cardanol treated cells, and doxorubicin treated cells were shown in 4.6A, 4.6B, and 4.6C, respectively. Duplication of experiments was done.

According to Figure 4.6, the percentage of cell death at 48 h whether by early apoptosis, late apoptosis, or necrosis was shown in Table 4.3 and Figure 4.7. In overall, it looked that both cardanol treated (15.45%) and doxorubicin treated (29.85%) cells were mainly dead by necrosis. Within the same treatment, significant difference was observed for % cell death between control and doxorubicin treated cells, but not cardanol treated cells. Comparing to control (86.3%), less viable cells was significantly found in doxorubicin treated cells (59.55%). In contrast, More % cell death could be found in doxorubicin treated cells (29.85%), comparing to control (8.55%).

Table 4.3 **The percentage of dead cells (48 h) which was analysed from the data of flow cytometry.**

BT-474 cells	Livable cells (V^-/PI^-)	Early apoptosis (V^+/PI^-)	Late apoptosis (V^+/PI^+)	Necrosis (V^-/PI^+)
Control	86.30±7.46	2.30±3.11	3.85±5.02	8.55±0.78
Cardanol treated	78.55±7.84	1.70±1.69	4.25±5.16	15.45±1.06
Doxorubicin treated	59.55±6.72	2.70±3.82	7.90±6.93	29.85±2.90

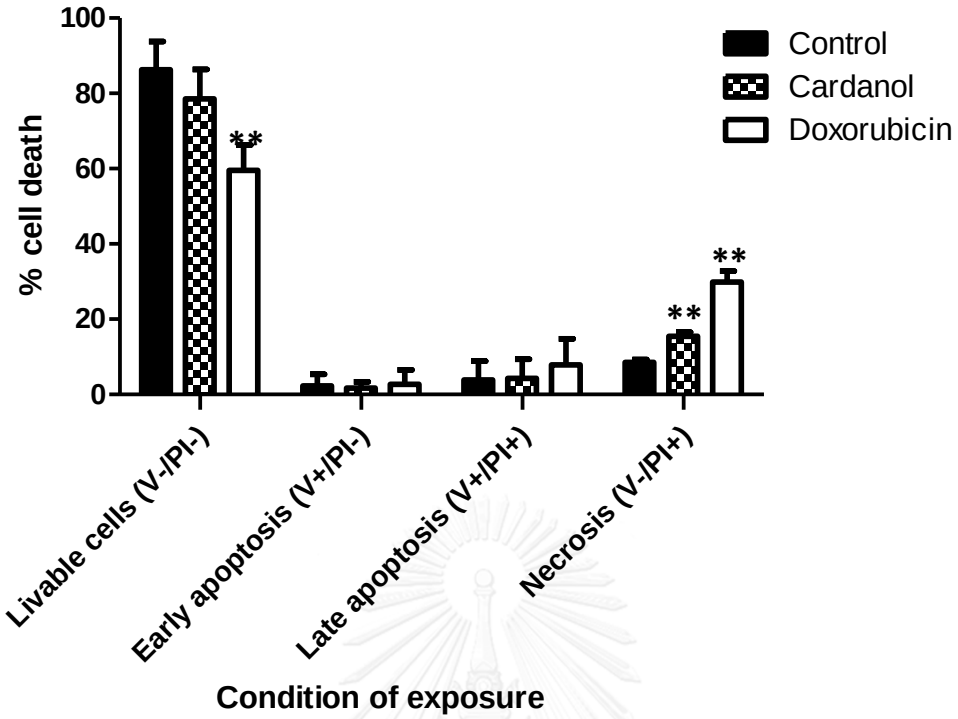


Figure 4.7 The percentage of cell death after 48 h treatment. Three groups of cells which were stained untreated cells, cardanol treated cells, and doxorubicin treated cells were used. The percentage of livable cells, early apoptosis, late apoptosis, and necrosis was shown. The significant difference was shown at **$p < 0.01$.

By using flow cytometry, the scattering of stained cells at 72 h of treatment was shown as followed.

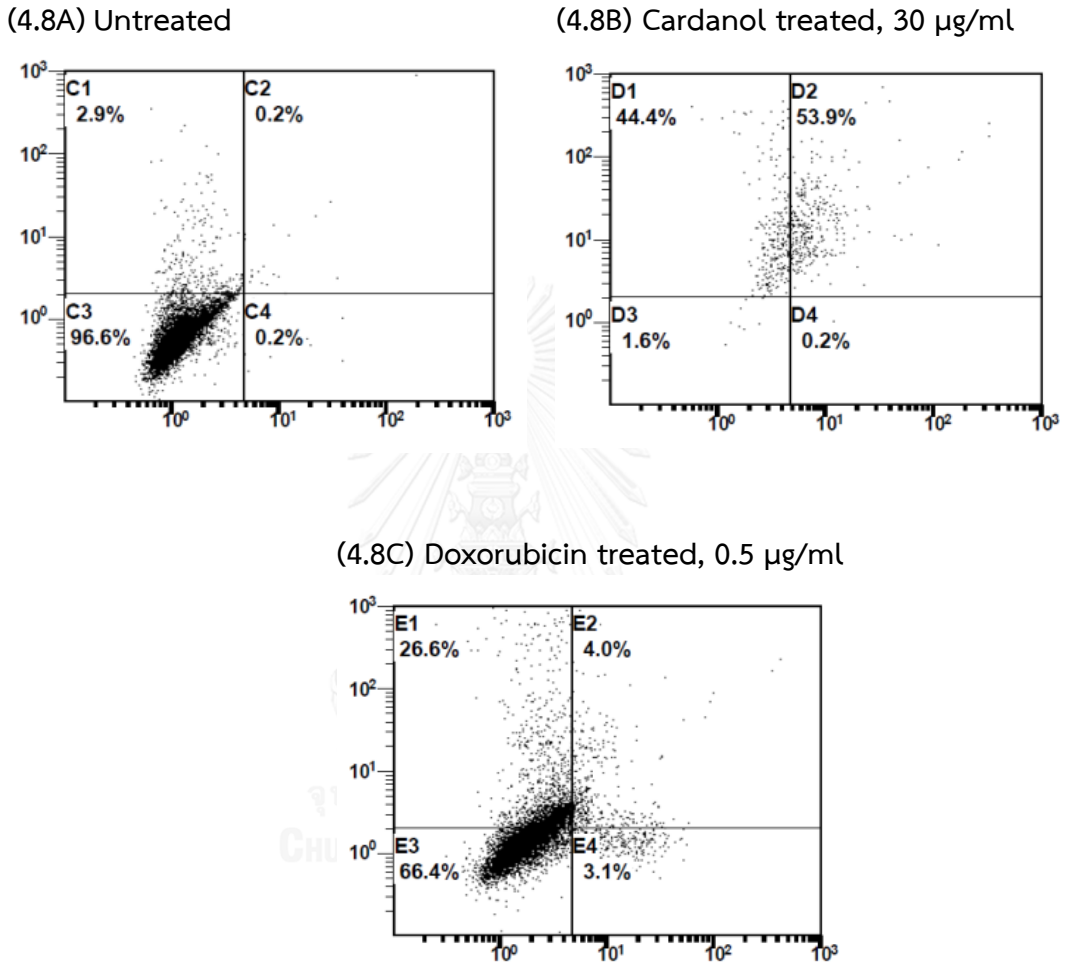


Figure 4.8 Program cell death. After 72 h incubation, the result of untreated cells, cardanol treated cells, and doxorubicin treated cells were shown in 4.8A, 4.8B, and 4.8C, respectively. Duplication of experiments was done.

According to Figure 4.8, the percentage of cell death at 72 h whether by early apoptosis, late apoptosis, or necrosis was shown in Table 4.4 and Figure 4.9. In overall, it looked that both cardanol treated (25.4%) and doxorubicin treated (35.8%) cells were mainly and significantly dead by necrosis. Considering livable cells, significant difference was observed among those three groups. The significance in decrease of viable cells after treated with two compounds was obvious (47.3% for cardanol and 58.4% for doxorubicin).

Table 4.4 **The percentage of dead cells (72 h) which was analysed from the data of flow cytometry.**

BT-474 cells	Livable cells (V^-/PI^-)	Early apoptosis (V^+/PI^-)	Late apoptosis (V^+/PI^+)	Necrosis (V^-/PI^+)
Control	87.15±13.36	0.50±0.42	0.85±0.92	11.45±0.64
Cardanol treated	47.30±0.99	0.10±0.07	27.20±1.13	25.40±1.41
Doxorubicin treated	58.40±11.31	1.55±2.19	4.30±0.42	35.80±13.01

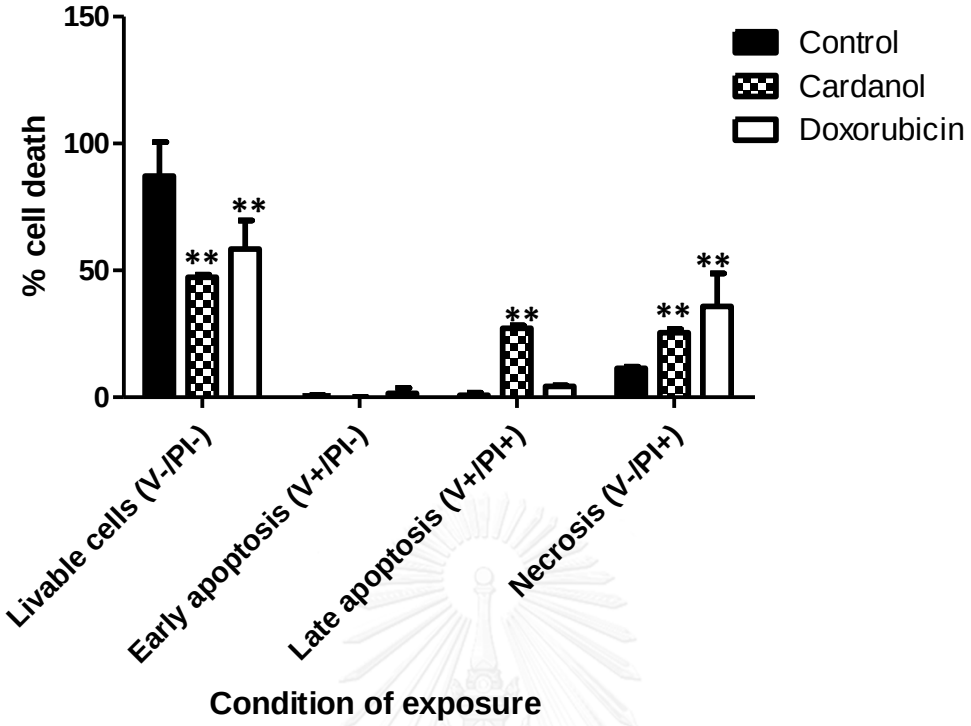


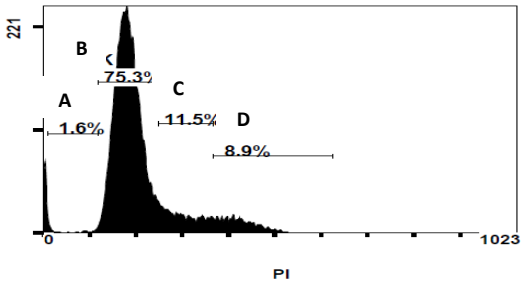
Figure 4.9 The percentage of cell death after 72 h treatment. Three groups of cells which were stained untreated cells, cardanol treated cells, and doxorubicin treated cells were used. The percentage of livable cells, early apoptosis, late apoptosis, and necrosis was shown. The significant difference was shown at **$p < 0.01$.

4.3.2 Cell cycle arrest

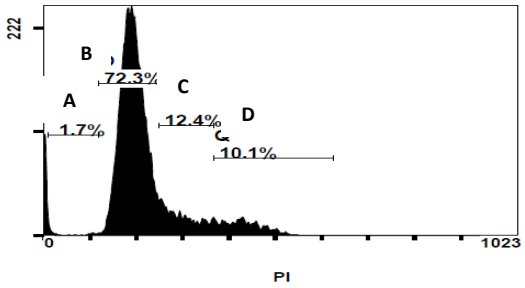
Three groups of BT-474 cells were prepared as 1) stained and untreated cells (control), 2) stained and cardanol treated cells, and 3) stained and doxorubicin-treated cells. All cells were incubated for total of 72 h. Cells were observed for cell cycle arrest at every 24, 48, and 72 h. Those target cells were stained with PI, cell cycle arrest was determined by flow cytometry. The results were shown in Figures 4.10 - 4.12 and appendix C. According to these reported Figures, the cell cycle was investigated at the interphase which was composed of 4 subphases, 1) pre G_1 subphase (A); 2) G_1 subphase (B); 3) S subphase (C); and G_2/M subphase (D).

For 24 h treatment, the cell cycle arrest was observed in Figure 4.10 as below.

(4.10A) Control



(4.10B) Cardanol treated, 30 µg/ml



(4.10C) Doxorubicin treated, 0.5 µg/ml

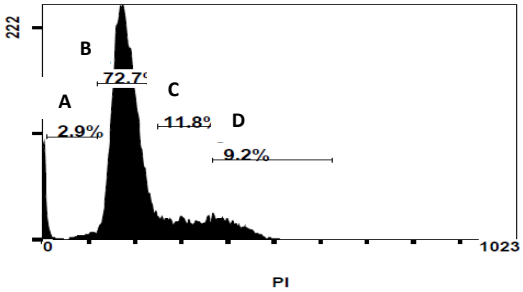
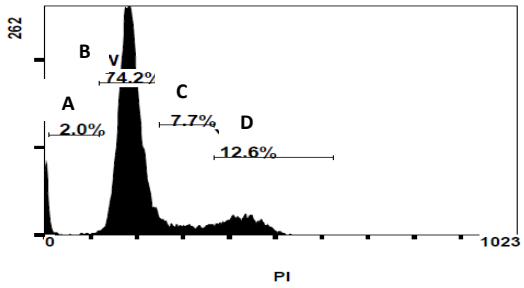


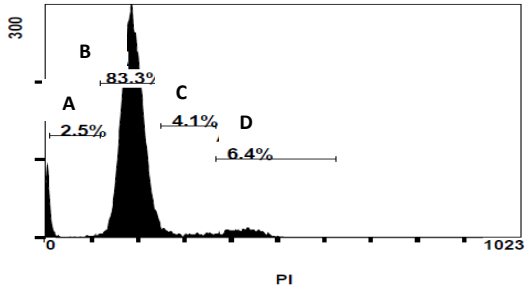
Figure 4.10 The cell cycle arrest of BT-474 cells after 24 h incubation. Control (4.10A), cardanol treated cells (4.10B), and doxorubicin treated cells (4.10C) were shown respectively. Duplication in experiments was done.

For 48 h treatment, the cell cycle arrest was observed in Figure 4.11 as below.

(4.11A) Control



(4.11B) Cardanol treated, 30 µg/ml



(4.11C) Doxorubicin treated, 0.5 µg/ml

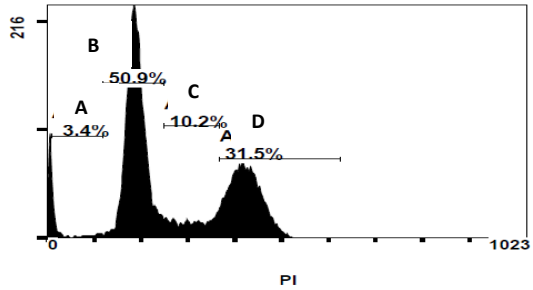
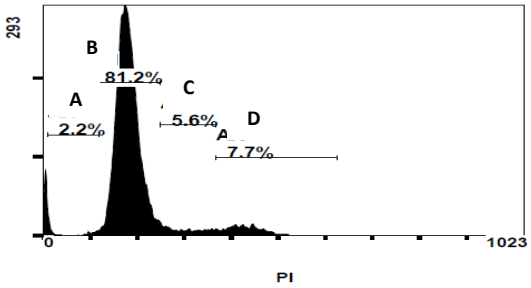


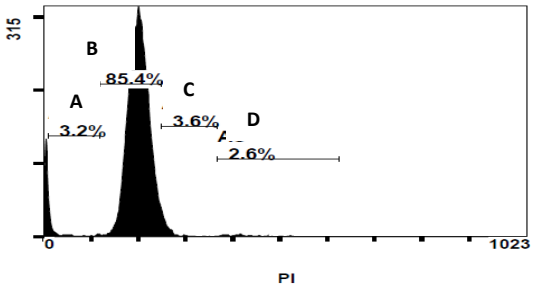
Figure 4.11 The cell cycle arrest of BT-474 cells after 48 h incubation. Control (4.11A), cardanol treated cells (4.11B), and doxorubicin treated cells (4.11C) were shown respectively. Duplication in experiments was done.

For 72 h treatment, the cell cycle arrest was observed in Figure 4.12 as below.

(4.12A) Control



(4.12B) Cardanol treated, 30 µg/ml



(4.12C) Doxorubicin treated, 0.5 µg/ml

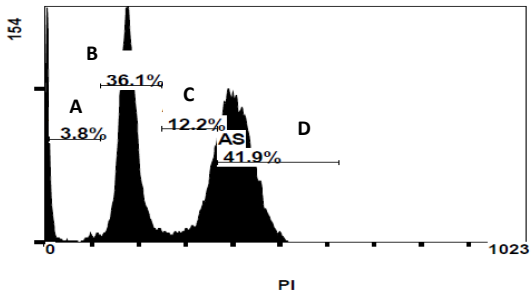


Figure 4.12 The cell cycle arrest of BT-474 cells after 72 h incubation. Control (4.12A), cardanol treated cells (4.12B), and doxorubicin treated cells (4.12C) were shown respectively. Duplication in experiments was done.

The cell cycle distribution was summarized and shown in Table 4.5. After 24, 48, and 72 h incubation, cardanol could increase the propotion of cells in the G_1 subphase of the cell cycle from 66.2% to 72.93%, 67.2% to 74.63%, and 71.47% to 80.70%, respectively. And doxorubicin could increase the propotion of cells in the G_2/M phase of the cell cycle from 19.27% to 20.57%, 20.13% to 30.00%, and 16.97% to 41.27%, respectively.

Table 4.5 Summary of the percentage of cell cycle arrest.

Cell cycle	Control			Cardanol treated			Doxorubicin treated		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Early G_1	1.1 ±0.70	1.3 ±0.89	1.13 ±0.97	1.73 ±1.15	2.47 ±0.55	1.97 ±1.30	2.03 ±0.85	3.5 ±1.05	5.27 ±3.91
G_1	66.2 ±11.41	67.2 ±6.30	71.47 ±8.71	72.93 ±10.17	74.63 ±3.44	80.70 ±4.12	59.47 ±11.46	46.93 ±3.44	31.77 ±.7.94
S	10.23 ±1.10	7.73 ±0.45	7.33 ±1.66	8.53 ±4.50	6.53 ±7.16	5.77 ±2.15	13.87 ±1.84	14.43 ±7.16	13.97 ±2.18
G_2/M	19.27 ±10.20	20.13 ±6.53	16.97 ±8.15	14.33 ±6.65	13.23 ±8.15	9.17 ±5.69	20.57 ±10.43	30.00 ±8.15	41.27 ±1.37

4.4 Change in gene expression

4.4.1 Transcriptional level

Total RNA extraction

Total RNA was extracted from three groups of BT-474 cells incubation at 72 h which were 1) untreated BT-474 cells (control), 2) cells treated with cardanol at 30 µg/ml, and 3) cells treated with doxorubicin at 0.5 µg/ml. The result was shown in Table 4.6. Considering the ratio of absorbance at 260 and 280 nm which indicated the purity of RNA, it was obvious that the obtained RNA with the ratio closed to 2.0

was good enough to continue for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Table 4.6 Summary of extracted total RNA.

Replicate	BT-474 cells	Abs260	Abs280	Ratio of Abs260/280	Conc. of total RNA (µg/ml)
# 1	Control	0.253	0.125	2.024	506
	Cardanol treated	0.337	0.176	1.915	674
	Doxorubicin treated	0.560	0.298	1.879	1120
# 2	Control	0.513	0.267	1.921	1026
	Cardanol treated	0.330	0.169	1.953	660
	Doxorubicin treated	0.532	0.274	1.942	1064
# 3	Control	0.602	0.316	1.905	1204
	Cardanol treated	0.460	0.250	1.840	920
	Doxorubicin treated	0.522	0.280	1.864	1044

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

To explore the effect of cardanol to the gene expression, primers used in this technique were classified into 2 groups. The first group belonged to the apoptosis regulating gene which contained, *b-cell lymphoma-2* (*Bcl-2*), *myeloid cell leukemia-1* (*Mcl-1*), *mitogen activating protein-kinase activating death domain* (*MADD*), *cellular FLICE-like inhibitory protein* (*c-FLIP*) and *human death receptor 5* (*DR5*). Due to Figure 4.13 and appendix D(1), the result showed cardanol could increase the *DR5* and *Bcl2* expression but decrease the expression level of *Mcl1 MADD and c-FLIP*. Doxorubicin could up-regulate the expression of *Bcl2* but down-regulate the expression of *Mcl1*, *MADD, c-FILP*, and *DR5*.

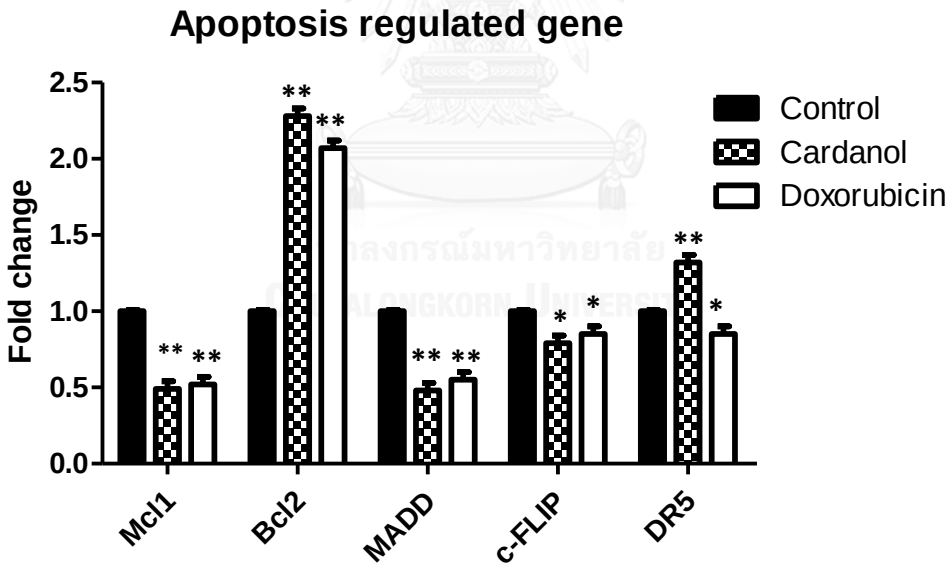


Figure 4.13 The change in expression of genes in death receptor group which were *human death receptor 5 (DR5), myeloid cell leukemia-1* (*Mcl-1*), *mitogen activating protein-kinase activating death domain* (*MADD*) and *b-cell lymphoma-2 (Bcl-2)*. Significant difference between untreated and treated cells (cardanol and doxorubicin) was shown at **$p < 0.01$ and *$p<0.05$.

The second groups belonged to cell cycle regulating genes. It contained *p2*, *cyclin D1*, *cyclin E*, *cyclin A*, *cyclin-dependent kinase 4* (*CDK4*), *cyclin-dependent kinase 6* (*CDK6*), and *cyclin-dependent kinase 2* (*CDK2*). According to Figure 4.14 and appendix D(2), the result showed that cardanol could increase the expression of *p21* and *E2F1* but could decrease the expression of *cyclin D1, cyclin E*, *CDK4*, and *CDK2*. In addition, doxorubicin could up-regulate *E2F1*, *p21*, *cyclin E*, *cyclin A*, *CDK4*, *CDK6* and *CDK2* gene expression.

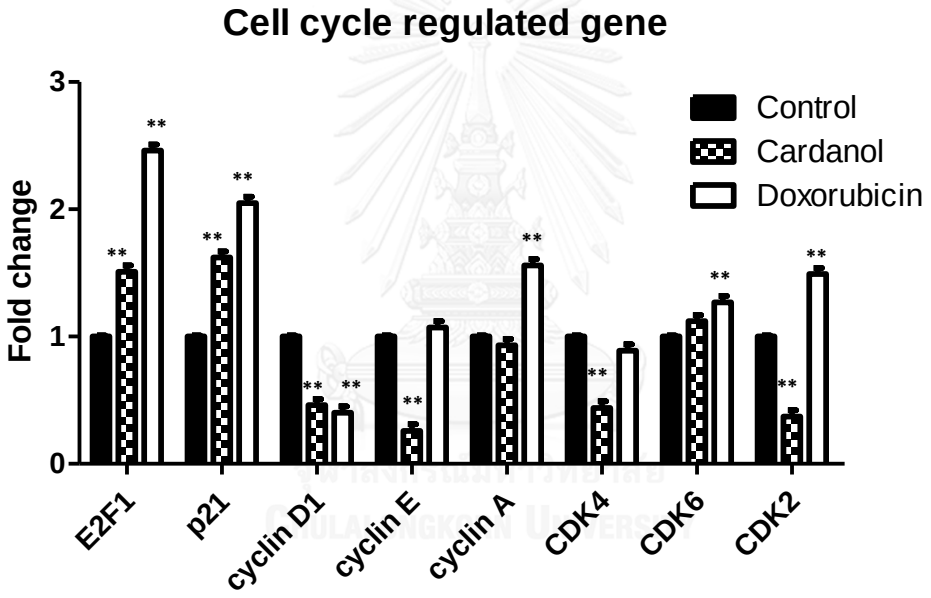


Figure 4.14 The change in expression of cell cycle regulating genes which were transcription factors important for cell cycle. The expression of *p21, cyclin D1, cyclin E*, *cyclin A, CDK4*, *CDK6*, and *CDK2* was investigated. Significant difference between untreated and treated cells (cardanol and doxorubicin) was shown at **$p < 0.01$.

4.4.2 Translational level

Effect of cardanol on ERK, JNK and p38 MAPK, p21 and cyclin D1 activity on BT- 474 cell line was focused by using Western blot. The activation of ERK, JNK, and p38 MAPK was measured in order to find out whether it was responded to cardanol. After BT-474 cells were treated for 24 h, the result could be revealed as shown in Figure 4.15. Cardanol could activate ERK, JNK, and p38 MAPK. By increasing phosphorylation of ERK, JNK, and p38 MAPK, it could increase p21 and cyclin D1 expression. In overall, the results strongly suggested that the G_1 subphase arrest by cardanol was mediated by activation of MAPK-p21 pathway.

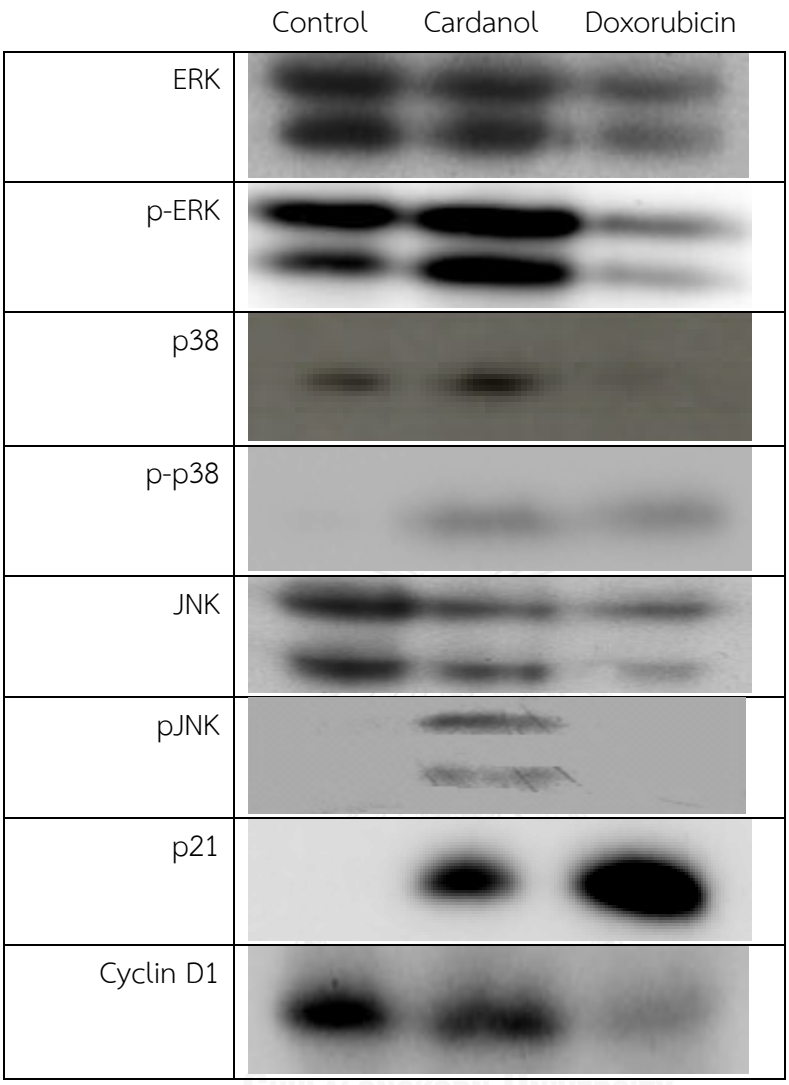


Figure 4.15 Western blot of target protein in untreated and treated BT-474 cell lines after 24 h incubation. “Control” was untreated cells. “Cardanol” represented cells treated with cardanol at 30 µg/ml and “doxorubicin” represented cells treated with doxorubicin at 0.5 µg/ml. Unphosphorylated and phosphorylated forms of protein were shown. For example, JNK was unphosphorylated form while pJNK was phosphorylated form.

CHAPTER V
DISCUSSION

Bees collected nectar, bee pollen, and resin from plants so many compounds found in bee products were found in plants as food source. For example, α-pinene was reported to be the major compound in European propolis. It was originated from many plant species like *Cupressus sempervirens*, coniferous species (Milos et al., 2002) [36].

Compounds with exact chemical structures and modified chemical structures were found in propolis. For example, galangin, chrysin, and pinocembrin were found to be main compounds in Serbian propolis. By using ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC-LTQ/Orbitrap/MS/MS), the spectrum presented that the obtained compounds were similar to the resin of poplar trees which were widely distributed in Europe (Ristivojević et al., 2014).[47]

Furthermore, Bankova et al. (2014)[2] reported that propolis from Hungary, Bulgaria, France, and Northern Italy contained resin from poplar trees also. However, one of major compounds found in those propolis types were non-terpenic compounds, benzyl alcohol and benzyl benzoate. Considering, benzyl benzoate, it was not detected in the volatile oils of poplar buds. The difference could be due to chemical variations of the volatiles of different poplar subspecies. Thus, it could be concluded that bud exudates even of the same species demonstrated quantitative variability in a wide range.

Previously, Teerasripreecha et al. (2012)[54] reported the major compounds in *Apis mellifera* propolis harvested from Nan province, Thailand were cardol and cardanol. Both of them could inhibit the proliferation of breast cancer BT-474 cell line but cardanol was ineffective to normal cells. Thus, that led to my interest to

continue the research on cardanol. Since cardanol was not commercially available, I had to purify it by following the method reported earlier.

Considering Figure 4.1, cardanol inhibited the growth of BT 474 cells with time and dose dependent manner. The manner like this was supported by many reports. Park et al. (2014)[43] reported that crude EtOH extract (CEE) of propolis harvested from many regions in Korea could inhibit the angiogenesis (tube formation of human umbilical vein endothelial cells or HUVECs with dose dependent manner (6.25 - 25 μg/ml). In addition, CEE from Uijeongbu and Pyoseon regions could significantly suppress the proliferation of HUVECs in a dose dependent manner (3.13 - 25 μg/ml). Thus, they purposed that Korean propolis was potential in the prevention and treatment of angiogenesis-related diseases such as cancer.

Considering the program cell death in Figure 4.5 and Table 4.2, cardanol killed BT-474 cells at late apoptosis (apoptosis and necrosis) which was similar to doxorubicin which was chemotherapeutic drug. The result of program cell death like this was commonly found in compounds purified from natural products including chemotherapeutic drugs. Besides propolis, it was found that Tualang honey could induce late apoptosis to human breast adenocarcinoma (MCF-7 and MDA-MB-231) and cervical (HeLa) cancer cell lines. With the EC_{50} value of 2.4-2.8%, MDA-MB-231 cells treated with Tualang honey at 24 h showed the highest percentage of late apoptosis at 37.8% while MCF-7 and Hela cells showed the highest percentage of late apoptosis at 55.6% and 56.2%, respectively (Fauzi et al., 2011).[10]

A mechanism of apoptotic cell has many pathways. In this research, expression of apoptosis-related genes including, *b-cell lymphoma-2* (*Bcl-2*), *myeloid cell leukemia-1 (Mcl-1*), *mitogen activating protein-kinase activating death domain* (*MADD*), *cellular FLICE-like inhibitory protein* (*c-FLIP*) and *human death receptor 5* (*DR5*) was determined. Cardanol affected expression of apoptosis-relating genes by

increasing *Bcl2* and *DR5* but decreasing *Mcl1*, *c-FILP*, and *MADD*. It was similar to doxorubicin (positive control). As known, DR5 was apoptosis inducing membrane receptor for tumor necrosis factor-related apoptosis-inducing ligand. Previously, apoptosis in human renal cancer cells could be induced by up-regulation of *DR5* and down regulation of *c-FLIP*. In addition, it also showed that the combined compounds between rosiglitazone and TRAIL could induce apoptosis in renal cancer cells. The combined compounds could induce *Bcl2* overexpression. Similarly, Yang et al. (2011) [63]reported that acrolein could effectively sensitize human renal Caki cells to TRAIL-induced apoptosis through down-regulating *Bcl-2* and up-regulating *DR5*. This was mediated by ROS generation and CHOP induction. Thus, lowering the TRAIL resistance or increasing the damage of tumor cells could help in cancer therapy. Moreover, it showed that knockdown of *MADD* and *c-FLIP* could reduce the resistance to TRAIL-induced apoptosis in SKOV-3 ovarian cancer cells. SKOV-3 cells with *MADD* and *c-FLIP* knock down turned to be more sensitive to apoptosis at 64.21 ± 3.03%.

As known, *Mcl1* was an anti-apoptotic member in the *Bcl2* family which was apoptosis regulating protein. In 2013, Zhou et al.[65] reported that benzyl isothiocyanate (BITC), an anti-cancer agent, could affect cell cycle arrest and apoptosis in human leukemia cell lines. The data showed the down-regulation of *Mcl-1* which resulted in cell cycle arrest at G_2/M phase and apoptosis mediated by BITC.

Cardanol could arrest the cell cycle at G_1 subphase. The potential like this was similar to propolin H which was purified from Taiwanese propolis, It was active against human lung carcinoma H460 cell line and significantly arrested the cell cycle at G_1 subphase. Treated H460 cells with 40 μM of propolin H resulted in increasing the percentage of cells at G_1 subphase from 57.8% to 75.1%. Hence, propolin H induced the cell cycle arrest at G_1 subphase (Weng et al., 2007).[60]

As known, cancer cells are uncontrolled in growth and present abnormal function in gene expression. Considering its cell cycle which is compared to normal cell cycle, there are more expression level of regulating genes such as cyclin and cyclin dependent kinase. Thus, they can induce the cell cycle to move to the next phase. Considering the effect of cardanol to cell cycle distribution of BT-474 cells, cardanol mostly afftected expression of many genes important for cell cycle because it could decrease p21, cyclin D1, cyclin E, CDK2 and CDK4 but increase p21 and E2F1. The obtained results here were coincided to He et al. (2006).[56] They reported that caffeic acid phenethyl ester (CAPE) at the concentration of 2.5, 5, 10, 20, 40, and 80 mg/l could increase the percentage of cells at G_1 subphase with the dose dependent manner. It could increase the expression of b-catenin but decrease the expression of cyclin D1 and c-myc. Also, Kuo et al. (2005) [29]reported that CAPE (50 μg/ml) could inhibit the growth of C6 glioma cells with the cell arrest at G_1 subphase after 24 incubation. Moreover, CAPE decreased CDK2/cyclin E and CDK4/cyclin D activity but inhibited Rb phosphorylation by increasing p21, p27, and p16 expression.

The way cardanol could induce G_1 cell cycle arrest by activating p21, p38 MAPK, JNK and ERK protein was similar to isothiocyanate sulforaphane (SFN) which was a chemotherapeutic drug. A mechanism of SFN on human colon carcinoma was that SFN could induce the expression of $p21^{CIP1}$ and cyclin D1 by activating MAPK pathways, including ERK, JNK, and p38 (Shen et al., 2006).[17]

With the background that propolis composition was complex and very much variable in different regions so it affected bioactivities of bee products directly. Also, both crude and purified forms were potential to use in traditional medicine, bio - cosmetics, and functional foods. The advantage of using crude form was that it was easily prepared, cheap, and practical to use. In 2013, Khacha-Ananda et al.[24] reported that the anti-oxidant activity and antiproliferative activity of crude propolis collected from Phayao province, Thailand. Crude propolis extracted by sonication showed the antioxidant activity by 3.30 ± 0.15 mg gallic acid equivalents/g extract.

Also, the same crude extract could give the antiproferative activity against HeLa cervival cancer cells with the IC_{50} value of 58.77, 58.66, and 59.61 μg/ml for 24, 48, and 72 h incubation, respectively.

In overall, it could be observed that doxorubicin was more efficient in inhibition of BT-474. However, cardanol can be developed as an alternative source.

Alternatively, synergistic effect between compounds chemotherapeutic drug and cardanol or between cardanol and other active compound purified from propolis like CAPE, apigenin, kaempferol, α-mangostin should be continued. Moreover, synergistic effect between a pure compound and crude extract should be tried as well. This hypothesis was supported by Oliveira et al. (2014) although no synergistic effect of their compounds were reported. They found that, at the crude level, the lowest IC_{50} values of Brazilian green propolis and *Baccharis dracunculifolia* extracts were 41.0 ± 4.5 μg/ml for U343 cancer cell line and 44.9 ± 7.1 μg/ml for $HepG_2$ cancer cell line, respectively. After Brazillian propolis was purified, artepillin C and baccharin were found to be dominant. The lowest IC_{50} values of artepillin C and baccharin were changed to be 20.1 ± 2.9 μg/ml for U343 cells and 13.0 ± 1.5 μg/ml for B16F10 cells, respectively. The data presented that the purified compounds were more potential than crude extracts. However, the combination of artepillin C and baccarin showed no synergistic effect since the lowest IC_{50} value was increased to be 35.2 ± 0.5 μg/ml for B16F10 cancer cells. Nonetheless, this combination were synergistic for the mutagenic effect of the ethyl acetate extract of *B. dracunculifolia* leaves (11.4-182.8 μg/plate) and artepillin C (0.69-10.99 μg/plate) by the Ames test using *Salmonella typhimurium* strains TA98, TA97a, TA100 and TA102 (Resende et al., 2012).

In this research, doxorubicon was used as positive control. In 2012, Thorn et al.[55] reported doxorubicin action. It was an anthracyline drug extracted from *Streptomyces peucetius* var *caesivs* and was used for cancer treatment including

breast, lung, gastric, ovarian, thyroid, non-Hodgkin's, and Hodgkin's lymphoma. The mechanism of this drug on cancer inhibition was described in 2 pathways. First, it would bind to DNA and disrupt topoisomerase II – mediating DNA repair. Second, it produced free radicals and damaged cell membrane, DNA, and protein. Later, that resulted in DNA damage and cell death. In this research, the data also supported the effect of doxorubicin on BT-474 cell line. It showed cell cycle arrest at G_2/M subphase. It up-regureted expression of *Bcl2*, *E2F1, p21, cyclin A*, *CDK6,* and *CDK2* but down-regulated expression of *Mcl1, MADD, c-FLIP*, and *cyclin D1.* Thus, doxorubicin was purposed to have a mechanism on inhibiting DNA synthesis by p21 mediating gene and cyclin D1. Also, it decreased expression of anti-apoptotic genes like *Mcl1* and *c-Flip*.

In summary, a purposed mechanism of cardanol on how it could inhibit the growth of BT-474 breast cancer cells was shown (Figure 5.1). Cardanol increased phosphorylation of ERK, JNK, and p38 MAPK leading to p21. Then, p21 suppressed CDK4/cyclin D, and cyclin E/CDK2 so that caused retinoblastoma protein unable to be hyperphosphorylated. This led to obstruct DNA synthesis and the movement of cells ahead from S subphase.

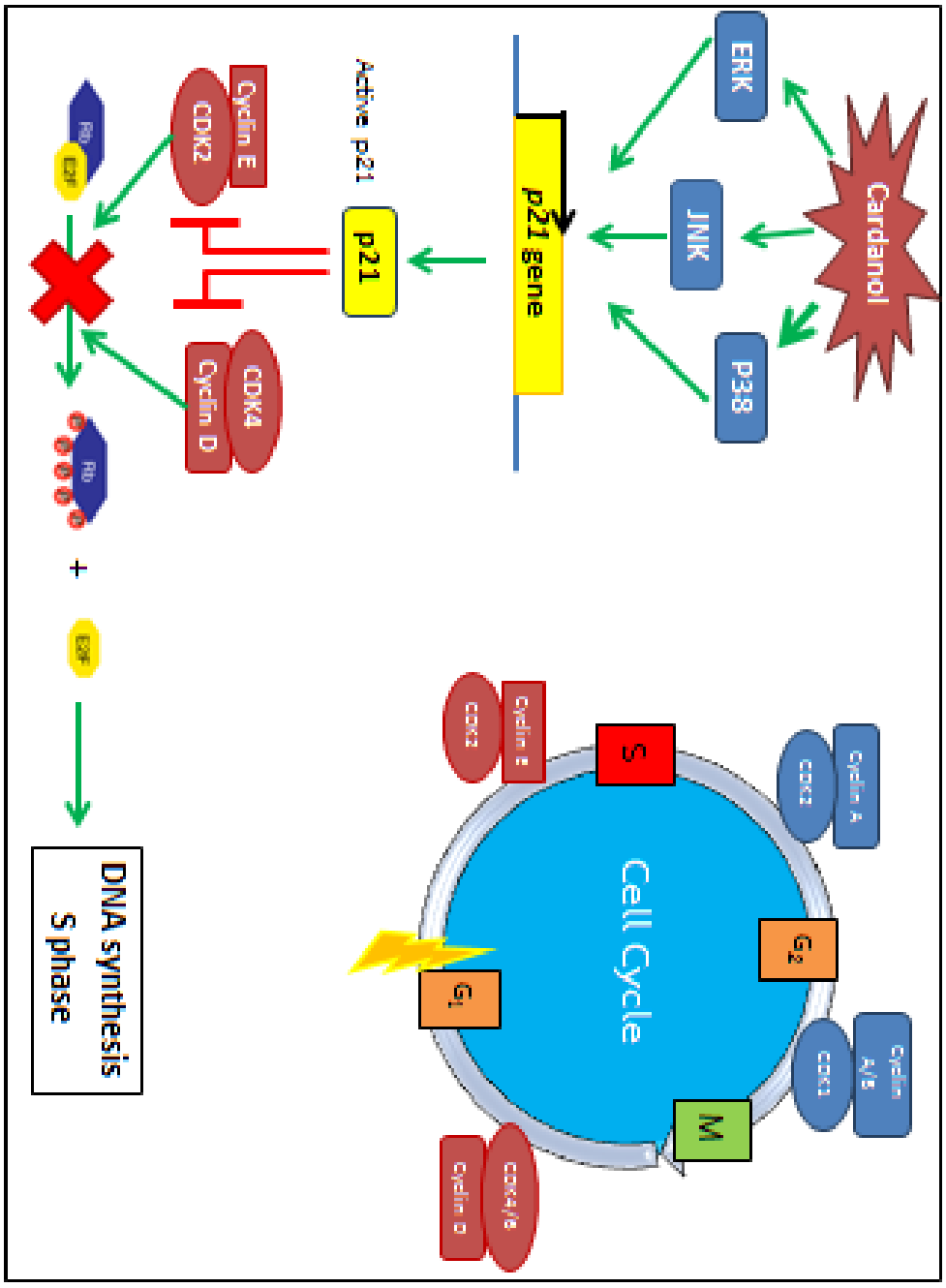


Figure 5.1 A purposed mechanism of cardanol on BT-474.

CHAPTER VI

CONCLUSION

1. *Apis mellifera* propolis from Nan province, Thailand was extracted by MeOH, CH_2Cl_2, and hexane so it yielded CME, CDE, and CHE. CDE was the best for anti-proliferative activity against BT-474 cells with the IC_{50} value of 29.97 ± 6.17 µg/ml.

2. CDE was purified by quick column and adsorption chromatography. Then, the obtained cardanol was proved by Rf value using thin layer chromatography and mass spectrometry. By MTT assay, cardanol showed the IC_{50} value of 15.57 ± 1.73 µg/ml.

3. For growth curve investigation, cardanol treated cells at the concenctration range of 0.001 - 100 µg/ml had been incubated for 7 days. The obtained growth curves showed the inhibition of cardanol was time and dose dependent manner.

4. Morphology of untreated and cardanol treated BT-474 cells were observed. The shape of cells in both groups was differently changed. For treated cells, cell floating, shrinking, large clump, and less cell number could be noticed.

5. In order to analyse program of cell death, stained cells with annexin V and propidium iodide (PI) was observed by using flow cytometry. It was obvious that cardanol treated cells at 72 h were significantly dead by late apoptosis (both of apoptosis and necrosis). The percentage of dead cells was increased from 0.85% to 27.2%.

6. Due to the distribution of PI stained cells, it was shown that the cell cycle was arrested at G_1 subphase. Cardanol could increase the percentage of cells in G_1

subphase from 66.2% to 72.93%, 67.2% to 74.63%, and 71.47% to 80.70%, respectively.

7. The effect of cardanol on gene expression was determined by qRT-PCR. Selected genes were classified into 2 sets. For the first set which genes belonged to the apoptosis regulating gene, cardanol could increase the *DR5* and *Bcl2* expression but could decrease the expression level of *Mcl1, MADD and c-FLIP*. And the second set, the genes belonged to cell cycle regulated gene. Cardanol could decrease the expression level of *p21* and *E2F1* but could decrease the expression of *cyclin D1, cyclin E*, *CDK4*, and *CDK2* significantly.
8. Finally, the expression change was confirmed by western blot. After BT-474 cells had been treated with cardanol for 24 h, it was found that cardanol had an effect on ERK, JNK and p38 MAPK, p21 activity. It could increase the amount of phosphorylated ERK, JNK and p38 MAPK and p21 expression Thus, the results strongly suggested that MAPK regulated p21-mediated G_1 phase cell cycle arrest was a mechanism underlying growth inhibitory effect of cardanol in BT-474 cells.

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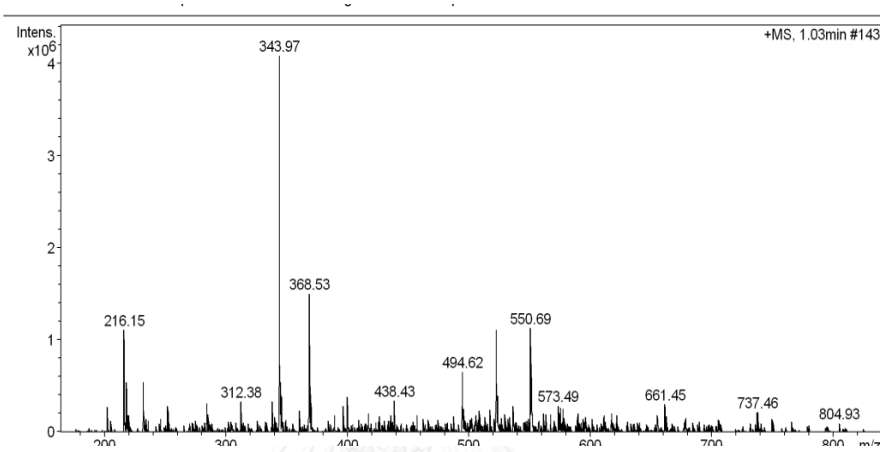
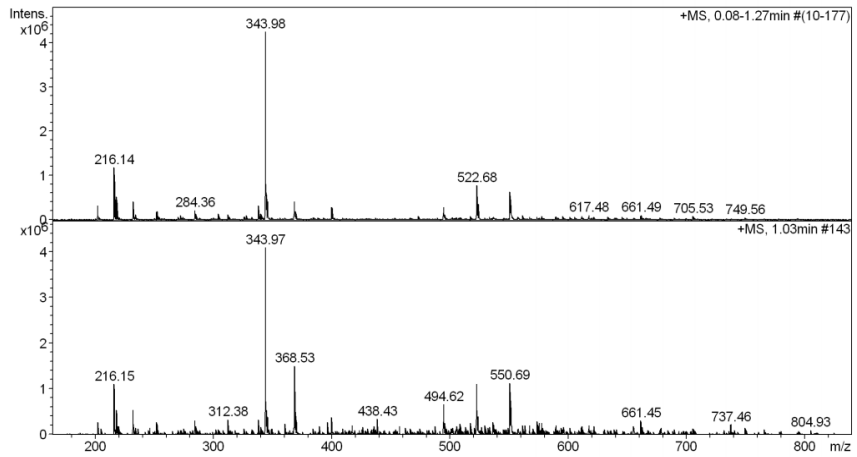
APPENDIX

APPENDIX A

Structure of cardanol by mass spectrometry

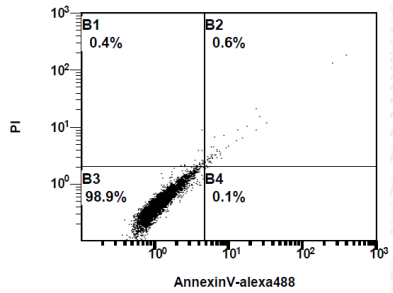
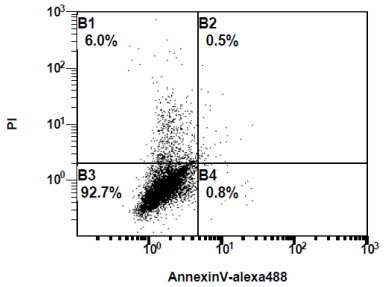
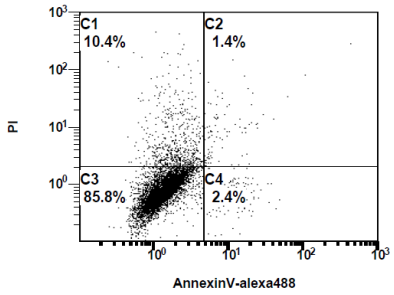
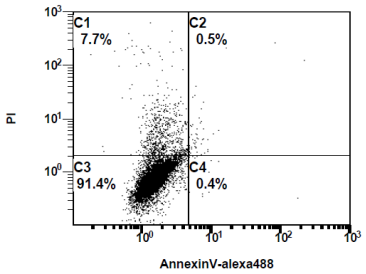
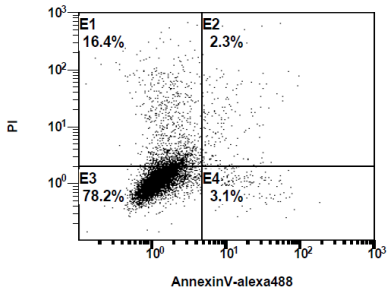
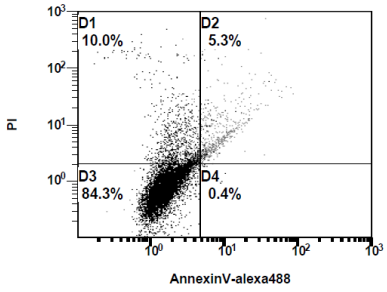




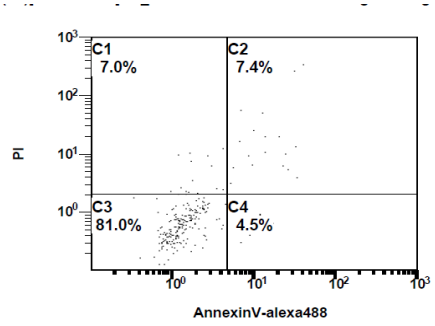
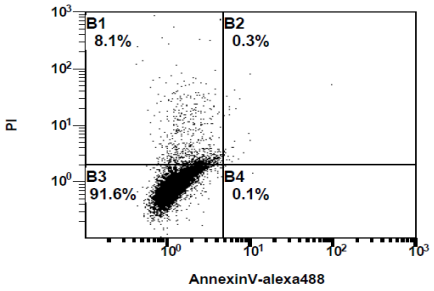
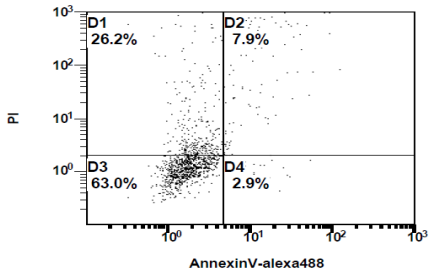
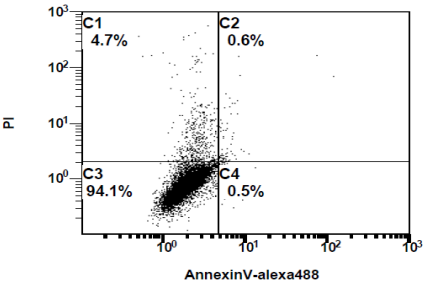
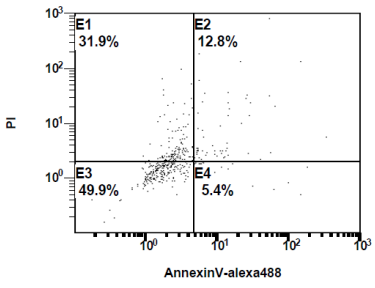
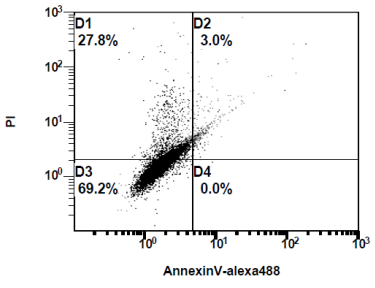
APPENDIX B

The results of program cell death using Annexin V and PI staining by flow cytrometry.

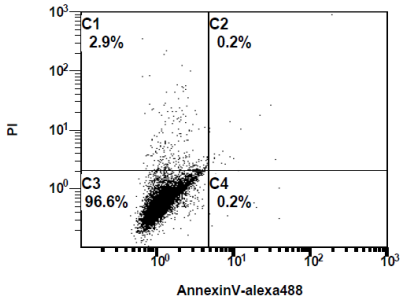
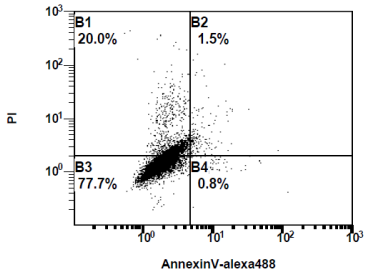
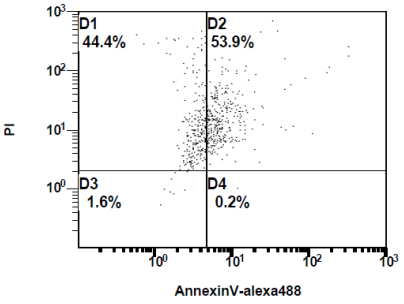
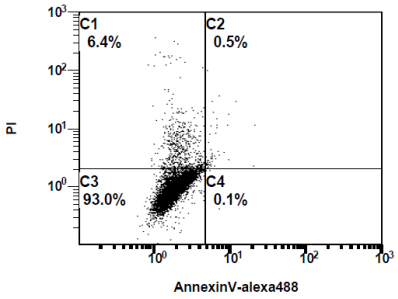
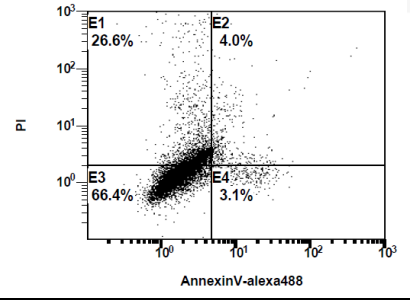
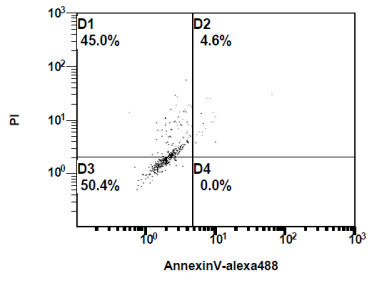
The results of program cell death after 24 h incubation using Annexin V and PI staining by flow cytrometry.

Repilcation 1	Repilcation 2
Untreated cell (control) 	Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 	Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 	Doxorubicin treated cell at 0.5 µg/ml 

The results of program cell death after 48 h incubation using Annexin V and PI staining by flow cytrometry.

Repilcation 1	Repilcation 2
Untreated cell (control) 	Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 	Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 	Doxorubicin treated cell at 0.5 µg/ml 

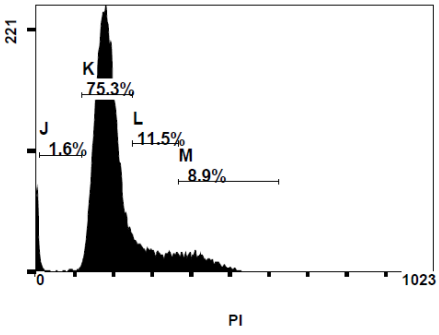
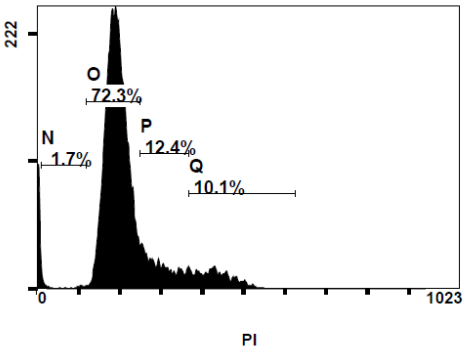
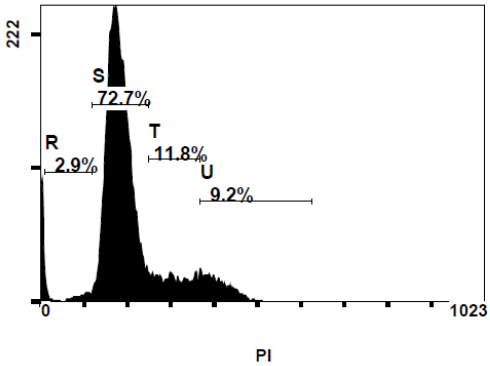
The results of program cell death after 72 h incubation using Annexin V and PI staining by flow cytrometry.

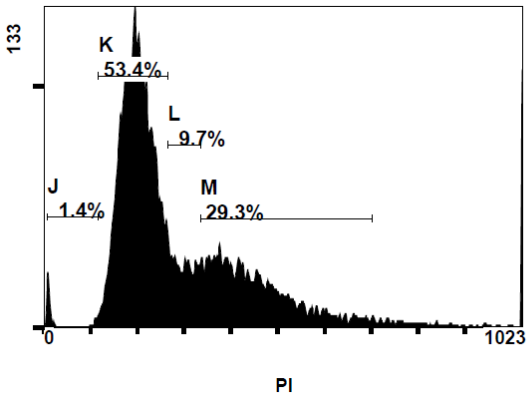
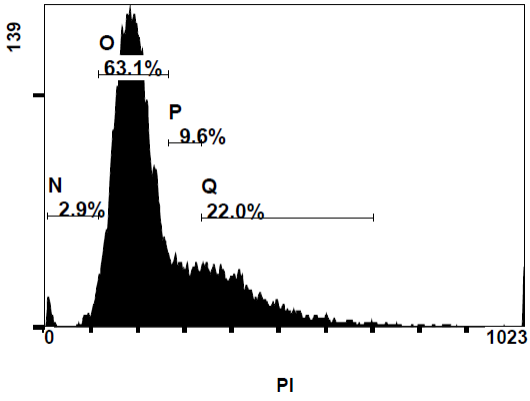
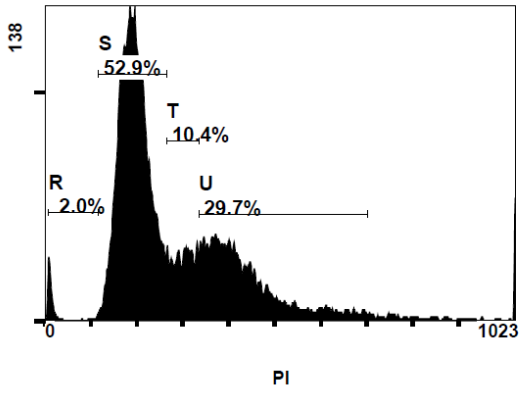
Repilcation 1	Repilcation 2
Untreated cell (control) 	Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 	Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 	Doxorubicin treated cell at 0.5 µg/ml 

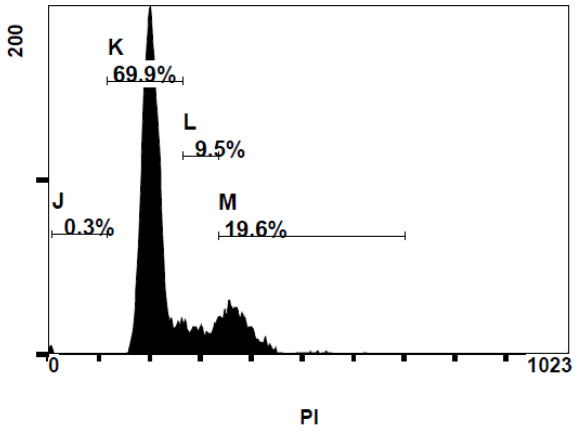
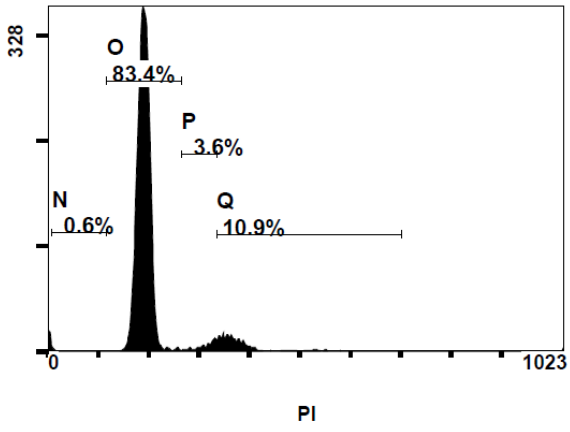
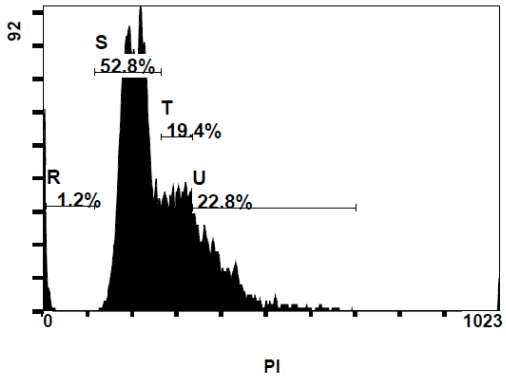
APPENDIX C

The results of cell cycle arrest using PI staining by flow cytrometry.

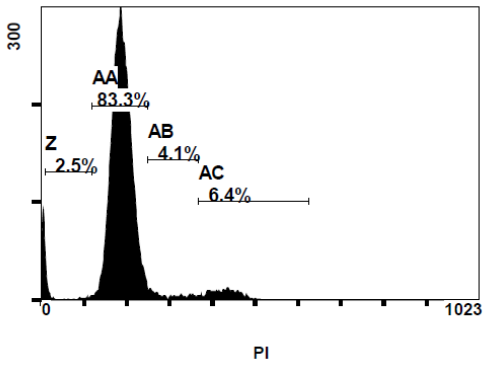
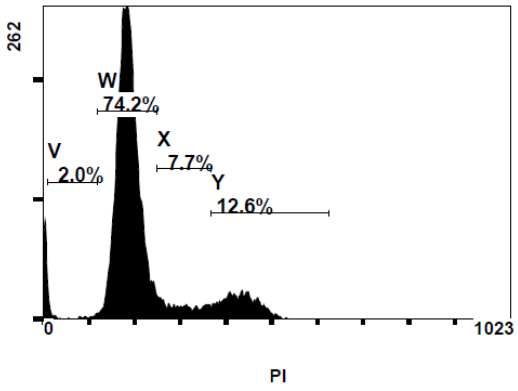
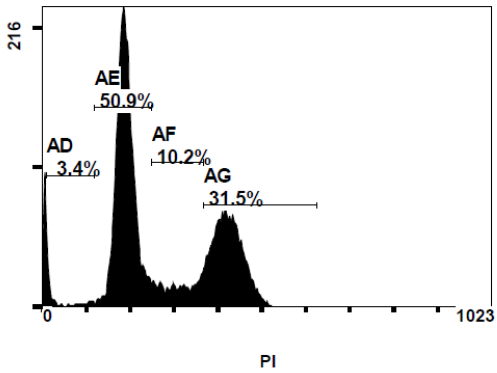
The results of cell cycle arrest after 24 h incubation using PI staining by flow cytrometry.

Repilcation 1
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

Repilcation 2
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

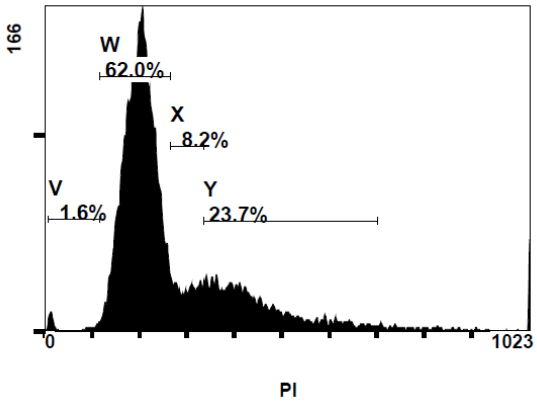
Repilcation 3
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

The results of cell cycle arrest after 48 h incubation using PI staining by flow cytrometry

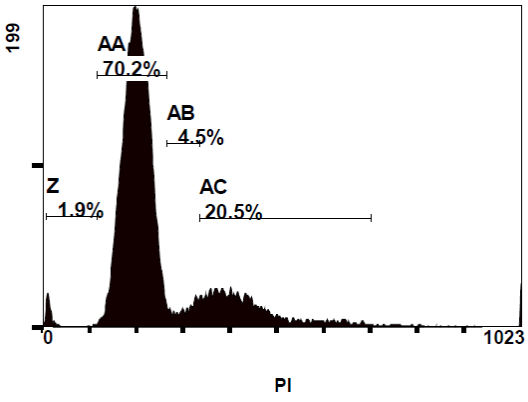
Repilcation 1
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

Repilcation 2

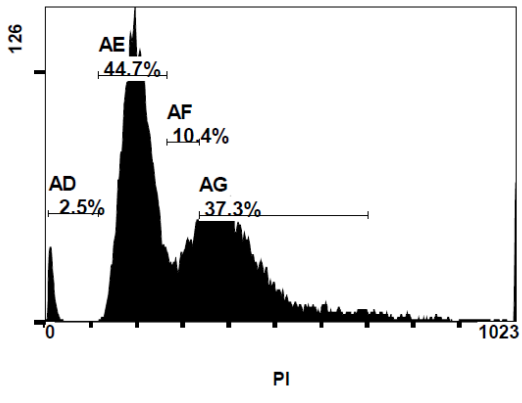
Untreated cell (control)

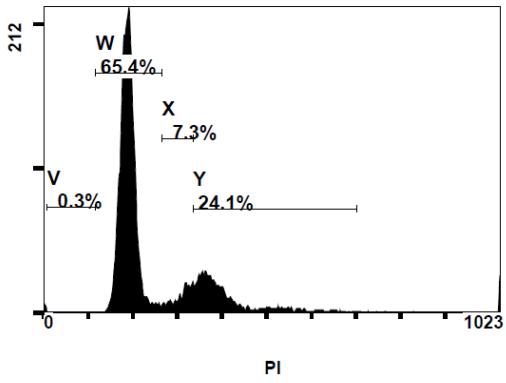
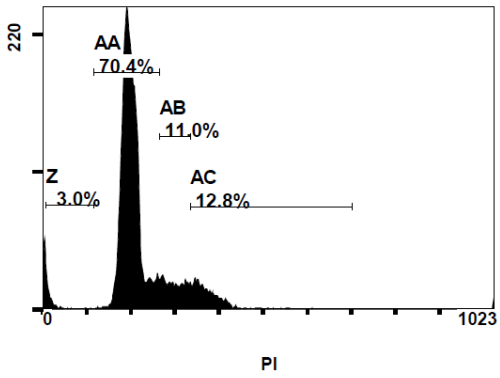
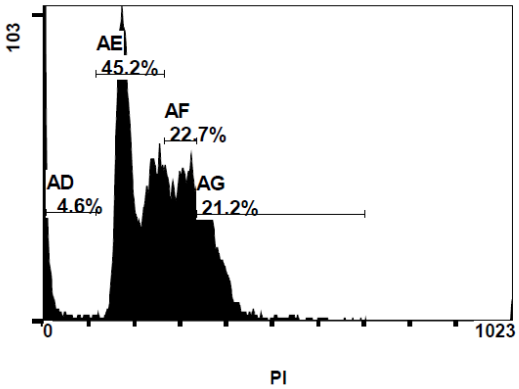


Cardanol treated cell at 30 µg/ml

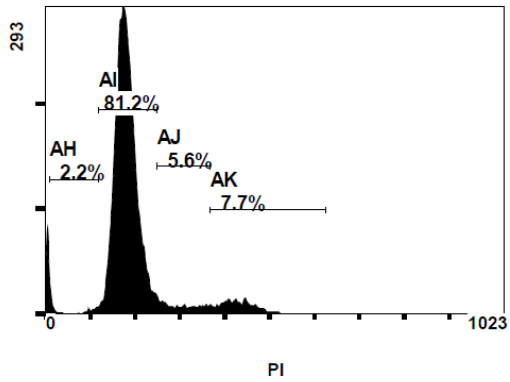
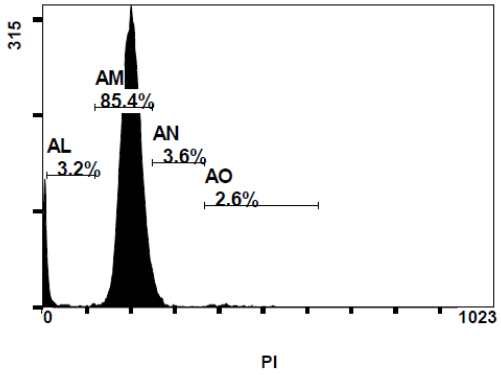
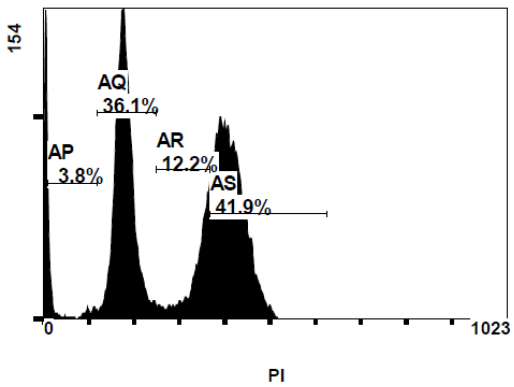


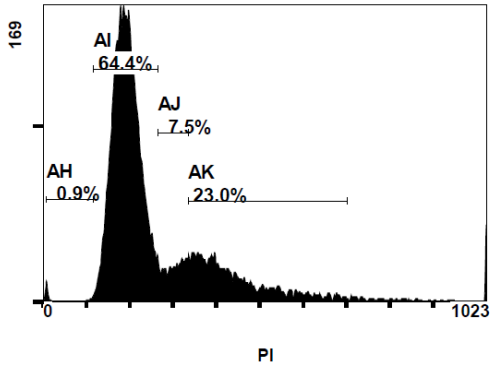
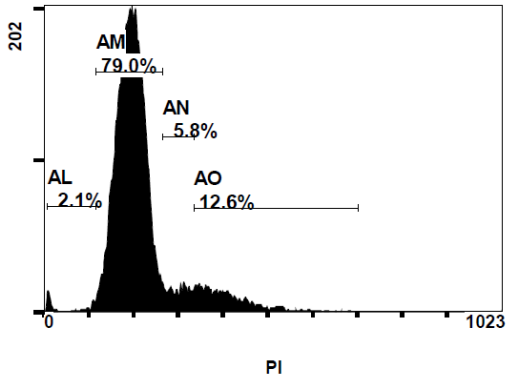
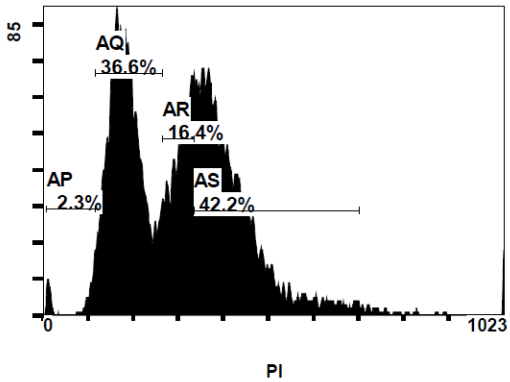
Doxorubicin treated cell at 0.5 µg/ml

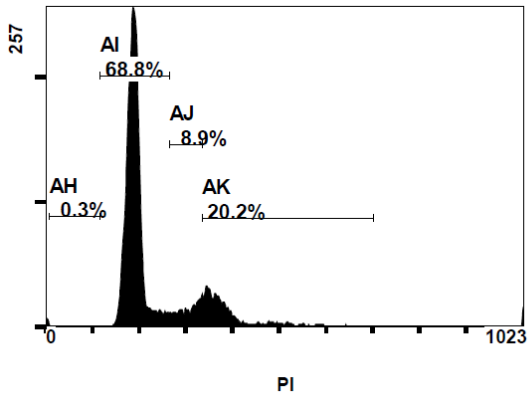
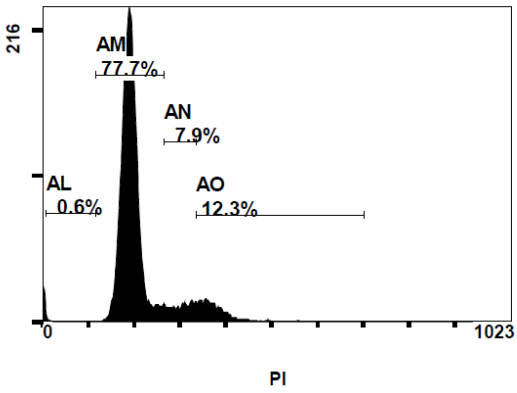
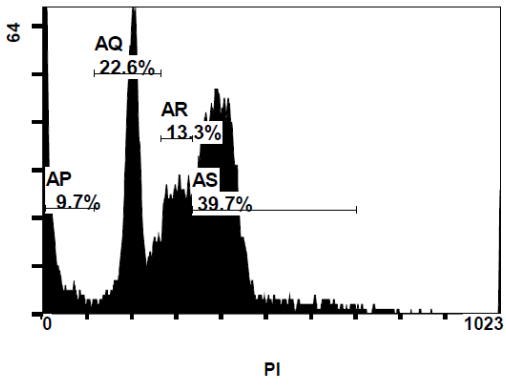


Repilcation 3
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

The results of cell cycle arrest after 72 h incubation using PI staining by flow cytrometry

Repilcation 1
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

Repilcation 2
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

Repilcation 3
Untreated cell (control) 
Cardanol treated cell at 30 µg/ml 
Doxorubicin treated cell at 0.5 µg/ml 

APPENDIX D

The results of gene expression using qRT-PCR

(1) Crossing point (Cp) value and the realative level of apoptosis related gene

Gene	Sample	Experiment			Average	The relative level = 2 (Cp actin-Cp target)
		Rep1	Rep 2	Rep3		
	Control	17.56	20.47	20.35	19.46	1
	Cardanol	20.26	19.70	21.54	20.50	0.49
Mcl1	Doxorubicin	21.64	20.22	19.36	20.41	0.52
	Control	18.03	20.7	23.88	20.87	1
	Cardanol	15.87	20.89	22.28	19.68	2.28
Bcl2	Doxorubicin	16.02	20.91	22.53	19.82	2.07
	Control+	18.71	20.46	20.32	19.83	1
	cardanol	20.36	20.7	21.57	20.88	0.48
MADD	Doxorubicin	20.63	21.23	20.35	20.74	0.53
	Control+	20.5	21.61	21.45	21.19	1
	cardanol	21.56	21.42	21.59	21.53	0.79
c-FLIP	Doxorubicin	21.08	21.32	21.86	21.42	0.85
	Control+	24.09	28.21	30.44	27.58	1
	cardanol	24.63	30.3	30.2	28.38	1.32
DR5	Doxorubicin	24.14	29.16	30.39	27.90	0.83

(2) Crossing point (Cp) value and the realative level of cell cycle related gene

Gene	Sample	Experiment			Average	The relative level = $2^{(\text{Cp actin-Cp target})}$
		Rep1	Rep 2	Rep3		
	Control	28.10	20.11	20.16	22.79	1
	Cardanol	24.02	23.42	24.20	23.88	1.62
p21	Doxorubicin	23.82	25.60	23.84	24.42	2.05
	Control	28.1	20.11	20.16	22.79	1
	Cardanol	23.02	23.18	24.2	23.47	1.51
E2F1	Doxorubicin	23.52	24.2	23.64	23.79	2.46
	Control+	20.64	19.49	19.4	19.84	1
	cardanol	19.33	20.37	20.16	19.95	0.93
Cyclin A	Doxorubicin	18.88	18.71	20.01	19.20	1.56
	Control+	23.24	23.42	23.11	23.26	1
	cardanol	26.88	24.24	24.39	25.17	0.26
Cyclin E	Doxorubicin	23.08	23.28	23.11	23.16	1.07
	Control+	17.46	17.91	17.86	17.74	1
	cardanol	18.83	19.81	17.94	18.86	0.46
cyclinD1	Doxorubicin	19.43	19.07	18.66	19.05	0.40
	Control+	19.68	19.75	20.04	19.82	1
	cardanol	21.6	20.89	21.26	21.25	0.37
CDK2	Doxorubicin	19.07	19.19	19.46	19.24	1.49
	Control+	19.56	20.01	19.4	19.66	1
	cardanol	21.52	20.92	20.08	20.84	0.44
CDK4	Doxorubicin	19.37	19.64	20.48	19.83	0.89
	Control+	18.78	17.79	19.82	18.80	1
	cardanol	18.56	19.04	18.32	18.64	1.12
CDK6	Doxorubicin	17.87	18.8	18.7	18.46	1.27

APPEXDIX E

Reagent preparation of western blot assay

10% Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE)

1) 10% Separating gel (for 2 gel)
   - Water (RO) 12.3 ml
   - 1.5 M Tris, pH 8.8 3.75 ml
   - 10% SDS 150 µl
   - 30.8% Acrylamide + 0.8% Bis 4.95 ml
   - 10% APS (10 mg/100 µl water) 75 µl
   - TEMED 15 µl
2) Stacking gel (for 2 gel)
   - Water (RO) 2.44 ml
   - 0.5 M Tris, pH 6.8 1 ml
   - 10% SDS 80 µl
   - 30.8% Acrylamide + 0.8% Bis 0.52 ml
   - 10% APS (10 mg/100 µl water) 20 µl
   - TEMED 4 µl

30.8% Acrylamide + Bis (100 ml)

- Acrylamide 30 g
- Bis acrylamide 0.8 g
- Water (MilliQ)

5x Running Buffer (1 L)

- Tris 15 g
- Glycine 72 g

- SDS 5 g
- Water (RO)

5x Blotting Buffer (1 L)

- Tris 15 g
- Glycine 72 g
- Water (RO)

10X TBS/T pH 7.6 (1 L)

- Tris 24.2 g
- Naci 80 g
- Water (MilliQ)

VITA

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Publication:

Buahorm, S., Chanchao, C., Phuwapraisirisan, P., Puthong, S., and Lirdprapamongkol,K. (2014) Effects of cardanol from Apis mellifera propolis on breast cancer BT-474 cell line. Burapha University International Conference 2014, Chon Buri, Thailand.

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