In vitro Cytotoxic and Apoptosis Effects of *Vatica diospyroides* Symington Type SS Fruit Extracts on Cervical Cancer Cells

Atchara Chothiphirat

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biomedical Sciences
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

..... Signature

(Miss Atchara Chothiphirat)

Candidate

ชื่อวิทยานิพนธ์ การศึกษาความเป็นพิษต่อเซลล์และชักนำให้เซลล์ตายแบบอะพอพโทสิสของสารสกัดจากผลจันทน์กะพ้อชนิดย่อยเอสเอส ต่อเซลล์มะเร็งปากมดลูกเพาะเลี้ยง
ผู้เขียน นางสาวอัจฉรา โชติพิรัตน์
สาขาวิชา ชีวเวชศาสตร์
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บทคัดย่อ

โรคมะเร็งปากมดลูกถูกจัดอยู่ในอันดับที่สี่ของอุบัติการณ์มะเร็งที่ตรวจพบในผู้หญิงทั่วโลก เป็นที่น่าตกใจว่าประเทศไทยถูกจัดอยู่ในอับดับที่สี่ของอุบัติการณ์มะเร็งที่ตรวจพบในผู้หญิงในแถบเอเชียตะวันออกเฉียงใต้ ซึ่งมีอุบัติการณ์ในประเทศเป็นอันดับที่สองและมีอัตราการเสียชีวิตในอันดับที่สี่ของผู้หญิงไทย จากรายงานดังกล่าวแสดงให้เห็นว่า โรคมะเร็งดังกล่าวยังคงเป็นปัญหาสำคัญทางสาธารณสุขทั้งในประเทศไทยและระดับโลก จากการศึกษาก่อนหน้าพบว่า จันทน์กะพ้อ (*Vatica diospyroides* Symington) ซึ่งเป็นพืชพื้นบ้านของประเทศไทย ถูกนำมาใช้ในการบำรุงโลหิตและหัวใจ สารสกัดจากผลจันทน์กะพ้อชนิดย่อยเอสเอส มีความเป็นพิษต่อเซลล์มะเร็งเต้านมเพาะเลี้ยงชนิด MDA-MB-468 ซึ่งในงานวิจัยนี้จะทำการศึกษาฤทธิ์ของสารสกัดจากผลของจันทน์กะพ้อชนิดย่อยเอสเอส ซึ่งแบ่งผลออกเป็นสองส่วนได้แก่ ผนังผลและใบเลี้ยง ทำการสกัดโดยใช้อะซีโตนและเมทานอลเป็นตัวทำละลาย โดยมีวัตถุประสงค์เพื่อศึกษาความเป็นพิษและรูปแบบการตายของสารสกัดจากผลของจันทน์กะพ้อชนิดย่อยเอสเอส ต่อเซลล์มะเร็งปากมดลูกเพาะเลี้ยง โดยการศึกษาความเป็นพิษของสารสกัดต่อเซลล์มะเร็งเพาะเลี้ยงถูกทดสอบด้วยเทคนิคเอ็มทีที (MTT assay) ซึ่งใช้ค่าความเข้มข้นที่ทำให้สามารถยับยั้งการเจริญเติบโตของเซลล์ได้ร้อยละ 50 (50% inhibitory concentration: IC_{50}) เป็นค่าที่บ่งบอกฤทธิ์ของสารสกัด ส่วนในการศึกษารูปแบบการตายของเซลล์ทั้งการชักนำการหยุดของวัฏจักรเซลล์ (Cell cycle arrest) และชักนำเซลล์ให้เกิดการตายแบบอะพอพโทสิส (Apoptosis) โดยวิเคราะห์ด้วยเทคนิคโฟลไซโตเมทรี (Flow cytometry) ต่อจากนั้นศึกษาการแสดงออกของโปรตีนที่เกี่ยวข้องกับวิถีของรูปแบบการตายชนิดดังกล่าวด้วยวิธีเวสเทิร์นบลอตติง (Western blotting) ผลจากการศึกษาพบว่า สารสกัดหยาบจากผลของจันทน์กะพ้อมีฤทธิ์ความเป็นพิษสูงต่อเซลล์มะเร็งปากมดลูกเพาะเลี้ยงชนิด HeLa มีค่า IC_{50} อยู่ในช่วง 7.69-17.93 ไมโครกรัมต่อมิลลิลิตร และในชนิด SiHa มีค่า IC_{50} อยู่ในช่วง 9.81-19.00 ไมโครกรัมต่อมิลลิลิตร และยังพบว่าสารสกัดอะซีโตนจากใบเลี้ยงมีฤทธิ์ความเป็นพิษสูงที่สุดเมื่อเปรียบเทียบกับสารสกัดชนิดอื่น จากนั้นศึกษาระดับความปลอดภัยของสารสกัดต่อเซลล์ปกติด้วยค่า Selectivity index (SI) พบว่ามีเพียงสารสกัดอะซีโตนจากใบเลี้ยงเท่านั้นที่มีความปลอดภัยต่อเซลล์ปกติด้วยค่า SI ที่มากกว่า

3 นอกจากนั้นได้ศึกษารูปแบบการตายของเซลล์ซึ่งเป็นผลมาจากฤทธิ์ของสารสกัดอะซีโตนจากใบเลี้ยง พบว่า สารสกัดสามารถชักนำให้เกิดการหยุดของวัฏจักรเซลล์มะเร็งปากมดลูกเพาะเลี้ยงในระยะ G2/M เมื่อทดสอบด้วยความเข้มข้นสูง ที่เวลา 48 ชั่วโมง และศึกษาการแสดงออกของโปรตีน Cyclin-B1 ซึ่งเป็นโปรตีนที่เกี่ยวข้องกับวัฏจักรเซลล์ในระยะ G2/M พบว่าโปรตีนดังกล่าวมีการแสดงออกเพิ่มขึ้น และ มีการแสดงออกเพิ่มขึ้นของ p-cdc2 ซึ่งเป็นโมเลกุลที่เกี่ยวข้องกับการยับยั้งการดำเนินของวัฏจักรเซลล์ในระยะ G2/M นอกจากนี้สารสกัดดังกล่าวยังสามารถชักนำให้เซลล์มะเร็งปากมดลูกเพาะเลี้ยงชนิด HeLa และ SiHa เกิดการตายแบบอะพอพโทสิสโดยแปรผันตรงกับความเข้มข้นของสารและมีการแสดงออกของโปรตีน Bax เพิ่มขึ้นที่เวลา 48 ชั่วโมง ส่วนโปรตีน caspase-8 ลดลงในขณะที่โปรตีน cleaved caspase-8 เพิ่มขึ้นอย่างต่อเนื่อง กล่าวโดยสรุปได้ว่าฤทธิ์การยับยั้งการเจริญเติบโตของเซลล์มะเร็งปากมดลูกของสารสกัดอะซีโตนจากใบเลี้ยงน่าจะเกิดจากความสามารถในการชักนำให้เกิดการหยุดของวัฏจักรเซลล์ในระยะ G2/M โดยอาจจะผ่านการสะสมของโปรตีน cyclin-B1 และชักนำให้เซลล์เกิดการตายแบบอะพอพโทสิสโดยผ่านการกระตุ้นการแสดงออกของโปรตีน Bax และ cleaved caspase-8 จากผลการศึกษาทั้งหมดอาจกล่าวได้ว่า สารสกัดอะซีโตนจากใบเลี้ยงน่าจะเป็นสารที่มีคุณสมบัติในการต้านมะเร็งและอาจเป็นทางเลือกใหม่ในการรักษาโรคมะเร็ง

คำสำคัญ: จันทน์กะพ้อ มะเร็งปากมดลูก เอ็มทีที โฟลไซโตเมทรี เวสเทิร์นบลอตติง

Thesis Title *In vitro* Cytotoxic and Apoptosis Effects of *Vatica diospyroides* Symington Type SS Fruit Extracts on Cervical Cancer Cells

Author Atchara Chothiphirat

Major Program Biomedical Sciences

Academic Year 2018

ABSTRACT

Cervical cancer is the fourth most common cancer diagnosed in woman worldwide in 2018. Astoundingly, the data base of GLOBOCAN 2018 has been reported that Thailand was ranked as fourth for most common woman cancer diagnosed of South-Eastern Asia. In Thailand, cervical cancer is the second of cancer incidence rate and fourth rank of mortality rate in female. The reports show that cervical cancers until remains a crucial also in Thailand and global public health problem. *Vatica diospyroides* Symington (VDS) has been used as a Thai medicinal herb for cardiac and haematonic therapy and shown greatly cytotoxic response on human breast cancer cells as showed in previous reports. In this study, fruit extracts (cotyledon and pericarp) of VDS type SS were obtained using acetone and methanol. The aim of this research is to determine the cytotoxic activity and mode of cell death of VDS type SS fruit extracts on cervical cancer cell lines. The cytotoxicity was performed by MTT assay and the 50% inhibitory concentration (IC_{50}) was used to indicate the cytotoxic effect. In order to investigate the mode of cell death including cell cycle arrest and apoptosis induction were subsequently measured using flow cytometry analysis. Protein expression involved with mode of cell death was elucidated by western blotting. The MTT result showed that the crude extracts exhibited highly cytotoxicity on HeLa with IC_{50} rank at 7.69-17.93 µg/mL and SiHa with IC_{50} rank at 9.81-19.00µg/mL, respectively. Surprisingly, the cotyledon-acetone extract notably showed the highest cytotoxicity on both cell lines. Furthermore, the selectivity index or SI was used to determine the harmless response of VDS extracts on normal cells. The result exhibited that only cotyledon-acetone extract shows high safety response on L929 cell with SI value more

than 3. Remarkable feature of anticancer agent as cell cycle arrest was performed on cervical cancer cells and result showed that the extract could induce G_2/M arrest in HeLa and SiHa with 2-fold IC_{50} at 48h through accumulation of cyclin-B1 expression and increasing of p-cdc2 CDK inhibitor in time-dependent manner. Additionally, the mode of cell death which effect by cotyledon-acetone extract was found that HeLa and SiHa treated cells were shown apoptosis induction in a dose-dependent manner. In addition, the expression of Bax was increased at 48 h and caspase-8 was declined together with increasing of cleaved caspase-8 expression. In conclusion, the anti-proliferation effect of cotyledon-acetone extract may be due to cell cycle arrest at G2/M phase via cyclin-B1 accumulation and apoptosis induction by stimulation of Bax and cleaved caspase-8 expression on cervical cancer cells. Based on our study, the cotyledon-acetone extract would be a candidate anticancer agent with promise anticancer properties and might be lead to alternative approach for cancer therapies.

Keywords: *Vatica diospyroides* Symington, Cervical Cancer, MTT, Flow cytometry, western blotting

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Atchara Chothiphirat

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LIST OF ABBREVIATIONS AND SYMBOLS

%	=	Percentage
β	=	Beta
µg/mL	=	Microgram per milliliter
µL	=	Microliter
Annexin V-FITC	=	Annexin V-Fluorescein isothiocyanate
APC	=	Anaphase-promoting complex
ATCC	=	American Type Culture Collection
Bax	=	Bcl-2-associated X protein
Cdk	=	Cyclin-dependent kinase
CO_2	=	Carbon dioxide
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
h	=	Hour
IC_{50}	=	Half-maximal inhibitory concentration
mg/mL	=	Milligram per milliliter

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NCI	=	National Cancer Institute
nm	=	Nanometer
NRF-2	=	Nuclear facter erythroid-related factor2
PI	=	Propidium iodide
QBG	=	Queen Sirikit Botanic Garden
SD	=	Standard deviation
SI	=	Selectivity index
VDS	=	*Vatica diospyroides* Symington

LIST OF PAPER AND PROCEEDING

1. Chothiphirat A, Srisawat T, Kanokwiroon K, Nittayaboon K, Navakanitworakul R. Anticancer potential of fruit extracts from *Vatica diospyroides* Symington type SS and their effect on program cell death of cervical cancer cell lines. **(Submitted manuscript)**

2. Chothiphirat A, Sangkhathat S, Srisawat T, Kanokwiroon K, Navakanitworakul R. Cytotoxic effect of cotyledon extract of *Vatica diospyroides* Symington type SS against colorectal cancer cell line. The 20th World Congress on Clinical Nutrition (WCCN), Bangkok, Thailand. December 14, 2016. **(Poster presentation)**

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Dear Dr. Navakanitworakul,

The Research Article titled "Anticancer Potential of Fruit Extracts fromVatica diospyroides Symington Type SS and Their Effect on Program Cell Death of Cervical Cancer Cell Lines," by Atchara Chotpirat, Kesara Nittayaboon, kanyanatt kanokwiroon, Theera Srisawat and Raphatphorn Navakanitworakul has been received and assigned the number 5491904.

All authors will receive a copy of all the correspondences regarding this manuscript.

Thank you for submitting your work to The Scientific World Journal.

Best regards,

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December 14-16, 2016: Rama Gardens Hotel, Bangkok, THAILAND
20th World Congress on Clinical Nutrition (WCCN)
" Traditional Medicine, Functional Food, Nutrition, Natural Health Product and Spiritual Healing : Additional Tools for Healthcare Delivery"

Acceptance Letter

the abstract entitled

"Cytotoxic effect of Cotyledon Extract of *Vatica diospyroides* Symington Type SS against Colorectal Cancer Cell Line"

To be presented by

Atchara Chothiphirat

has been accepted for a poster presentation at the 20th World Congress on Clinic Nutrition (WCCN), Rama Gardens Hotel, Bangkok, 14-16 December 2016

31 October 2016

Assoc. Prof. Preecha Wanichsetakul, MD

Chairperson

CHAPTER 1
INTRODUCTION

Cancer is a major cause of human deaths worldwide reported by World Health Organization (WHO). Cervical cancer is the fourth most common cancer diagnosed in woman worldwide with 569,847 new cases and 311,365 deaths in 2018. Cervical cancer is also frequently occurring in Asia female with rank as the third most common cancer. Astoundingly, the data base of GLOBOCAN 2018 has been reported that cervical cancer incidence in Thailand was ranked as fourth most common cancer diagnosed in woman of South-Eastern Asia. In Thailand, cervical cancer is the second of cancer incidence rate and fourth rank of mortality rate in female (1). The data demonstrate that cervical cancer remains a serious health problem worldwide.

Although, the most conventional treatment such chemotherapy that cause to kill cancer cells, but induce the systemic side effect in cancer patients. Several anticancer chemotherapy drugs are effect due to their mechanisms of action. First line drug of chemotherapy in cervical cancer patient is cisplatin. The cisplatin and various chemotherapy drugs induce DNA damage and subsequently causing cancer cell death, but also in normal cells (2). Therefore, the patients with conventional chemotherapy treatment are received the side effect such as gastrointestinal problem, hearing loss, diarrhea and also related with severe side effect such as nephrotoxicity, cardiotoxicity and hepatotoxicity (3).

Natural products have been used as the major source of illness therapy including cancer. Various natural sources, such as fungus, marine life and especially natural plants have been accepted by universal as an efficient material for world healthcare system (4). The medicinal or plant derived compounds are well characterized as anticancer properties, such as inhibition of cell proliferation, anti-angiogenic activity and induction of apoptosis (5). This is consistent with report was explained that 25% of modern drugs are developed from traditional folk medicines (6). The medicinal herbs have consistent some therapeutic agents that are called secondary metabolites or natural products (7). At present, natural product has a significant role and is interesting in drug discovery process for treatment of various

diseases. The most cases of synthetic drugs cause many side effects, but the phytochemical agents that discovered from plants and other natural sources may represent less side effects (8). Therefore, researchers try to seek out the alternative treatment from natural plant as an anticancer agent with less or no side effects to the patients. Many plants have potential compounds containing several biological activities to treat various ailments such as cardiotonic, anti- inflammatory, analgesic and antitumor agent (9). Thailand is a rich in biodiversity, consisting of plant families that used as medicinal herbs. Among the variety of plant families, there is one family that has been used as a folk remedy which Dipterocarpaceae. The family Dipterocarpaceae majority found in Asian rain forests and Thailand includes 9 genuses, 64 species (10). The phytochemical and biological activities testing in Dipterocarpaceae plans have been discovered continuously for many years (11). The family contains many plants that have biological activities such as antioxidant, antimicrobial, antifungal and including anticancer activity (9, 11). *Vatica diospyroides* Symington (VDS) is one of the Dipterocarpaceae. In 2017, VDS was identified in endangered population on the IUCN red list of International Union for Conservation of Nature and Natural Resources (IUCN) (12). In Thailand, flower and stems of VDS have been used as Thai ethnobotanical medicine such as ingredients in cardiac and blood tonic treatments (13). VDS contains major compound that called resveratrol. Resveratrol derivatives which is a plant-derived polyphenolic phytoalexin that produced by enzyme stilbene synthase in response to its infection. This polyphenol has many biological activities including anticancer property. The resveratrol dimers from stem bark extract of *Hopea gregaria* one of Dipterocarpaceae that called ε-viniferin, showed highly active cytotoxicity on murine leukemia P-388 cells (14). The VDS can be easily observed into two subtypes with not only discriminated of leaf morphology, but also in differentiated on color of flower. There are two subtypes of VDS which are large size of leaf (LS type) and small size of leaf (SS type). The previous works have been screened the phytochemical from root, leaf, branch, pericarp and cotyledon extract of subtype LS and showed cytotoxic activity against breast cancer cell lines (15). The extract from cotyledon showed highly active cytotoxicity against MDA- MB- 468 cell line. The results from this research indicated that VDS fruit is the best source of active compounds for cytotoxic effect on

cancer cell lines. The later research by Srisawart *et al* (2014). showed the highest cytotoxic response and induced apoptosis in breast cancer cell line from VDS subtype SS fruit extracts (16).

Therefore, this study was interested in cytotoxic activity of the extracts and their mode of cell death induction on cervical cancer cell lines which are HeLa and SiHa. Cytotoxic activity was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Mode of cell death of extract was performed by flow cytometry and western blot analysis. This research is the first to investigate VDS crude extract against cervical cancer cell lines from VDS type SS fruit. Based on their effective biological activity, the extracts could be serves as a potential agent against cancer and lead to therapeutic development for those anticancer therapies.

CHAPTER 2
OBJECTIVES

2. 1 To determine the cytotoxic activity of *V. diospyroides* type SS fruit extracts on cervical cancer cell lines.

2. 2 To investigate the mode of cell death on cervical cancer cell lines by *V. diospyroides* type SS fruit extracts induce apoptosis and cell cycle arrest.

2.3 To elucidate the mode of action of *V. diospyroides* type SS fruit extracts by protein expression of molecules involve in apoptosis induction and cell cycle arrest of pathway by crude extracts.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Scope of study

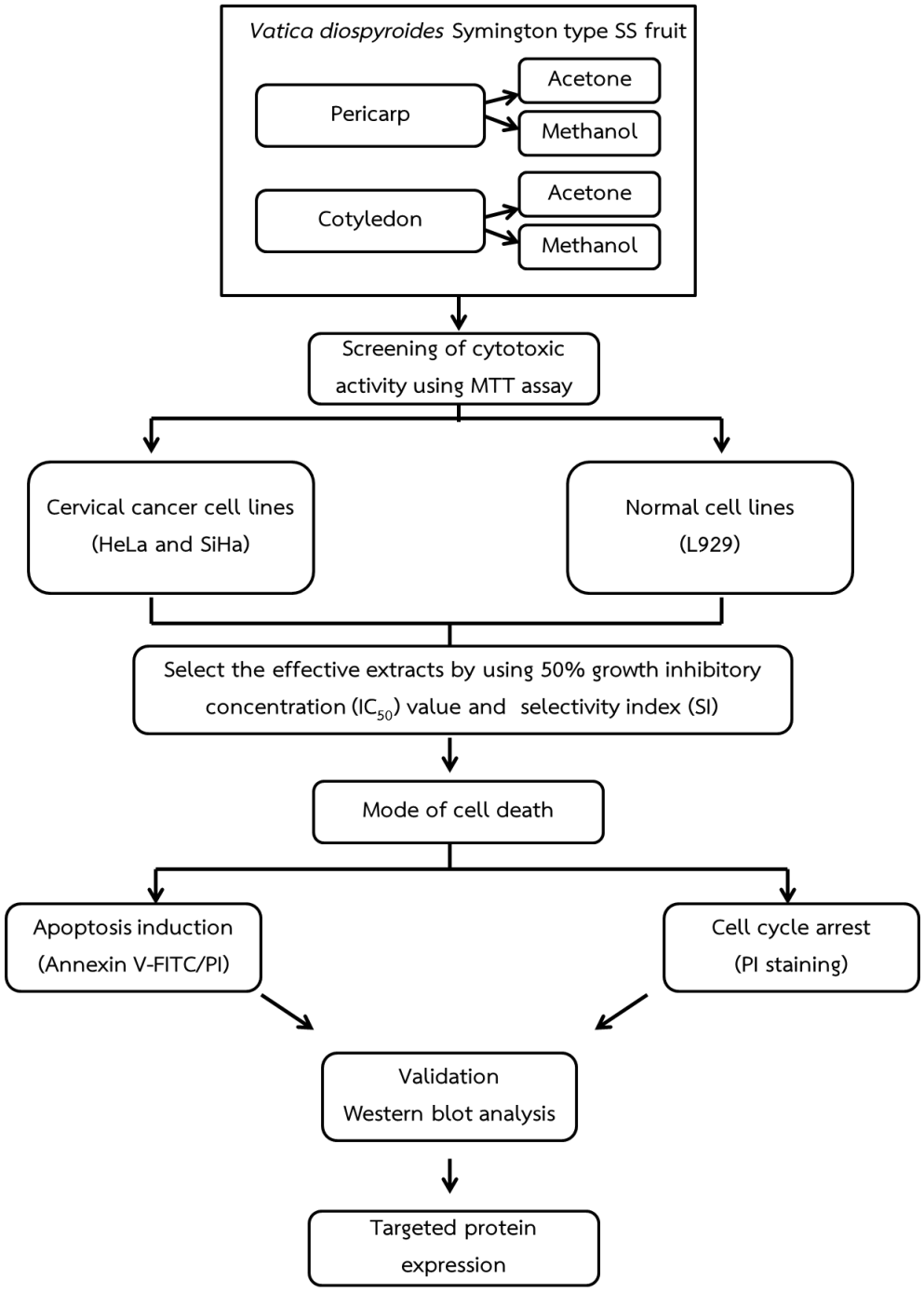


Figure 1 Scope of study

3.2 Results

3.2.1 *In vitro* cytotoxicity of VDS crude extracts on cervical cancer cell lines by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The VDS crude extracts were extracted by Asst. Prof. Dr. Yaowapa Sukpondma, Prince of Songkla University and kindly provided by Asst. Prof. Dr. Theera Srisawat, Surat Thani campus. Sample of SS fruit of *V. diospyroides* was collected from Nong Thung Thong non-hunting area, Kiansa, Suratthani Province, Thailand. Voucher specimens (Collector number T. Srisawat 002) were deposited in the Herbarium of Queen Sirikit Botanic Garden (QBG), Maerim, Chiang Mai, Thailand. The samples were authenticated by Dr. Charun Maknoi of QBG. The dry fruit was separately extracted in methanol (CH_3OH) and acetone ($(CH_3)_2CO$). Then, extracts were dissolved in 100% dimethyl suloxide (DMSO) to final concentration of DMSO in extracts less than 0.5% (17). Two human cervical cancer cell lines HeLa (ATCC CCL-21) and SiHa (ATCC HTB-35) were purchased from the American Type Culture Collection (ATCC) and mouse fibroblast cell L929, which kindly provided from Prof. Dr. Teerapol Srichana, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, was used as a normal cell line. The cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, streptomycin (100 µg/mL) and penicillin (100 U/mL). All cells were maintained in humidified air with 5% CO_2 at 37 °C incubator. The cytotoxic effect of crude extracts was performed by using MTT assay. This method is measurement of cell viability. Cells were cultured to exponentially stage and trypsinized. The cells were seeded into 96-well plates (5×10^3 cells/well) and incubated for 24 hours to reach 70% confluency. After incubation, concentration of each extracts were diluted with medium to 5, 10, 20, 40 and 80 µg/mL and treated with these extracts for 72 hours. At the end of incubation, cells were washed with 100 µl of 1x PBS and added 100 µl of 1 mg/mL MTT solution (Invitrogen, California, USA) to each well and incubated at 37°C for 90 minutes. The yellow tetrazolium MTT was reduced by mitochondrial reductase in viable cells and resulting in intracellular purple formazan crystal (18). The solution was

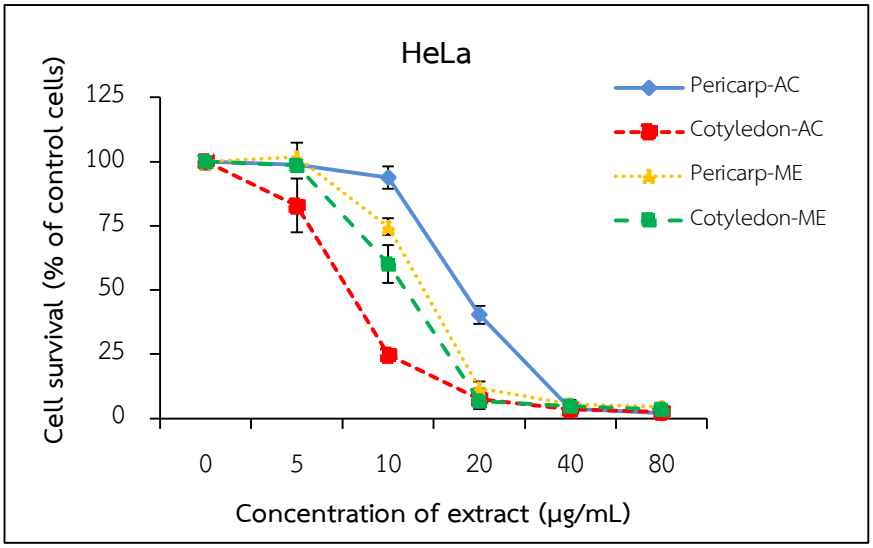
carefully removed and formazan crystal was dissolved with organic solvent DMSO to each well and incubated at 37°C for 30 minutes. The absorbance was measured at 570 nm and 650 nm using multi-well plate reader (SpectraMax M5 Multi-Mode Microplate Reader). The absorbance value associate with viable cell counts. The result showed that four of the VDS crude extract exhibited antiproliferation on HeLa and SiHa cells. The cotyledon-acetone extract was the highest effect in term of antiproliferation with decreasing of cell survival approximately 25% at 10 µg/mL. On the other hand, the inhibition of survival rate by other extracts showed merely decreased rank at 60.20-94.00% at 10 µg/mL treatment (Figure 2A). The extracts were also screened in SiHa cell for cytotoxic effect. The result showed that the cotyledon-acetone extract as well displayed inhibitory effect on SiHa cell with decreasing the percentage of cell survival to 45.95% at lower concentration (10 µg/mL). However, three other extracts showed slightly declined the cell survival same as HeLa cell with 68.74-74.24% at 10 µg/mL treatment (Figure 2B). After that, the data was calculated for percentage of inhibition and plot the graph for cytotoxic effect in term of the half maximal inhibitory concentration (IC_{50}) value.

The IC_{50} value was used for preliminary screening following the criteria of crude extracts decided by the American National Cancer Institute (NCI) and Geran *et al*, which IC_{50} less than 20 µg/mL will be defined to highly active (19). The cytotoxic result of VDS extracts that induced anti proliferation of cancer and normal cell lines and the IC_{50} values were expressed as mean ± SD in three independent experiments were shown in Table 1. The lower IC_{50} values considered the extract represented the higher potent cytotoxicity to the cells. After treatment for 72 h, pericarp-acetone extract showed highly cytotoxic response on both HeLa and SiHa cervical cancer cell lines at IC_{50} 17.93±0.81 and 19.00±3.37 µg/mL, respectively. The pericarp-methanol treated cancer cells also displayed no different of the IC_{50} values between cervical cancer (HeLa and SiHa: 13.40±0.71 and 14.55±0.69 µg/mL, respectively). For cotyledon-methanol extract, the extract was shown the highly active cytotoxic against HeLa, and SiHa cell lines with IC_{50} value at 11.60±0.96 and 14.54±1.10 µg/mL, respectively. Notably, the cotyledon-acetone extract showed very strong cytotoxic effect on cervical cancer cell lines with the lowest IC_{50} values ranged between 7-9 µg/mL. Although the

extracts showed potent cytotoxicity against cervical cancer cell lines, the cytotoxic effect of the normal cell also required in order to evaluate safety of extracts, *in vitro*. Thus, the MTT assay was performed using L929 mouse fibroblast cell line. The results showed that pericarp- methanol and cotyledon- methanol extracts showed highly cytotoxic on L929, while the pericarp-acetone and cotyledon-acetone exhibited the cytotoxic response with IC_{50} more than 20 µg/mL (IC_{50} of 33.12±0.82 and 34.41±2.05 µg/mL, respectively).

As part of anticancer drug finding out, the safety response of the extracts on the normal cell should be considered. The selectivity index (SI) was used to determine the harmless response of VDS extracts which dividing the IC_{50} of the normal cell by the cancer cell in same extract (Appendix A1) as represented in Table 2. The SI value which more than 3 were considered to high selectivity or less toxic on the normal cell lines (20). Unfortunately, only cotyledon-acetone extract shows high safety response on L929 cell with ranked of SI value between 3.51-4.47 which followed in safety criteria of Prayong *et al*., 2008 (20) with greater than 3 of SI value, whereas the other extracts were shown critical effect (SI<3) on cancer cell line. Hence, these results suggested that the cotyledon-acetone extract showed highly active cytotoxic against cervical cell lines or without harmful on normal fibroblast cell line.

(A)



(B)

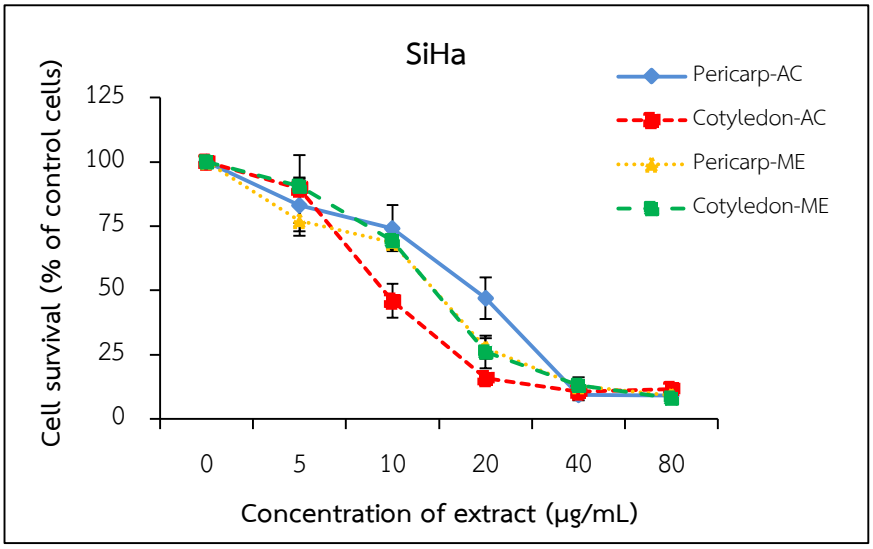


Figure 2 Cell survival curve by dose response of VDS extracts on HeLa (A) and SiHa (B) cells after 72 h treatment. Data were expressed as mean±SD.

Table 1 The cytotoxic activity in 50% growth inhibitory concentration (IC_{50}) of *V. diospyroides* type SS fruit extracts on cancer and normal cell lines. The result showed as a mean ± SD from triplicate independent experiments.

Cell types	Cell lines	Pericarp		Cotyledon	
		Acetone	Methanol	Acetone	Methanol
Cervical cancer	HeLa	17.93 ± 0.81	13.40 ± 0.71	7.69 ± 0.44	11.60 ± 0.96
	SiHa	19.00 ± 3.37	14.55 ± 0.69	9.81 ± 1.38	14.54 ± 1.10
Normal fibroblast	L929	33.12 ± 0.82	14.10 ± 0.47	34.41 ± 2.05	24.32 ± 1.81

Activity criteria IC_{50} of crude extract: ≤ 20 µg/mL = highly active; 21-200 µg/mL = moderately active; 201-500 µg/mL = weakly active; > 501 µg/mL = inactive

Table 2 The safety response of *V. diospyroides* type SS fruit extracts in selectivity index (SI) on cancer cell lines.

Cell lines	Selectivity Index (SI)			
	Pericarp		Cotyledon	
	Acetone	Methanol	Acetone	Methanol
HeLa	1.85	1.05	4.47[a]	2.1
SiHa	1.74	0.97	3.51[a]	1.67

[a] SI value > 3 is considered to be high selectivity

3.2.2 Cotyledon-acetone extract on cell cycle arrest induction in HeLa and SiHa human cervical cancer cell lines using propidium iodide (PI) and flow cytometry analysis

The cell cycle machinery and components of checkpoint pathways have already provided the targets for novel antitumor (21). Since the cotyledon-acetone extract seemed to be the most effective extract. So, the extract was chosen for further analysis to identify its cytotoxic mechanism. Mode of cancer cell death by cell cycle arrest induction of the cotyledon-acetone extract was measured by flow cytometry. This method is a cell cycle analysis using propidium iodide (PI). The PI is fluorescent nucleic acid dye to identify the population of cells in cell cycle phase by using flow cytometry to measure their relative DNA content. In this study, cell cycle of HeLa and SiHa were analyzed by Amnis® ImageStreamX Mark II imaging flow cytometer. The single cell for 10000 events was acquired with flow cytometer and data analysis using IDEAs image data exploration and analysis software. The results from software were shown as a histogram. The fluorescence intensity of the stained cells was correlated with the amount of DNA content. This approach reveals the distribution of cells in three major phases of the cycle including G1, S and G2/M (Appendix A3).

In order to investigate the mode of cell death of cotyledon-acetone on cell cycle progression, PI staining analysis was measured on treated cells with IC_{50} and 2-fold IC_{50} for 24 and 48 h and the results were expressed as the mean of three independent experiments as shown in Figure 3A and 3B. As presented in Figure 4A, after 24 h of the treatment, IC_{50} cotyledon-acetone-treated HeLa cells were slightly decreased of cell population in G1 and G2/M phase as 52.71% and 21.35%, respectively. Whereas high dose extract at 2-fold IC_{50} induce the percentage in the cell cycle distribution of G1 phase was decreased from 20.67% to 17.68% when compared to control. When extending the treatment time to 48 h, the cell population in sub-G1 phase was increased in dose-dependent manner. Interestingly, the distribution of S and G2/M phase were significantly increased in 2-fold IC_{50} treatment with 12.93% to 25.08% and 14.50% to 27.59% when compared with control. For SiHa cell, number in

G1 slightly decreases and S and G2/M increased in IC_{50} treatment for 24 h. Meanwhile, the percentage of the treated SiHa cells in S and G2/M phase was augmented from 7.34% in control to 11.49% and 16.98% to 23.73%, respectively, in IC_{50} treatment at 48 h. The 2-fold IC_{50} treated cell seemed to be similar with the IC_{50} treatment (Figure 4B). From these data, we conclude that the cotyledon-acetone extract had the ability to anti-proliferation on cervical cancer cell lines especially in HeLa by cell cycle arrest induction at G2/M, but the initiation stage was occurred in the S phase.

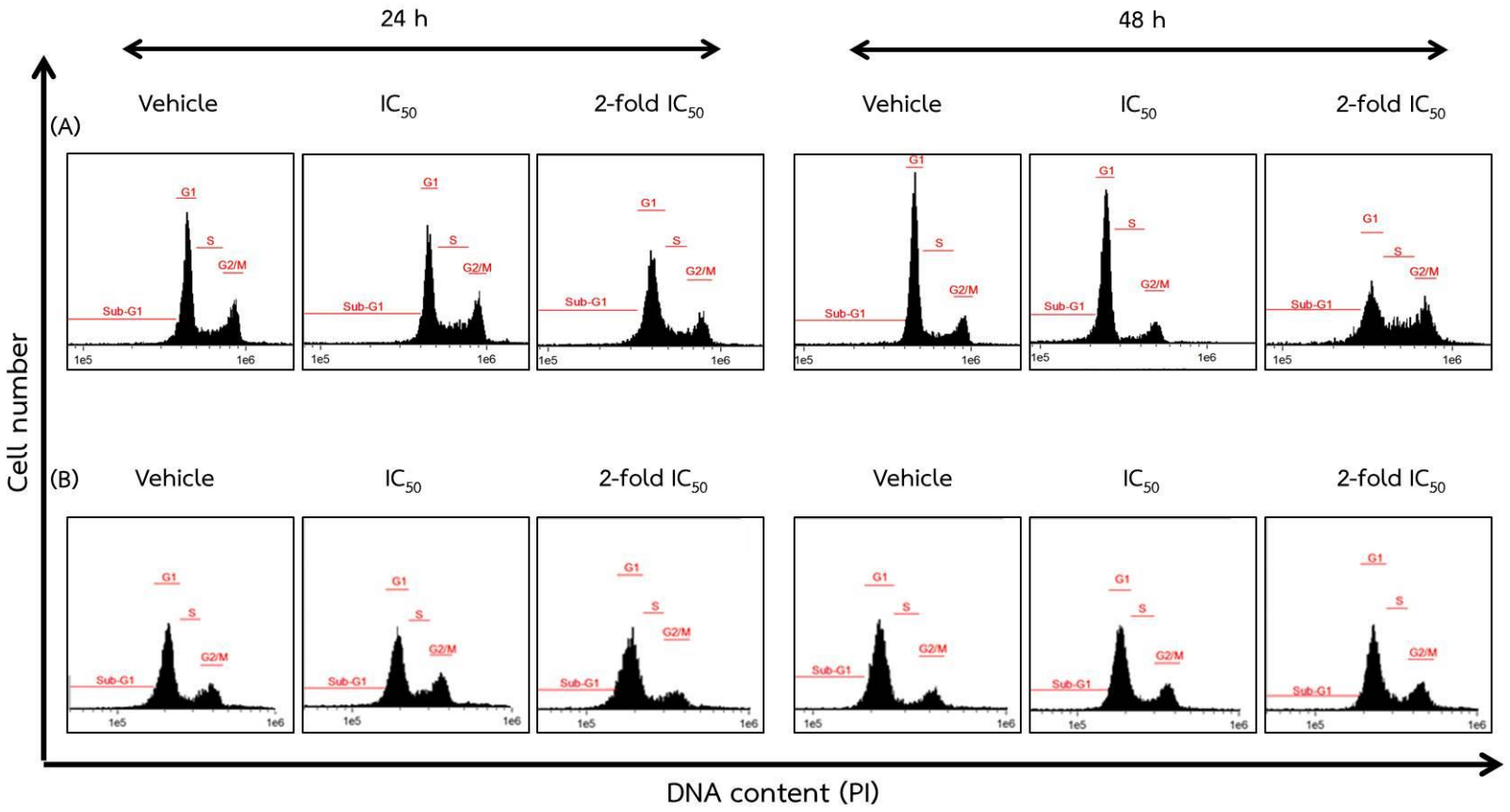


Figure 3 DNA distribution histograms on cell cycle profile of cotyledon-acetone extract-treated HeLa (A) and SiHa (B) human cervical cancer cell lines. DNA content was analyzed by PI staining and flow cytometry analysis

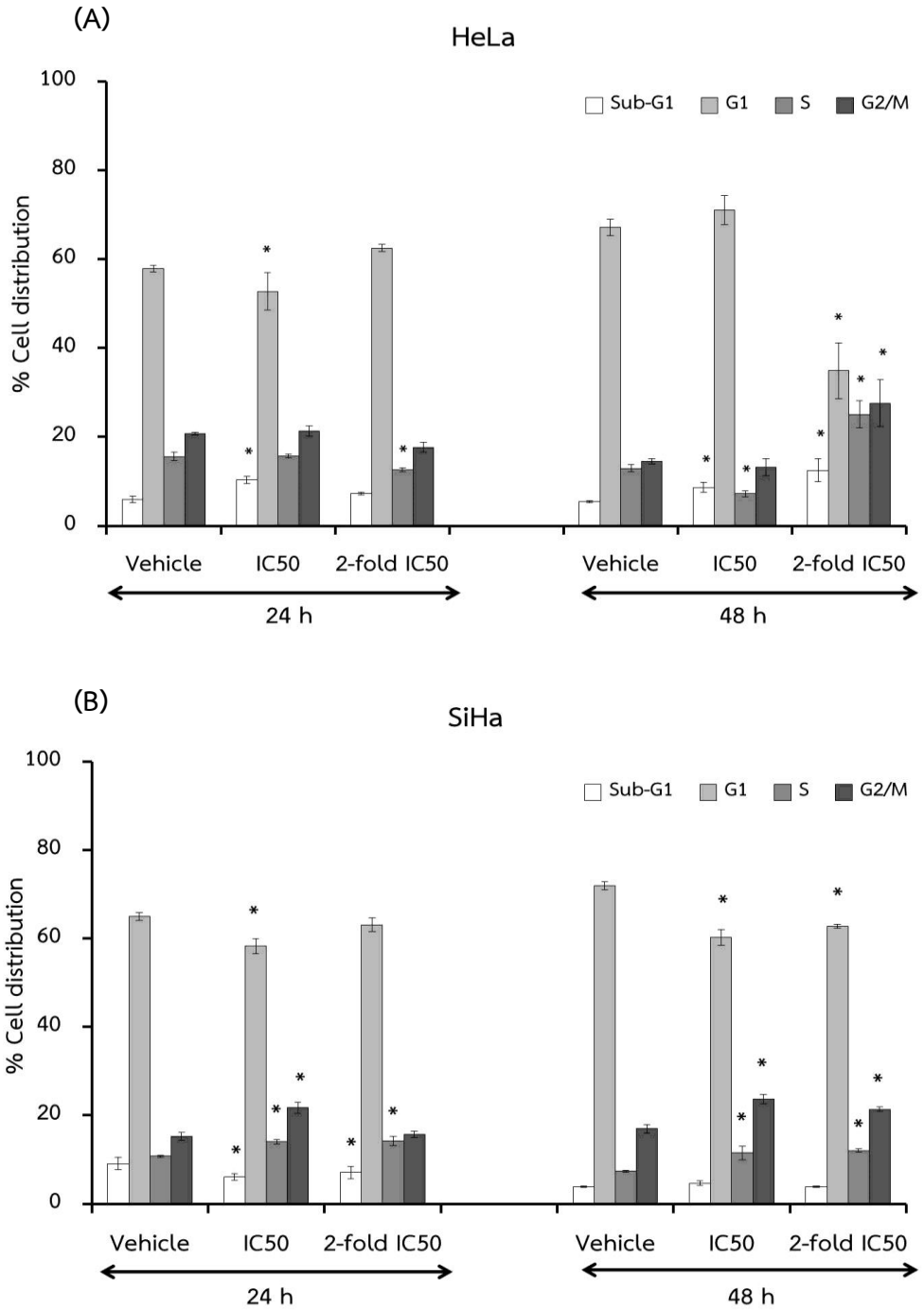
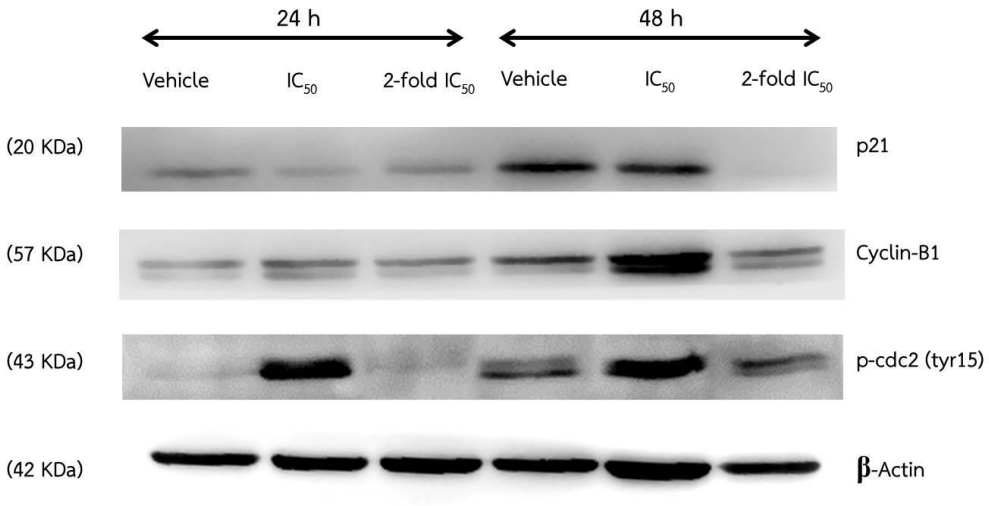


Figure 4 Percentage of cycle distribution in each cell cycle phase on HeLa (A) and SiHa (B) cells with cotyledon-acetone extract treatment. The data was showed to triplicate independent experiments. (*$p < 0.05$, the mean difference is significant at the 0.05 level compared to control by one-way ANOVA)

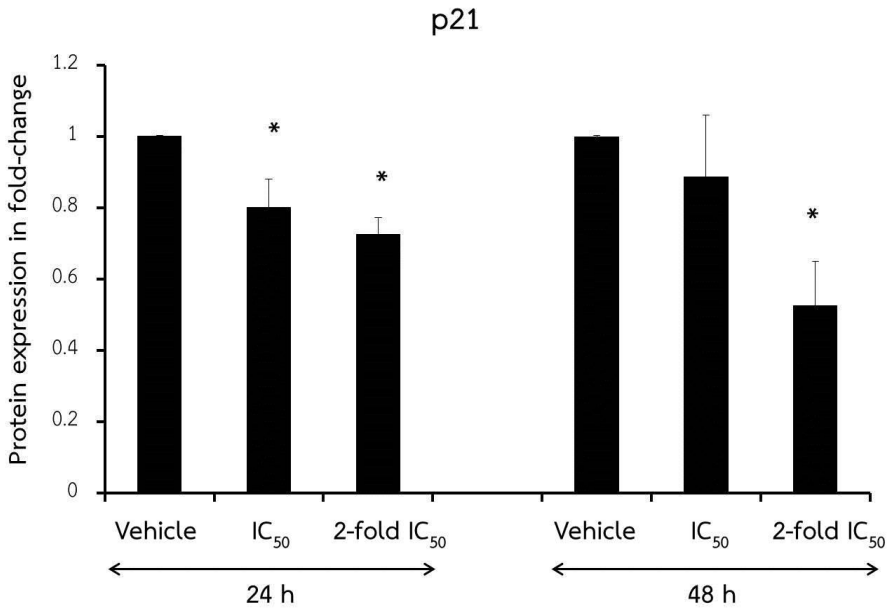
3.2.3 Effect of cotyledon-acetone extract on the protein expression involved in cell cycle arrest induction in cervical cancer cell lines

Since the cotyledon-acetone extract was determined to induce the cell cycle arrest in G2/M phase at 48 h particularly on HeLa cell, we further performed its effect on the expression of protein related in G2/M arrest molecule which are p21, cyclin-B1 and phosphorylative form of cdc2 (p-cdc2 or CDK1). To better understand how the extract induces G2/M arrest, the cell cycle arrest related molecules in protein level were performed by western blot analysis (Figure 5A). The key factor protein of G2/M phase is driven by cyclin-B1. The cyclin/Cdk families play a critical role or cell cycle progression. The expression of cell cycle inhibitor which is p21 was determined on HeLa cells. The p21 expression was decreased in dose-dependent manner both 24 and 48 h treatments (Figure 5B). For cyclin-B1, the extract induced the expression of this protein for 24-48 h (Figure 5C). Similarly, p-cdc2 protein also increased in time–dependent manner, but decreased expression in term of dose dependent manner (Figure 5D). These results may convince that the cotyledon-acetone extract-modulated cell cycle arrest at G2/M phase. Although, p21 play critical role in G2/M checkpoint, in our result, the extract did not regulate the expression on HeLa cells at G2/M arrest, suggesting that the extract may cause G2/M arrest through other molecules such as p27 and p57.

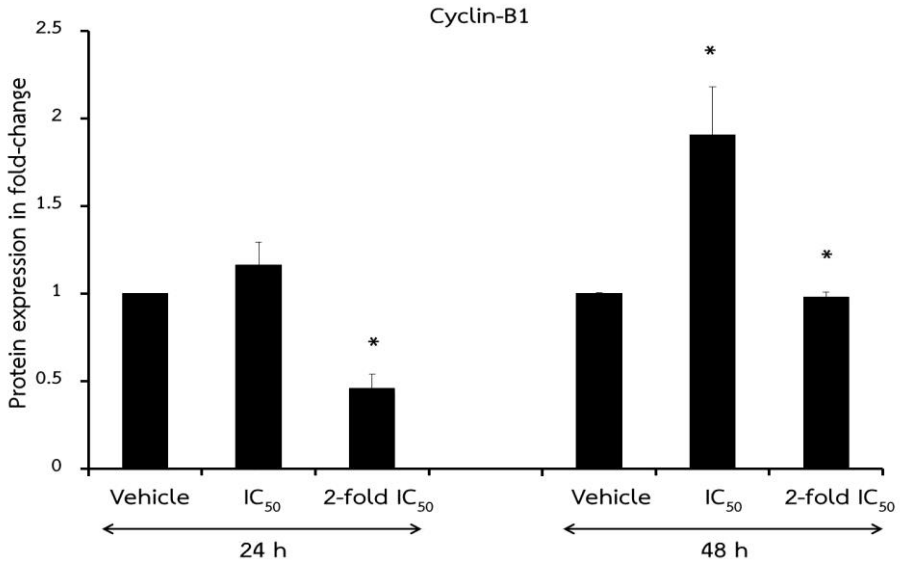
(A)


24 h
48 h
Vehicle
IC50
2-fold IC50
Vehicle
IC50
2-fold IC50
(20 KDa)
p21
(57 KDa)
Cyclin-B1
(43 KDa)
p-cdc2 (tyr15)
(42 KDa)
β-Actin

(B)


p21
Protein expression in fold-change
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Vehicle
IC50
2-fold IC50
Vehicle
IC50
2-fold IC50
24 h
48 h

(C)



(D)

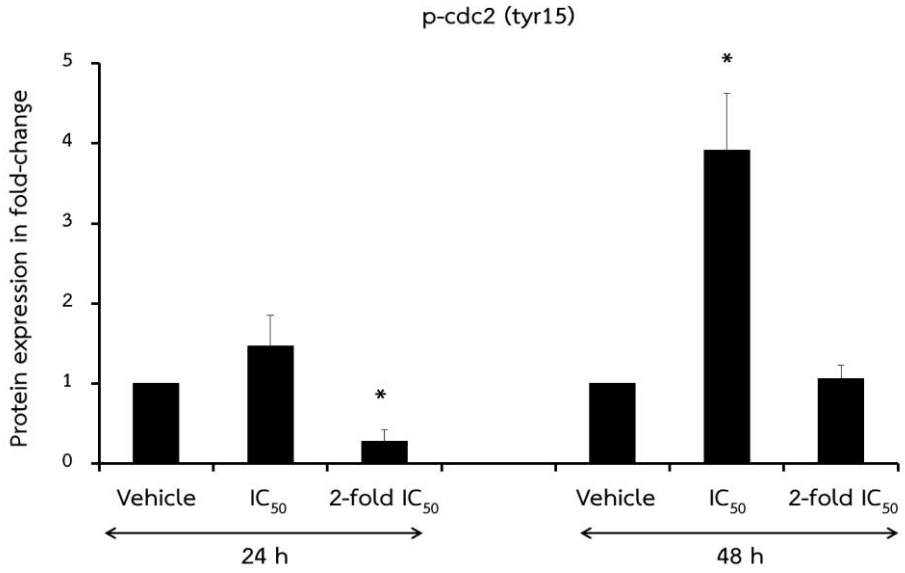


Figure 5 Effect of cotyledon-acetone extract on cell cycle arrest induction related-proteins in HeLa cells were measured by western blot analysis (A). Cells were treated with the extract at IC_{50} and 2-fold IC_{50} for 24 and 48 h. Quantification of protein expression levels were shown for p21 (B), cyclin-B1 (C) and p-cdc2 (Tyr15) (D). (*p < 0.05, the mean difference is significant at the 0.05 level compared to control by one-way ANOVA with LSD multiple comparisons)

3.2.4 Apoptosis induction of cotyledon-acetone extract on HeLa and SiHa human cervical cancer cell lines using Annexin V-FITC/PI binding and flow cytometry analysis

The mechanisms of action of plant-derived anticancer drugs are numerous and most of them induce apoptotic cell death that may be intrinsic or extrinsic (22). The data from flow cytometry analysis was analyzed by WinMDI software and represented as a density plot diagram. The cell populations were discriminated by the positions in the plot (Appendix A2).

This experiment, HeLa and SiHa cells were used as a model for apoptosis induction by cotyledon-acetone extract. After 48 h of various concentration of the extract at half- IC_{50}, IC_{50} and 2-fold IC_{50} treatment, we found that cotyledon-acetone extract decreased viability from 98.50% to 7.15% in HeLa cells as shown in Figure 6A and Figure 7A. Importantly, the percentage of apoptotic cells which is a total of lower and upper-right quadrants was considerably increased from 0.80% in control to 85.98% in 2-fold IC_{50} treatment. Noteworthy, the extracts showed the efficiency for apoptosis induction in a dose-dependent manner. On the other hand, the extract showed slightly decreased the SiHa live cells and apoptotic cell population of half- IC_{50}, IC_{50} and 2-fold IC_{50} were not different when compared to those groups (Figure 6B and Figure 7B). However, the apoptosis induction of SiHa cell was seemed to be slightly increased in dose-dependent manner. It is noticeable that the extract at half-IC_{50} exhibited negligible apoptosis induction by increased the apoptotic cells from 1.15% to 32.90%, while the viable cells were declined from 98.85% to 65.90% when compared to control. Although, apoptosis induction was also shown in SiHa cells, but expressed to the lower rate of apoptotic cells when compared with HeLa cells. In summary, we could initially conclude that the cotyledon-acetone extract shows promising apoptosis induction in cervical cancer cell lines, especially in HeLa cell.

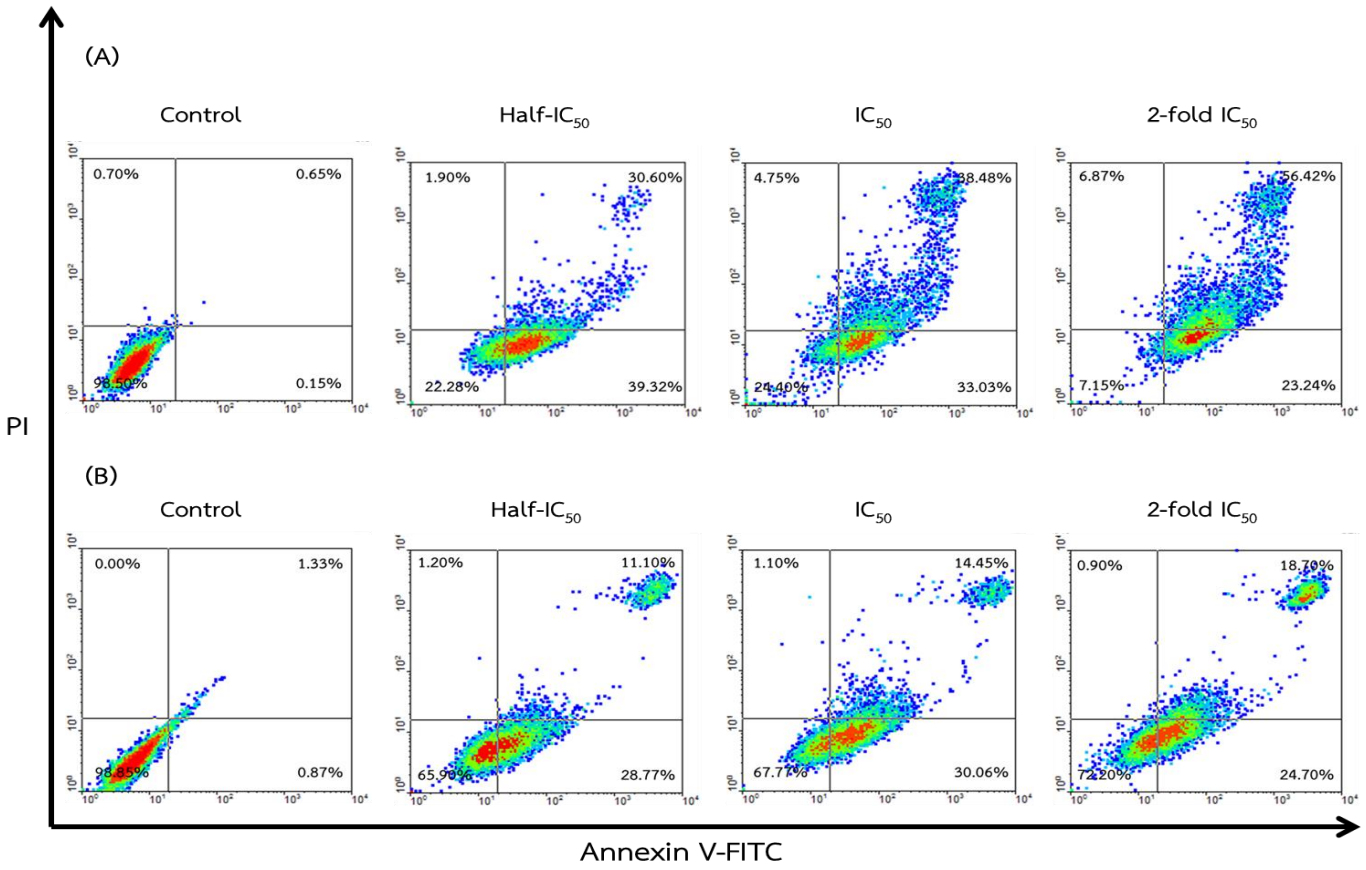
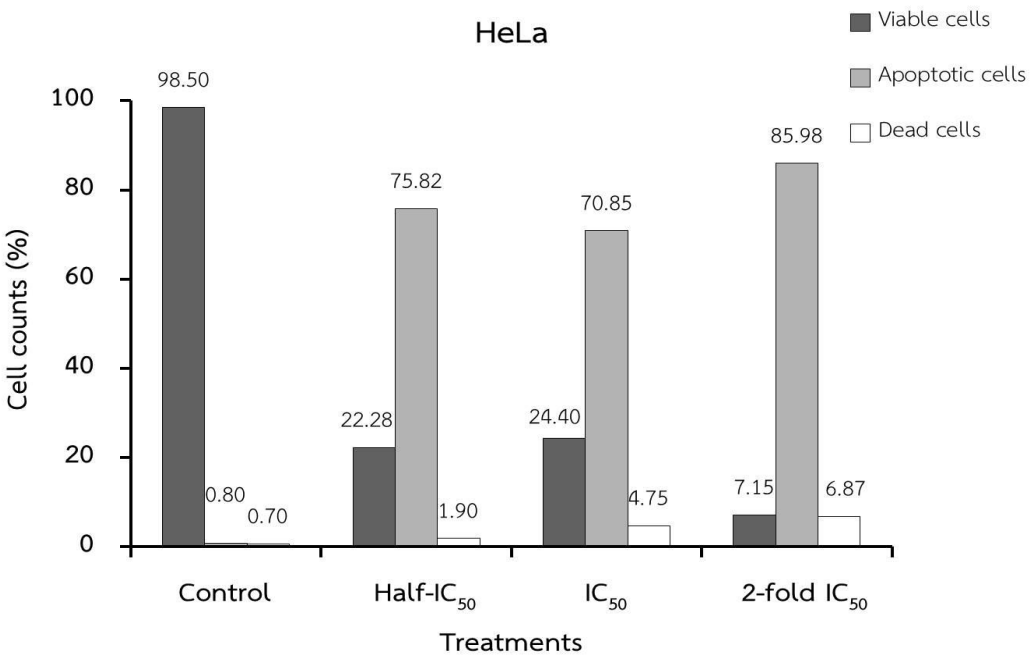


Figure 6 Effect of cotyledon-acetone extract on HeLa (A) and SiHa (B) cervical cancer cell lines using flow cytometry analysis with Annexin V-FIC/PI binding. The data were represented in viable cells (lower-left quadrant), apoptotic cells (lower-right quadrant and upper-right quadrant) and dead cell (upper-left quadrant).

(A)



(B)

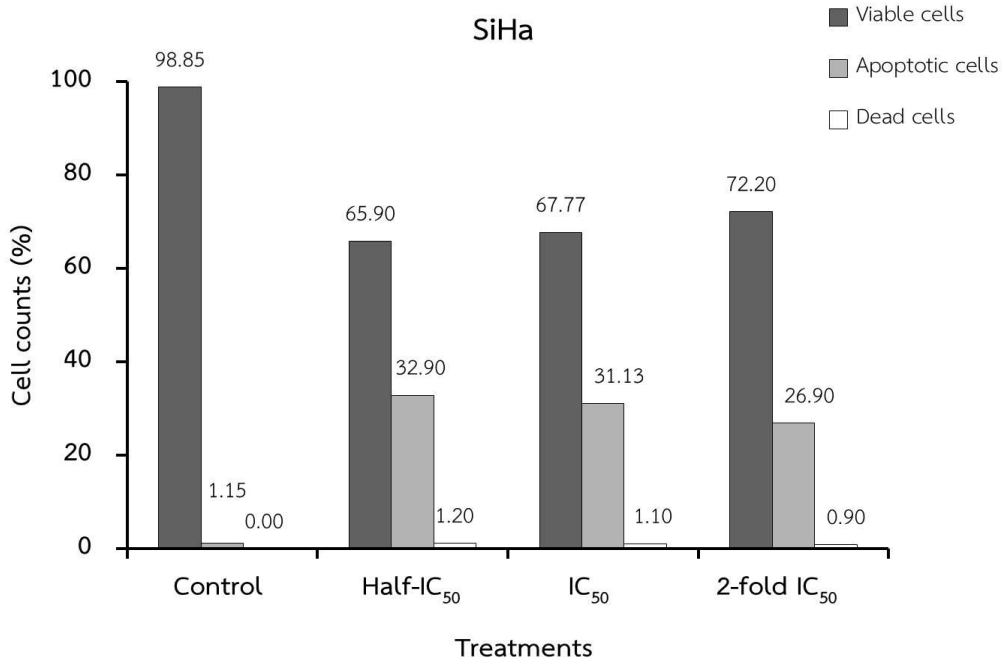
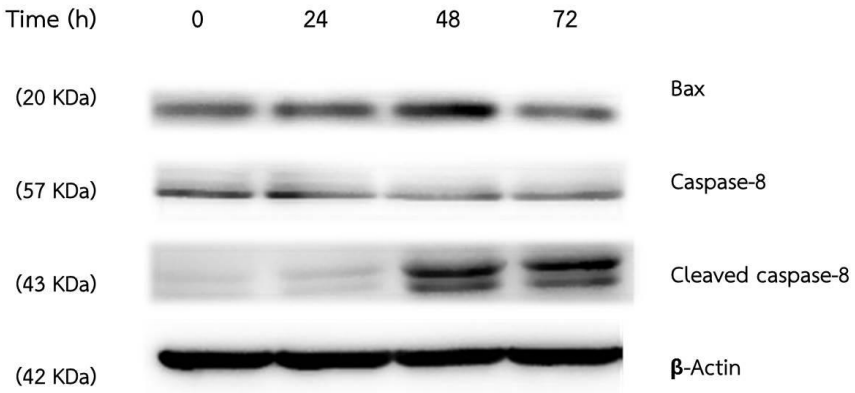


Figure 7 Cell populations by cotyledon-acetone extract-induced apoptosis on HeLa (A) and SiHa (B) cell lines using flow cytometry.

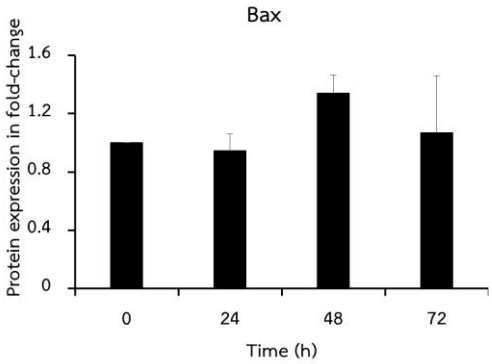
3.2.5 Effect of cotyledon-acetone extract on the protein expression involved in apoptosis induction in cervical cancer cell lines

To investigated molecular events underlying cotyledon-acetone extract on apoptosis induction in HeLa cervical cancer cell line. Bax as a pro-apoptotic protein that involved in mitochondrial apoptosis or intrinsic pathway was measured under the treated-cell at half-IC_{50} for 0, 24, 48 and 72 h. Bax induction were more detectable with expanding time up to 72 h (Figure 8A and 8B). As shown in Figure 5C, while the expression of caspase-8 was reduced (Figure 8C), the cleaved caspase-8 as an active form was continuously escalated in time-dependent manner (Figure 8D). From these results, we could assume that the cytotoxic activity of the extract on HeLa cell maybe possibly through apoptosis induction via Bax induction and encouragement of active caspase-8.

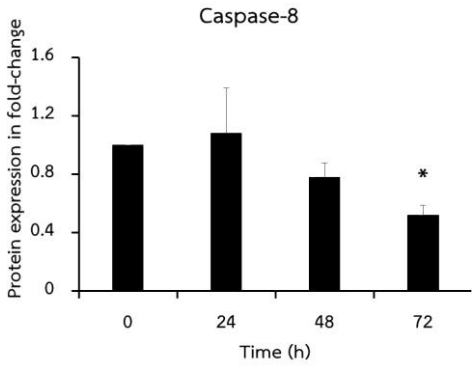
(A)



(B)



(C)



(D)

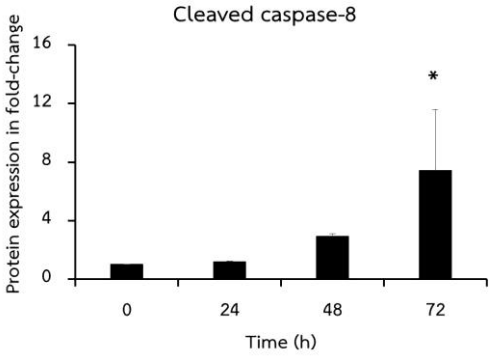


Figure 8 Cotyledon-acetone extract on related-apoptosis induction proteins in HeLa cells were performed by western blot analysis (A). Cells were treated with the extract at half- IC_{50} for 0, 24, 48 and 72 h. Quantification of protein expression levels were shown for Bax (B), caspase-8 (C) and cleaved caspase-8 (D).

3.3 Discussion

Based on medical record, natural source has long been reported to be a precious and trustworthy origin of drug development, including antitumor therapies (23). It is well known that the natural resource serves as immense medicinal products which originate from marine, microorganism, especially plants. Throughout medicinal background, plants have been used as a valuable source with their bioactivities inducing anticancer properties (24) . However, several steps were required for anticancer drug discovery which initiate by preclinical screening. The aim of screening process is to indicate the agent which contains the cytotoxic or anticancer effects (25). This research was determined the cytotoxic activity of the crude extracts and their mode of cell death induction on cervical cancer cell lines which are HeLa and SiHa. All extracts showed highly cytotoxic effect on both cervical cancer cell lines with ranked of IC_{50} values between 8-19 µg/mL (Table 1). In addition, the cytotoxic values of each extract in both cell lines were similar. Surprisingly, the cotyledon-acetone extract demonstrated the highest cytotoxicity with lowest IC_{50} value in HeLa and SiHa. Regrettably, pericarp-methanaol and cotyledon-methanol extracts were represented not only the lower IC_{50} value on cancer cell lines, but also cytotoxic to L929 murine fibroblast cells. The different anti-proliferation effect of these extracts was possibly relied on cellular characteristics of each cell line. According to the different cellular histological of those cell lines, these extracts showed specifically cytotoxic activity against HeLa less aggressive cancer. This hypothesis was supported by previous research, the researchers has been reported that the up regulation of nuclear factor erythroid-related factor2 (NRF-2) which is an oncogene in SiHa cell resulting in inducing anti apoptosis, metastasis and resistance to therapy. Following this evidence, it implies that SiHa cell contain more aggressive characteristic when compare to HeLa (26). The excellent cytotoxicity of cotyledon- acetone extract was maybe based on the phytochemical constituents that contain in the extract. However, the phytochemical component of SS fruit extract has not been screened. Previous research has been tested the phytochemical in cotyledon- acetone extract of LS fruit subtype of *V. diospiroides*. Terpenoid, anthraquinones and saponins which has been reported as potential anticancer agents (27-29) were the main component that found in the extract

(30). Ground on the phytochemical data of cotyledon-acetone extract of LS subtype, we could deduce that the cytotoxic effect on cancer cells by the cotyledon-acetone extract of SS subtype maybe through the bioactivity effects of those phytochemical compounds. The best cytotoxicity against cancer cell lines in this study was consistent with previous report (16). Moreover, *in vitro* screenings of cytotoxic activity of the cotyledon-acetone extract has shown on MDA-MB-468 human breast cancer cell line with highly active effect (16). Although, the cytotoxicity of these extracts on cancer cell lines was the key of anticancer screening, but the safety response on normal cell also required. The safety effect of these extracts was determined by selectivity index (SI) which calculated from IC_{50} value of normal cell versus cancer cells (31). The result showed that only the cotyledon-acetone extract demonstrated the safety response on both colorectal and cervical cancer cell lines with SI value more than 3 (Table 2). Based upon the cytotoxic activity, the cotyledon-acetone extract not only showed highly cytotoxicity, but also presented the safety response on both groups of cancer as mentioned above which specific toxicity against cancer cells. Therefore, cotyledon-acetone extract was used to investigate the mode of cell death and mode of action for further experiments. It is well known that uncontrolled cell proliferation cause to cancer, so inhibiting this process will result in the positive response in cancer therapy. In this study, we determined the cell cycle arrest induction on cervical cancer cells using PI staining analysis. For IC_{50} treatment, the cell population in G2/M phase was increased at 24 h, while the population was decreased at 48h. The result may hypotheses that this alteration may cause by normal process in cell cycle progression. As shown at 48 h after treatment, the cell population in G1 phase was increased. The extract clearly induced the accumulation of HeLa cells in G2/M phase, while declined the G1 phase population of 48 h after 2-fold IC_{50} treatment (Figure 3A and 4A). For SiHa, the alterations of cell populations were similar occurring as HeLa (Figure 3B and 4B), but showed slightly altered. These results suggested that the extract could induce G2/M arrest in HeLa and SiHa cells at 48 h for 2-fold IC_{50} treatment. Many studies have shown the accumulation of G2/M phase together with decreasing of G1 phase was identified to G2/M cell cycle arrest (32, 33). In accordance with our results, the G2/M arrest was occurred in human gastric carcinoma by deoxypodophyllotoxin (34).

Consequently, to confirm cell cycle arrest induction of the extract, the protein that involved in G2/M arrest were performed in HeLa cell using western blotting. The result showed that p21 cyclin-dependent kinase inhibitor was decreased in dose-dependent manner both 24 and 48h treatments in dose-dependent manner (Figure 5B). This result suggested that p21 maybe not a key regulator in mode of action by cotyledon-acetone extract. The G2/M arrest induction on HeLa cells may cause through other molecules such as p27 and p57. There are many molecules involved in cell cycle progression and two key molecules that drive the cell cycle in eukaryotic cell are cyclin and cyclin dependent kinase (Cdk). Main protagonist protein related G2/M transition is cyclin-B1/Cdk1 complex (35). The cyclin-B1 and phosphorylated form of cdc2 (p-cdc2 or CDK1) protein was also measured in HeLa cells. The result showed that the extract at IC_{50} induced the expression of cyclin-B1 for 24-48 h which consistent with flow cytometry result (Figure 5C). The extract showed the accumulation of cyclin-B1 expression which is crucial marker for mitotic arrest (36). Meanwhile, p-cdc2 protein also increased in time–dependent manner (Figure 5D). Generally, the phosphorylation of cdc2 at Tyr15 resulting in kinase activity inhibition (37). The result suggested that prolonged treatment may up-regulate the p-cdc2 leading to G2/M arrest. This result was supported with the G2/M arrest induced by *Origanum majorana* extract on breast cancer cells (38). Generally, cyclin-B1 accumulation peak in metaphase and it degrade before entry to anaphase by anaphase-promoting complex (APC) (39). In case of this study, the augmentation of cyclin-B1 cause to G2/M arrest as shown by G2/M population was increased (Figure 3A and 4A). These results indicated that the extract may be able to induce G2/M arrest by inhibiting of kinase activity with p-cdc2 and activating of cyclin-B1 accumulation. Many anticancer drug discovery researches reported that apoptosis induction and cell cycle arrest are used as a target in cancer therapeutics. In this study, we measured the apoptosis induction function by flow cytometry analysis. Apoptosis or programmed cell death is a crucial pathway for eradicating abnormal cells. When this process fails, it can cause various diseases, especially cancer (40). This experiment, the mode of cell death of cervical cancer cell lines including HeLa and SiHa were performed by flow cytometer. Interestingly, HeLa-treated cells showed increased of apoptotic cells, while decreased the viable cells in

dose-dependent manner (Figure 6A). On the other hand, the extract was slight induced apoptosis on SiHa cells (Figure 6B) as compared with HeLa in dose-dependent manner. Similarly, a previous work reported that the extract could inhibit human breast cancer cell line through the apoptosis induction by time- dependent manner (16) . Furthermore, the apoptosis induction activity on HeLa cells were confirmed by protein expression using western blotting. Main two different pathways of apoptosis are mitochondrial or intrinsic and death receptor or extrinsic pathway (41). Our study, we selected the representative apoptosis-related protein in both pathways namely Bax (intrinsic pathway), Caspase-8 and cleaved caspase-8 (extrinsic pathway). We found that Bax, a pro-apoptotic protein was increased after 48 h of treatment (Figure 8A and 8B). On the other hand, caspase-8 was measured in HeLa cells and the result showed that the expression of caspase-8, an inactive form, was continuously declined (Figure 8C), together with consecutively increasing of active form cleaved caspase-8 (Figure 8D). Intrinsic apoptosis induction is well related with accelerate in Bax protein expression and Bax can induced the cytochrome c release from mitochondria and form with Apaf-1 and caspase-9 as called Apoptosome complex and it can cleave caspase-9 to active form and then cleaved the effector caspases such as caspase-3 and caspase-7 lead to apoptosis. For extrinsic pathway, cleaved caspase-8 is a key molecule that acts as initiator caspase for this process and lead to apoptosis. Meanwhile, cleaved caspase-8 can cleave BID to tBID and tBID fragment then translocates to mitochondria, causes to cytochrome c release, resulting in formation of apoptosome complex, thereby activating a cascade of caspase pathway, and cell death (42). Our results demonstrated that the cytotoxic activity of the extract on cancer cell lines maybe possibly through apoptosis induction via both intrinsic and extrinsic pathways. This hypothesis is supported by a previous research that showed intrinsic and extrinsic activation of *Emilia sonchifolia* extract on colorectal cell line (43) and *Solanum lyratum* extract on leukemia cell line by increased Bax and cleaved caspase-8, while decreased the caspase-8 (44). Although, the p21 is not related with cell cycle arrest, there is evidence was reported that decreasing of p21 expression resulting in apoptosis induction. Piccolo and Crispi (2012) reported that in case of prolonged treatment, p21 was inactivated through cleavage caspase-3 and decreasing

of p21 resulting in apoptosis induction (45). Reduction of p21 expression was consistent with the apoptosis result which showed apoptosis induction in dose- dependent manner. This hypothesis was supported with the decreasing of p21 expression in prostate cancer cells to sensitization for doxorubicin and lead to apoptosis induction (46). Finally, it may be concluded that the cytotoxic activity of cotyledon- acetone extract maybe through both of cell cycle arrest and apoptosis induction on cervical cancer cell as shown in Figure 9. To our research, the cotyledon-acetone extract would be a candidate anticancer agent with promise anticancer properties and might be lead to alternative approach for cancer therapies.

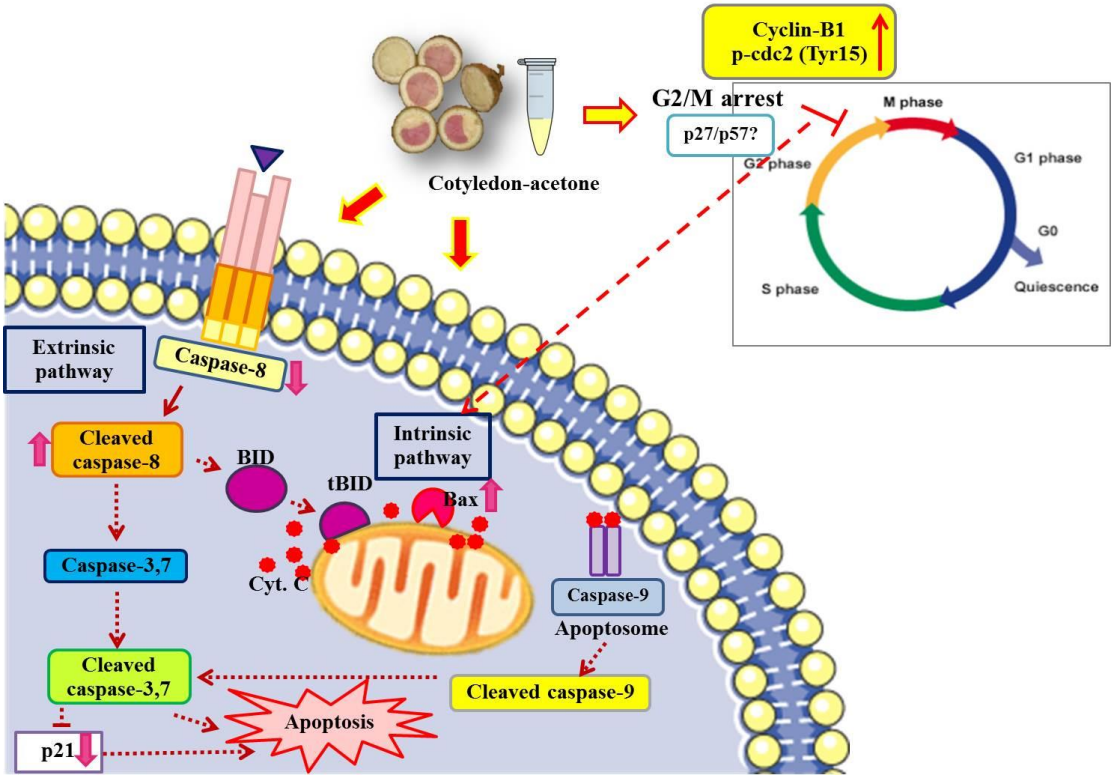


Figure 9 The possible pathway for anticancer property of cotyledon-acetone extract on cervical cancer cell line

CHARPTER 4
CONCLUDING REMARKS

1. The crude extracts of VDS type SS fruit showed highly active against both cervical cancers which are HeLa and SiHa cell lines.

2. The cotyledon-acetone extract notably showed the highest cytotoxicity on cervical cancer and exhibited high safety response on normal fibroblast L929 cell.

3. The cotyledon-acetone extract induced cell cycle arrest at G2/M phase and apoptosis.

4. Cell cycle arrest at G2/M phase effect by the cotyledon-acetone extract on HeLa and SiHa cells due to accumulation of cyclin-B1 protein expression.

5. The cotyledon-acetone extract induce apoptosis on HeLa and SiHa cell lines via increasing of Bax (pro-apoptotic protein) and caspase-8 was declined together with increasing of cleaved caspase-8 expression in HeLa cells which indicated that the cytotoxic activity of the extract on cervical cancer cell lines may be possibly trough apoptosis induction via both intrinsic and extrinsic pathways.

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APPENDIX A

1. Cytotoxicity screenning of VDS crude extracts on human cancer cell lines using (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) MTT assay

Cell viability was performed by colorimetric technique MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT is the widely used method for determination of cell viability and cytotoxic effect in drug screening process. In viable cell, the mitochondrial reductase enzyme can generate the NADH and NADPH that act as reducing equivalent resulting in reduce yellow tetrazolium salt [3-(4, 5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] to purple formazan crystal (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan. The crystal is dissolved with dimethyl sulfoxide (DMSO) and the absorbance value is measured at 570 and 650 nm. Therefore, the viable cell shows high intensity of purple formazan, while the dead cell that has inactive mitochondrial reductase enzyme resulting in fail to reduce the yellow tetrazolium indicate with lower absorbance value. These data are calculated for percentage of viability as showed in equation (1) and plot the graph for the half maximal inhibitory concentration (IC_{50}) value. Additionally, selectivity index (SI) is calculated which dividing the cytotoxicity as IC_{50} of the normal cell by the cancer cell in same extracts as showed in equation (2).

$$\text{Percentage of cell viability} = \frac{\textbf{OD 570–650 nm of treated cells}}{\textbf{OD 570–650 nm of untreated cells}} \times 100 \qquad (1)$$

$$\text{Selectivity index (SI)} = \frac{\textbf{IC50 of A extract on normal cells}}{\textbf{IC50 of A extract on cancer cells}} \qquad (2)$$

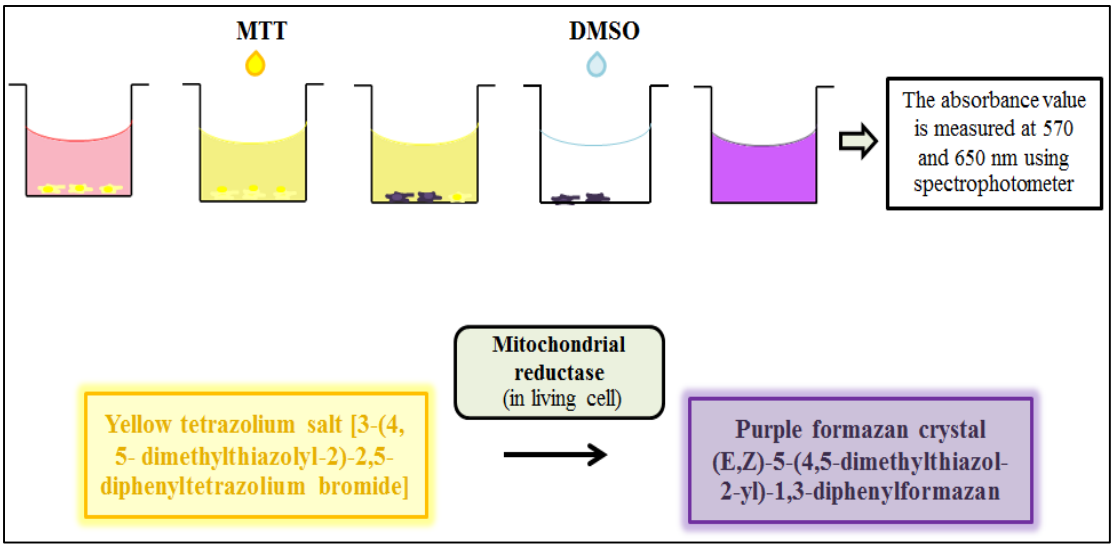

Figure 10 Representative diagram of MTT assay

2. Apoptosis induction using Annexin V-FITC/PI binding and flow cytometry analysis

The data which derived from flow cytometry analysis and WinMDI software was displayed as a density plot diagram and the results were interpreted by the position of the cell population in each quadrant of the diagram. In healthy cell, phosphatidylserine (PS) that represented as purple circle is located at inner cell surface membrane resulting in viable cells can exclude both dyes with the complete plasma membrane and showed rid of both Annexin V-FITC and propidium iodide (PI) intensity as showed in lower-left quadrant. Meanwhile, the early apoptosis is occurred, initially altered by cell surface flip-flop of PS to outer membrane. Whereupon, Annexin V which a dye that conjugated with Fluorescein isothiocyanate (FITC) and high affinity binding with PS is used as a detector to determine the early apoptotic cell as showed in lower-right quadrant. Following the altered of membrane position, the PS membrane is changed and the cell also loss of membrane integrity in late apoptosis process resulting in the late apoptotic cells not only stained with Annexin V-FITC, but also labeled with PI as appeared in the upper-right quadrant. In death cells, whereas the cell that loss of plasma and nuclear membrane, PI which used as a DNA binding dye can readily enter to the cell and bind with DNA in the nucleus which showed as the upper-left quadrant.

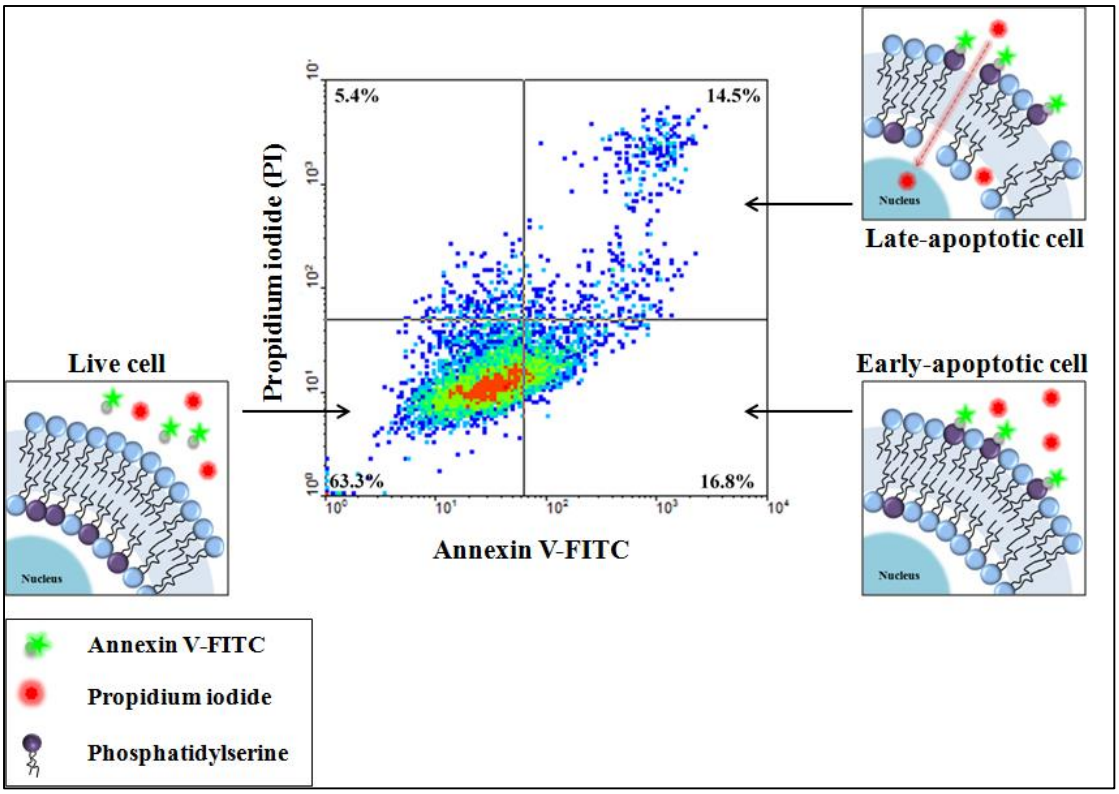

Figure 11 The density plot diagram representative cell population in apoptosis assay using Annexin V-FITC and propidium iodide (PI) staining and flow cytometry analysis

3. Cell cycle distribution analysis by propidium iodide (PI) and flow cytometry analysis

The propidium iodide (PI) is fluorescent nucleic acid dye to discriminate the cell population in cell cycle phase by using flow cytometry to measure their relative cellular DNA content. Cell cycle was analyzed by Amnis® ImageStreamX Mark II imaging flow cytometer. The single cell for 10,000 cell counts was acquired with flow cytometer and data analysis using IDEAs image data exploration and analysis software. The result from software was shown as a histogram. The fluorescence intensity of the PI-stained cells was correlated with the amount of DNA content. This approach reveals the distribution of cells in three major phases of the cycle including G1, S and G2/M. PI intensity is used for describe the cell proportion in each phase of cell cycle. In G1 phase, this phase is called that synthesis phase because the RNA and protein molecules that is equipped for cell division were synthesized in this process (2N DNA content) as showed the lower PI intensity as G1 peak. The S phase is a duration time for DNA synthesis resulting in altered the cellular DNA content to 2N and 4N as showed the higher PI intensity in S peak. In G2/M phase, this phase show 4N DNA content from continuous growth cell and mitosis stage as indicated in G2/M peak.

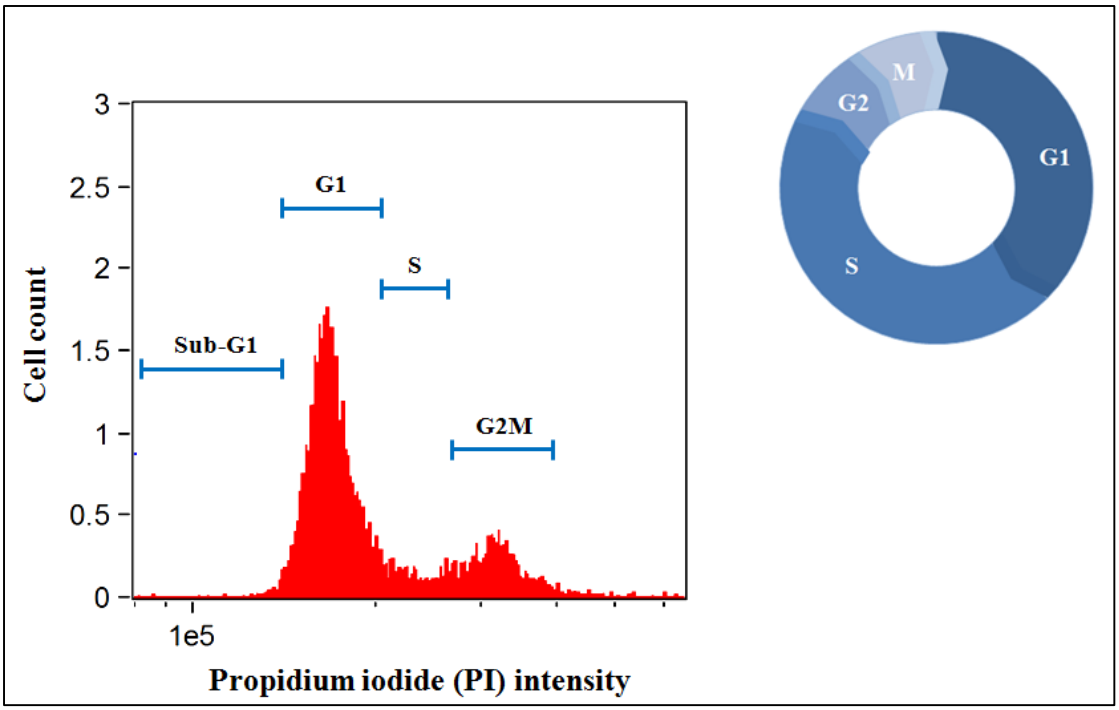

Figure 12 The histogram of cell cycle distribution using propidium iodide (PI) and flow cytometry analysis

APPENDIX B

Submitted Manuscript

Chothiphirat A, Srisawat T, Kanokwiroon K, Nittayaboon K, Navakanitworakul R. Anticancer potential of fruit extracts from *Vatica diospyroides* symington type SS and their effect on program cell death of cervical cancer cell lines.

Anticancer Potential of Fruit Extracts from *Vatica diospyroides* Symington Type SS and Their Effect on Program Cell Death of Cervical Cancer Cell Lines

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Abstract

Vatica diospyroides Symington or locally known as Chan-ka-pho in Thailand. An ancient people have been used as therapeutical plant for cardiac and blood tonic cure. The anticancer property of the plant extracts has been reported with greatly cytotoxicity and apoptosis induction on breast cancer cell lines. The purpose of this research was to investigate the potential cytotoxic effect and mode of its action of VDS extracts on human cervical cancer HeLa and SiHa cell lines. The cytotoxicity of the extracts was observed using MTT colorimetric assay. Our results revealed that the extracts showed inhibition of cell survival in dose-dependent manner and exhibited highly cytotoxic activity against both HeLa and SiHa cells with IC_{50} value less than 20 µg/mL (7.69 ± 0.44 - 19.00 ± 3.37 µg/mL). Moreover, the cytotoxicity of these extracts also determined on L929 normal cell line. Unfortunately, only the acetone cotyledon extract was shown safety response on normal cells. Therefore, acetone cotyledon extract was further evaluated apoptosis induction characterizing by Annexin V-FITC and propidium iodide (PI) staining and flow cytometry analysis. The results suggested that the extract has indicated ability of apoptosis induction by increasing of apoptotic cells and decreasing of viable cells compared to control. Interestingly, HeLa cells treated showed clearly expanding of apoptotic cell population by dose-dependent manner. In addition, the apoptosis feature was confirmed by DNA fragmentation. The

DNA ladder were appeared after 48 h and obviously showed laddering pattern for 72-120 h of treatment. Furthermore, the extract also induced an increasing of Bax protein, concordant with the induction of cleaved caspase-8. Our finding indicated that the apoptosis pathway related in acetone cotyledon extract-promoted antiproliferation may be through the intrinsic pathway. Based on our results from this study, the extract could be carried out of its potential biological activity to alternative approach for cancer treatment.

Keywords: *Vatica diospyroides Symington, cervical cancer, cytotoxicity, Flow cytometry, apoptosis*

Introduction

Cervical cancer is a serious cause of death in women. It is the fourth worldwide ranking of common cancer incidence and mortality rate among females. According to the 2012 GLOBOCAN report, cervical cancer has been diagnosed as 528,000 new cases and 266,000 deaths (49). In Thailand, the cervical cancer is the second major type of cancer in women with 8,184 new cases and 4,513 deaths in 2012 (50). The significant risk factor of cervical cancer are smoking or environmental tobacco smoke, oral contraceptive use, nutrition and parity including sexually transmitted infections (51). Human Papillomavirus (HPV) infection is related with cervical cancer development. Nowadays, cervical cancer treatments were radiotherapy and chemotherapy. However, these treatments were reported to have mild to severe side effects. These conventional treatments will destroy to tumor cells and normal cells and will cause adverse drug reaction such as skin problems, hair lose, bone marrow suppression, gastrointestinal abnormalities and neuropathies. (52). Consequently, the research and development of anticancer therapeutic with lower adverse drug reaction are necessary to improve patient's quality of life. The major resources of therapeutic origin is natural sources especially plants with a lot of ancient medical reports (53).

The family Dipterocarpaceae grown in the Asian rain forests and Thailand includes 9 genuses, 64 species (54). The phytochemical and biological screening in Dipterocarpaceae plans has been discovered continuously for many years (55). Various species of this family have been shown biological activities, such as anti-inflammatory,

antioxidant, anti HIV and antimicrobial activity together with against on several cancer cell lines (56). For example, hopeaphenol was isolated from *Shorea hopeifolia* shown anti-proliferation on hepatocarcinoma cell line (HepG2) (57). Ampelopsin H which is phytochemical constituent in stem bark extract of *Hopea mengarawan*, showed cytotoxic effect against human cervical cancer cell line (HeLa-S3) (58). *Vatica diospyroides* Symington belonging to Dipterocarpaceae, is an endemic plant in southern part of Thailand. Thai people used stem and flower of *V. diospyroides* as folklore medicine in cardiac and blood tonic remedies (59). The main phytochemical that found in Dipterocarpaceae is resveratrol. This compound also has been isolated from ethyl acetate of *V. diospyroides* stem extract, and has been shown against human oral epidermoid carcinoma (KB) (60). Cytotoxic activity screening from leaf, branch, root and fruit of *V. diospyroides* type LS were previous reported that the fruit extracts showed highly active against breast cancer cells (59, 61, 62). Fruit of *V. diospyroides* type LS and SS were appropriated to compare cytotoxic activity. These report indicated that *V. diospyroides* fruit type SS is a potential source of active agent. The extracts had indicated highly active cytotoxicity and induced apoptosis on human breast cancer cell line (63). Furthermore, there were continued screening of phytochemical from crude extract of *V. diospyroides* type LS fruit. Terpenoids, anthraquinones and saponins were found in the fruit extracts. These compounds have been reported for many plant species with show anticancer effect against various cancers. Terpenoids are a group of secondary metabolites with various structures and biological functions. For instance, Lupane terpenes, a member terpenoid that found in *Hopea odorata*, showed cytotoxicity against several cancers such as prostate and colorectal cancer cell lines. More than 25 terpenoids that has been isolated from *Dipterocarpus costatus* were evaluated anticancer on human cancer cells (56). Isolated anthraquinones from genus *Cassia* also showed for antitumor properties (64). Cytotoxic effect of several plant saponins were shown cytotoxic effects on several cancer cell lines such as lung, colorectal, breast and liver cancer through apoptosis induction (65). Most popularity organic solvent for plant compound extraction are medium and high polarity such as methanol, ethanol, acetone, ethyl acetate or combination which commonly used for extracting anticancer agents from plant materials (66). Previous result for

phytochemical extraction indicated that acetone and methanol are the most appropriated solvents for extraction (59).

However, the cytotoxic activity of fruit extracts on human cervical cancer cell lines has not been investigated yet. Therefore, in this research was determined to cytotoxic effects and mode of action of *V. diospyroides* fruit type SS crude extracts on cervical cancer cell lines. The cytotoxic activity on cervical cancer cell lines was evaluated by MTT assay. Mode of cell death was analyzed using flow cytometry and western blot analysis which used to investigate the cell death pathway proteins. Our research is the important preliminary data to guide the potential anticancer resource and could be applied to alternative drugs development in future studies.

Materials and Method

Cell culture conditions

Three cell lines were used in this experiment, including two human cervical cancer cell lines HeLa (ATCC CCL-21) and SiHa (ATCC HTB-35), and fibroblast cell L929 which kindly provided from Asst. Prof. Dr. Jasadee Kaewsichan was used as normal cell line. The cell lines were purchased from the American Type Culture Collection (ATCC). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, streptomycin (100 μg/mL) and penicillin (100 U/mL). All cells were maintained in humidified air with 5% CO_2 at 37 °C incubator.

Preparation of extracts

The fruit samples were separated for two segment including cotyledon and pericarp and were separately extracted with methanol (CH_3OH) and acetone ($(CH_3)_2CO$), following the procedure described previously (59). Then, extracts were dissolved with dimethyl suloxide (DMSO) with a final concentration of DMSO in extracts less than 0.5% (15)

***In vitro* cytotoxicity assay**

Cytotoxic activity of cotyledon and pericarp extracts were performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The HeLa and SiHa cell lines were seeded with 5×10^3 cells per well in 96-well plates (150 µL of medium cell suspension) and cultured overnight. After incubating, the crude extracts were diluted to the concentration at 5, 10, 20, 40 and 80 µg/mL with complete medium, and cells were treated with each extracts for 72 hours. Each cell line was washed with 100 µL of PBS buffer, before treated cells were detected with 100 µL of 0.5 mg/mL MTT solution and incubated for 30 minutes. The crystal formazan was dissolved with DMSO and incubated for 30 minutes. Cell viability was measured the by optical density absorbance with wavelength at 570 nm and 650 nm using multi-well plate reader (SpectraMax M5 Multi-Mode Microplate Reader). The cytotoxic data or the half maximal inhibitory concentration (IC_{50} value) was calculated from growth inhibition curves.

Apoptotic cells analysis by flow cytometry

The cells were seeded in 6-well plates at 1.5×10^5 cells per well. After incubating for 24 hours, cells were treated with extracts at 0, 0.5 IC_{50}, IC_{50} and 2-fold IC_{50} with culture periods were 0, 24, 48 and 72 hours. After discard the old medium, cells were washed with PBS buffer, and trypsinized with 300 µL of 0.25% trypsin-EDTA. The cell suspension was harvested by centrifugation and washed with PBS. Pellet cells were diluted with PBS to 1×10^6 cells per mL. After centrifugation, 100 µL of 1X binding buffer (0.1 M Hepes, 0.1 M NaOH pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) was added to the pellet and these solutions were transferred to 5 mL clear tube. Each Annexin V-FITC and Propidium iodide (PI) was added for 5 µL. The solutions were vortexed softly. After incubation at room temperature for 15 minutes in the dark condition with cover by aluminums foil, 400 µL of 1X binding buffer was added into tube before the flow cytometric analysis. The FACSCalibur flow cytometer (Becton Dickinson Biosciences [BDB], San Jose, CA) was used in apoptotic cells analysis. The single cell for 5000 events was acquired with FACS and data analysis using WinMDI version 2.9 software.

DNA fragmentation

For DNA fragmentation assay, HeLa cells were seeded in 100×20 mm. cell culture plates with 3×10^6 cells for 0.01% (v/v) Triton X-100 (positive control) and 2×10^6 cells for extract treatment respectively. After 24 hours of incubation, the old medium in the plate was replaced by new medium that contained 0.01% Triton X-100 and the acetone cotyledon extract (0.5 IC_{50}). At the end of treatment in each condition (1 hour for positive control and 0-120 hours for treatment), both attached cells and suspension cells were collected before DNA extraction. Cell pallets were lysed in lysis buffer (50 mM Tris-HCl; pH 8.0, 20 mM EDTA; pH 8.0, and 0.5% Triton X-100). Low molecular weight DNA and genomic DNA were collected from supernatant after centrifugation. RNA and protein were removed by treated with 1 mg/mL RNase A and 1 mg/mL Proteinase K respectively. DNA was extracted by phenol: chloroform: isoamyl alcohol (25:24:1). After centrifugation, the DNA were collected in upper-phase and DNA were precipitated by isopropanol (Labscan, Thailand), and resuspendednin DEPC-treated H_2O (Applichem, Germany). All samples were run on 2.5% (w/v) agarose gel in 0.5x Tris acetate-EDTA (TAE) buffer, pH 7.0 with 50 volt for 2 hours, then gel was stained with ethidium bromide. The characteristic of apoptosis was show in DNA laddering in multiples of 200 bp.

Western blot analysis

Cells were treated with a half concentration of IC_{50} value (0.5 IC_{50}) extracts for 0, 24, 48 and 72 hours. The cells were trypsinized and harvested by centrifugation. The cell pellets were washed in cold phosphate buffer saline (PBS), follow by lysis with RIPA buffer (Peirce Biotechnology, IL, USA). The extracted protein samples were measured by Bradford assay (Biorad, CA, USA). Quantitation of protein samples with 40 µg was separated by 12% SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), and transferred to a PVDF (polyvinylidene difluoride) membrane using electrophoretically supporting. The membranes were blocked in 5% non-fat milk in TBS-T (Tris-*buffered* saline, 0.1% Tween 20) on a shaking incubator at room temperature for 1 hour. After blocking, the membrane was washed twice with 1X TBS-T for 10 minutes. Each membrane was incubated for overnight at 4°C, shaking continuously in primary antibodies (1:1,000; diluted with 1% non-fat milk in TBS-T)

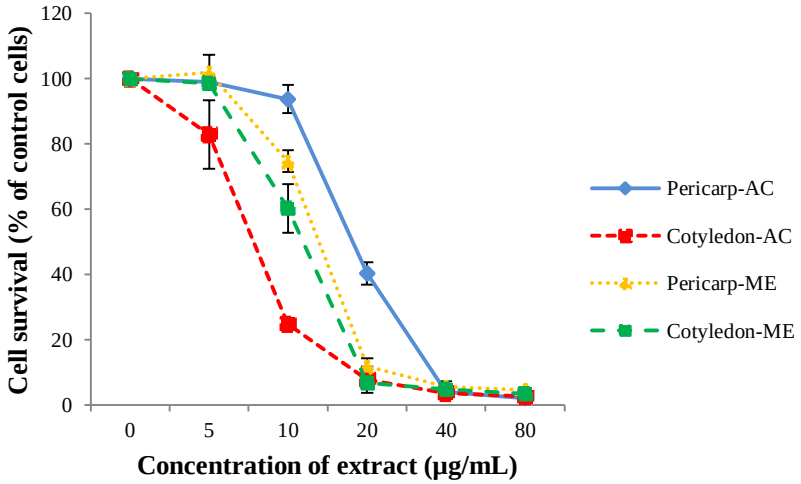
specific with Bax, caspase-8, cleaved caspase-8 and β-actin (used as the internal control). After incubation, the membrane were washed with 1X TBS-T for three times (10 minutes/time) and incubated with secondary antibody (anti-rabbit IgG horseradish peroxidase) at 1:5,000 in 1% non-fat milk in TBS-T for 2 hours. Then, membranes were washed in 1X TBS-T for three times and the last washing using 1X TBS for 10 minutes. Finally, the protein expressions were detected by a chemiluminescent detection kit (Peirce, IL, USA).

Results

In vitro cytotoxicity on cervical cancer cell lines

The cytotoxic effects of *V. diospyroides* fruit type SS crude extracts on cervical cancer cell lines were determined by MTT assay. After 72 hours of treatment with various concentrations, the overall results showed highly cytotoxicity which IC_{50} value less than 30 µg/mL (67). HeLa and SiHa cells were continuously decrease in dose-dependent manner in term of cell survival (Fig. 1A and 1B). The IC_{50} value of all extracts were similar effect on both HeLa and SiHa cell lines. The acetone cotyledon extract showed very strong activity of cell growth inhibition on HeLa and SiHa cells with low IC_{50} values at 7.69 ± 0.44 and 9.81 ± 1.38 µg/mL respectively (Table 1) and showed a significant cytotoxicity by low concentration at 20 µg/mL against both cancer cell lines comparing with control. Moreover, these extracts indicated cytotoxic against HeLa and SiHa cells in a dose-dependent manner. Selectivity index (SI) is a value that indicates the cytotoxic or safety of the crude extract against cancer cells versus normal cells, and was calculated from the IC_{50} of the crude extract in normal cell line versus IC_{50} of the same extract in cancer cell line. SI value >3 is indicating to high selectivity and more safety to normal cells (18). The methanol cotyledon, methanol pericarp and acetone pericarp extracts were shown lower SI value (<3) on L929 cell. The results indicated that these extracts showed cytotoxic on normal cell line. On the other hand, the acetone cotyledon extract showed higher SI values on HeLa and SiHa cells with 4.47 and 3.51, respectively. This result was demonstrated that the acetone cotyledon extract not only showed the highest cytotoxicity against HeLa and SiHa cells, but also showed highest selectivity value in L929 cell (Table 2).

A



B

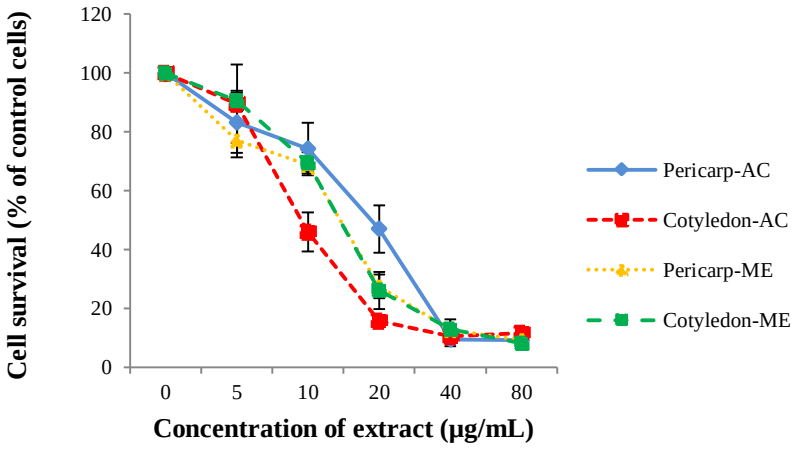


Fig. 1 Cell survival curves on HeLa (A) and SiHa (B) cell lines are determined by MTT assay for 72 h of treatment with V. diospyroides fruit type SS crude extracts concentrations at 0, 10, 20, 40 and 80 µg/mL (Data are show as mean ±SD).

Table 1 Cytotoxic effects for different extracts of *V. diospyroides* fruit type SS on HeLa and SiHa cells. The IC_{50} results are represented by the mean ± SD of three independent experiments (n=3) of the replicates.

IC_{50} value for cytotoxicity (µg/mL)					
Cell type	**Cell line**	**Pericarp**		**Cotyledon**	
		Acetone	**Methanol**	**Acetone**	**Methanol**
Cervical cancer	**HeLa**	17.93 ± 0.81	13.40 ± 0.71	7.69 ± 0.44	11.60 ± 0.96
	SiHa	19.00 ± 3.37	14.55 ± 0.69	9.81 ± 1.38	14.54 ± 1.10
Normal cell line	**L929**	33.12 ± 0.82	14.10 ± 0.47	34.41 ± 2.05	24.32 ± 1.81

Table 2 Selectivity index (SI) of *V. diospyroides* fruit type SS extracts on HeLa and SiHa cells.

Selectivity Index (SI)				
	Pericarp		**Cotyledon**	
Cell line	**Acetone**	**Methanol**	**Acetone**	**Methanol**
HeLa	1.85	1.05	4.47[a]	2.10
SiHa	1.74	0.97	3.51[a]	1.67

[a] SI value > 3 is considered to be high selectivity

Apoptotic cells analysis

The occurrence of apoptosis on cancer cell is determined as an importance model to seek out and develop the anticancer drugs. In this study, mode of cell death of cotyledon- AC was performed by morphological investigation and Annexin V-FITC/ Propidium iodide (PI) staining, follow by flow cytometry analysis. HeLa and SiHa cells were treated with cotyledon- AC extract at half IC50, IC50 and 2- fold IC50 concentrations for 48 hours. The morphology of HeLa and SiHa cervical cancer cell lines was observed under phase- contrast microscopy. Acetone cotyledon extract induced cellular changes which are cell shrinkage, round shape of cell and detachment of non- viable cells, especially in 2- fold IC50 treatment when compared with control (Fig. 2A and B). This morphological observation provides evidence for apoptosis induction by the extract on cervical cancer cells. However, apoptosis induction efficiency was carried out to ensure by flow cytometry analysis. After analyze with flow cytometry, the results were showed in a dot plot diagram. The modes of cell death were determined from the transitions of cells in the plots. One of the characteristics of early apoptotic cell is phosphatidylserine (PS) translocation which specific for Annexin V- FITC binding as shown in lower- right quadrant. For late apoptotic cell, the loss of plasma membrane is permeable to PI that viability probe to distinguish viable from dead cell as located in upper-right quadrant (3). HeLa cell that treated with the extract at half IC50 was reduced viable cells and increased apoptotic population with slight dead cells (Fig. 2A). By increasing concentration to 2-fold IC50, the viable cells were dramatically decreased from 98.50% to 7.15% as compared with control. Additionally, the acetone cotyledon extract was shown induction of apoptosis with increasing of apoptotic cells at half IC50 to 2- fold IC50 (60.92 % to 79.66 %). After 48 hours of treatment, the apoptotic cells of SiHa were slightly increased (Fig. 2B). However, there is no different in apoptosis induction among three concentrations.

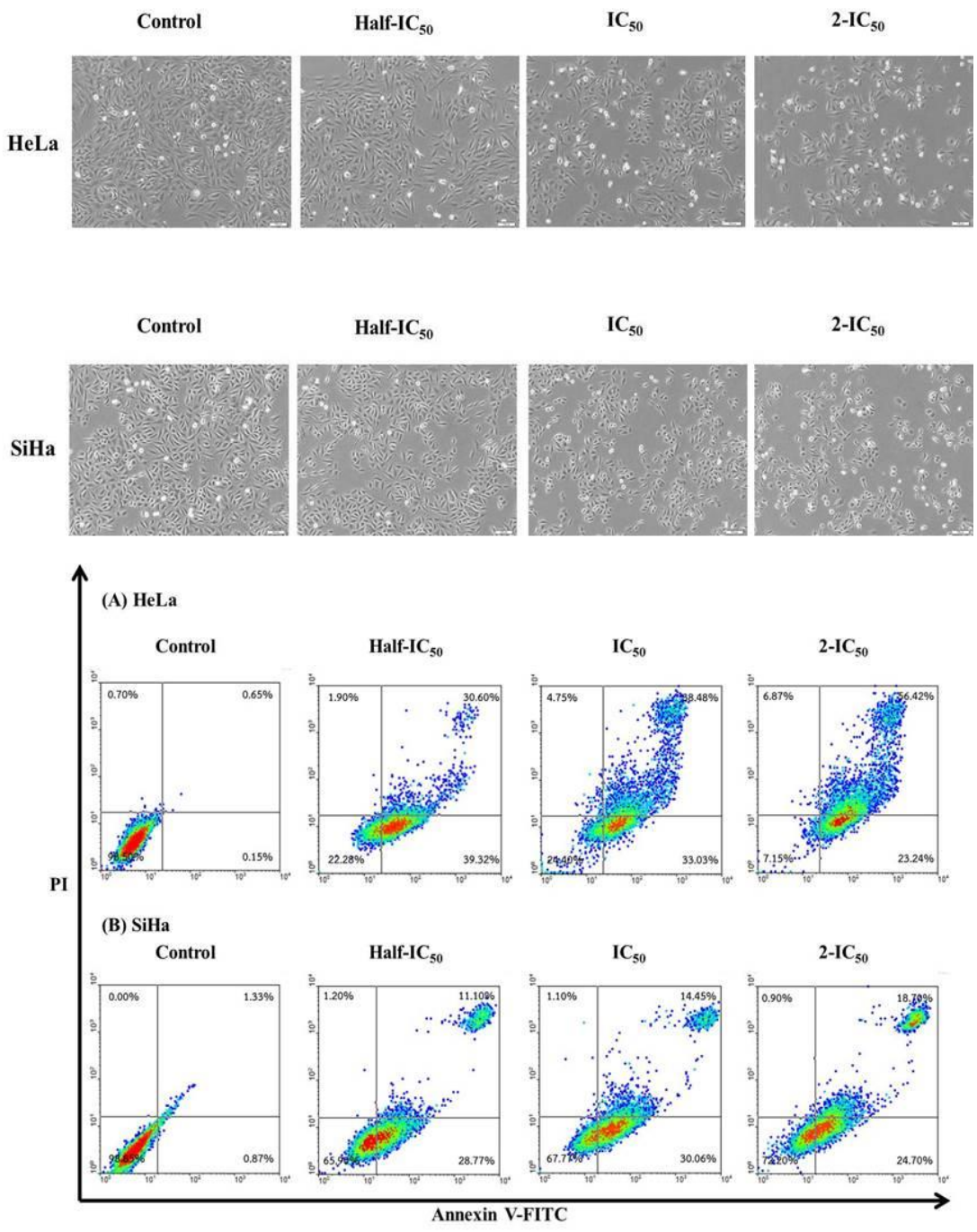


Fig. 2 Apoptosis induction effect of cotyledon-acetone extract at half IC50, IC50 and 2-fold IC50 on HeLa and SiHa cells for 48 hours by morphological investigation (A) under phase contrast microscopy (magnification 10x). Flow cytometry analysis by Annexin V-FITC and PI staining (B). Dot plot diagrams show percentage of cell populations which divide to viable, early apoptotic, late apoptotic and dead cells.

DNA fragmentation

To further evaluated insights of apoptosis induction by cotyledon-AC. The agarose gel electrophoresis was used for observation of DNA fragmentation which a crucial marker of apoptosis. HeLa cells were treated with the extract at half IC_{50} for various time intervals (0, 24, 48, 72, 96, 120 h). As the genomic DNA pattern in control (lane 3) was without appearance of fragment ladder. Interestingly, agarose showed a laddering pattern which initially occurred at 48 h after treatment (lane 5). The ladder of DNA as 72-120 h (lane 6-8) were found to be a clearly ladder as compared to 0.01% triton-x 100 (positive control: lane 2) which 200-1000 base pair as shown in figure 3. The result indicated that cotyledon-AC extract induced apoptosis in HeLa cells.

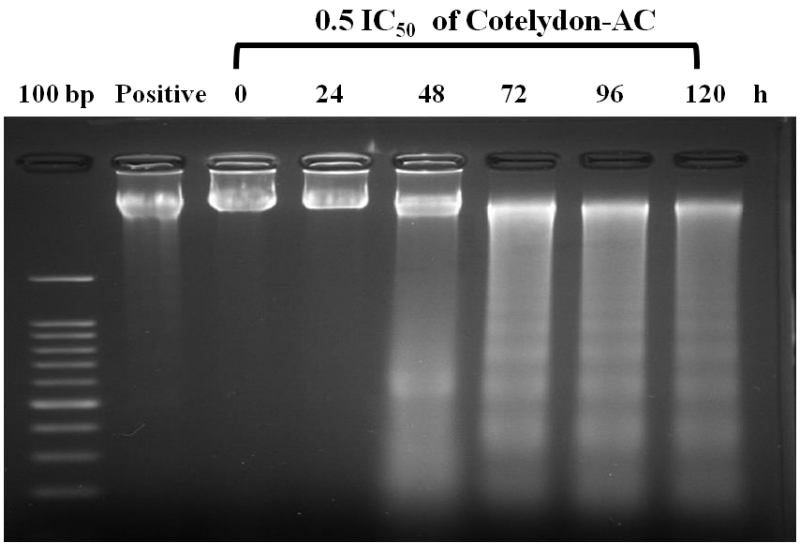


Fig. 3 DNA fragmentation pattern in HeLa cells treated with cotyledon-acetone extract by 2.5% agarose gel electrophoresis. From left lane1; 100 bp DNA ladder, lane 2; positive control (0.01% triton-x 100), lane 3-8; treated cells with cotyledon-acetone extract at half-IC_{50} for 0, 24, 48, 72, 96,120 h, respectively.

Western blot analysis

To validate the apoptosis induction effect of cotyledon-AC extract on HeLa cells. The Bcl-2-associated X protein (Bax), caspase-8 and cleaved caspase-8 were examined using western blot analysis. HeLa cells were exposed the extract at half IC_{50} for 48 h. The western blotting result showed that the extract induced the expression of Bax at 48 h after treatment. The protein expression of caspase-8 was decline. Meanwhile, the cleaved form of caspase-8 was increased in time dependent manner (Fig. 4). These results suggested that the induction of apoptosis of cotyledon-AC extract in HeLa cell line could be due to up-regulation of pro apoptotic (Bax) protein and initiated cascade caspase (cleaved caspase-8).

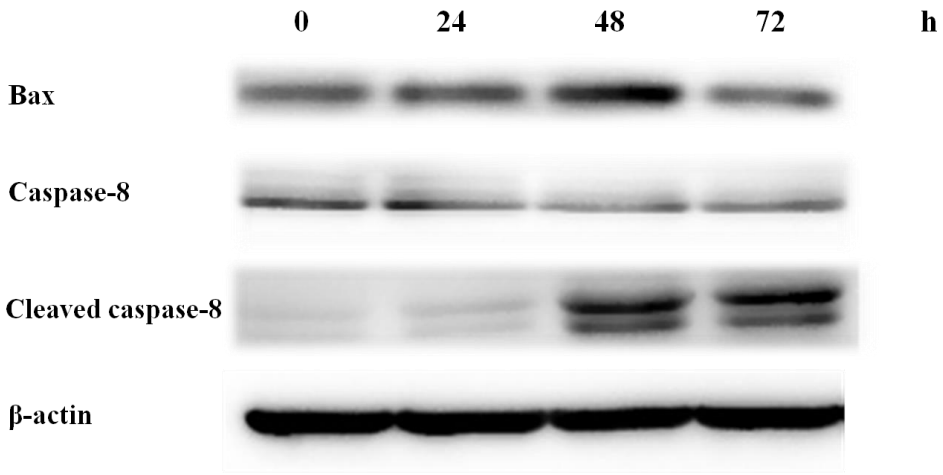


Fig. 4 Apoptosis induction effect of cotyledon-acetone extract at half-IC_{50} on protein expression of Bax, caspase-8 and cleaved caspase-8 in HeLa cells was observed using western blot analysis. β-actin served as an internal control.

Discussion

Preclinical screening is an essential process for new anticancer drug development (69). The purpose of screening step is to selecting the extracts that has anticancer activities. Cell proliferation is labeled feature in cancer development (70). Therefore, the antiproliferation property is required in anticancer agents. In vitro cytotoxicity test is importantly evaluated to seek out the active compounds that have cytotoxic effect on cancer cells. In our study, *V. diospyroides* fruit type SS crude extracts (VDS) were divided by fruit ingredients (pericarp and cotyledon) and organic solvents (acetone and methanol) which four agents including pericarp- acetone, pericarp-methanol, cotyledon-acetone and cotyledon-methanol. Cell growth inhibition on cervical cancer cell lines was performed using MTT assay and the half maximal inhibitory concentration (IC_{50}) was used to classified effect. The IC_{50} that less than 30 µg/mL were considered to high cytotoxic effect (67). We found that HeLa and SiHa cells that exposed with extracts at various concentrations were significantly declined in term of cell survival in dose- dependent manner as compared to control. In vitro cytotoxic activity was reported as IC_{50} value which the lower of value intended to higher cytotoxic response in cancer cells. Among four crude extracts from VDS also showed highly active cytoxic against both cervical cancer cell lines with IC_{50} value less than 20 µg/mL. In agreement with previous report, the VDS extracts has previously been screened the cytotoxicity on MDA-MB 468 breast cancer cell line and showed strong activity with IC_{50} at 1.60-17.45 µg/mL (63). Noteworthy, acetone extracts of cotyledon had shown greatly affect than other extract in HeLa and SiHa cells with IC_{50} at 7.69 ± 0.44 and 9.81 ± 1.38 µg/mL respectively. On the contrary, Srisawat *et al*. (63) found that the ethanol extract of cotyledon also showed strong activity against MDA-MB-468 cell with IC_{50} 3.50 ± 0.06 µg/mL. This dissimilarity can be described by the variation in cellular responses of the cell types. Our data explained that the extracts which used the acetone as an organic solvent was more effective in terms of cytotoxic effect than the methanol. It is known the most of the anticancer drugs not only harmful on cancer cells but also damaging on normal cells (71). Thus, the goal of anticancer agent is could be safe on normal cells. In consequence, the safety responses of VDS extract on normal cell lines were investigated on L929 cell. L929 is a mouse normal

fibroblast cell line which commonly used as a normal cell model in cytotoxicity screening (72). The value of selectivity index (SI) was used to determine the harmless response of VDS extracts and SI value which more than 3 were considered to high selectivity (18). Unfortunately, only cotyledon-acetone extract was shown high safety response on L929 cell with greater than 3 of SI value on both cells. Therefore, these results imply that the cotyledon-acetone extract showed the highest cytotoxic effect on cervical cancer cells and also showed utmost safety response on normal cell. Subsequently, the mode of cell death which induced by the extract at half IC_{50}, IC_{50} and 2-fold IC_{50} were investigated using flow cytometry analysis. As is well known, somatic cells are produce by mitosis cell division and all of them will eradicate by apoptosis. An imbalance of cell proliferation and apoptosis is occurring can leading to tumorigenesis (73). Programmed cell death or apoptosis is a key mechanism which relates the genetically elimination of cells (74). For apoptosis detection, we numbered the proportion of apoptotic cells which the transition of cell populations in the density plots. The apoptotic cells were located in lower and upper quadrants (68). As shown in figure 2A, this result showed that HeLa cells treated cotyledon-acetone extract at half IC_{50} for 48 h were shoed apoptosis induction with diminished viable cells and extended apoptotic populations. The treatment of HeLa cell with the extract at IC_{50} showed higher apoptotic cells. Moreover, exposure to highest concentration (2-fold IC_{50}) resulted in an augment of apoptotic cells from 60.92% to 71.51% and 79.66%, respectively which confirm by morphological changing of HeLa and SiHa cell lines (Fig. 2A and B). Our results are also confirmed with the investigation of Srisawat *et al* (63) breast cancer cells exposed to acetone extract of cotyledon at half IC_{50} for 48 h induced apoptosis by increased apoptotic count to 20.82%. Meanwhile, the SiHa cell also showed apoptosis induction in dose-dependent manner, but lower rate of apoptotic cells when compared with HeLa cells. Therefore, we selected the HeLa cell for further experiment in this study. In apoptotic cell death, the one of remarkable characteristics of apoptosis is DNA fragmentation. Nuclear DNA was cleaved by endonuclease resulted in DNA degradation which oligonucleosomal fragment about 180-200 bp length or multiple of it (75). In this study, the mode of cell death was confirmed by DNA fragmentation using agarose gel electrophoresis. The DNA ladder obtained from agarose gel clearly showed that the cotyledon-acetone extract at half IC_{50} caused initially apoptosis

induction after 48 h of treatment. The DNA ladder of HeLa cell showed 500-600 bp of fragments, while DNA pattern of untreated showed as a single band of genomic DNA. Interestingly, nuclear fragmentations after treatment for 72-120 h were observed with clearly band at 200-1000 bp size. In agreement with previous result on the HL-60 treated with betulinic acid from *Melalenca cajuput* showed ladder in 100-1500 bp fragment (76). Apoptosis pathway can be identified by the adapters and cellular stimulation. These programs are divided into extrinsic (death receptors pathway) and intrinsic (mitochondrial pathway). Caspase or endoprotease are playing important role in cell death and inflammatory regulation (77). Cleaved caspase-8 is an initiator caspase which have a death receptor domain and the response of death receptor-mediated activation of cleaved caspase-8 rely on cell type (78). HeLa cell was classified in type II cells which first activate to intrinsic pathway (79). Thus, cleaved caspase-8 was induced regulation of pro-apoptosis Bax protein resulting in intrinsic apoptosis induction. The protein-involved apoptosis pathway each other confirmed with appearance of DNA fragmentation. These results are also in corroboration with the data from Peng *et al* (80). Our data also suggested that the anticancer activity of the extract on HeLa cell may act via apoptosis induction. In summary, cotyledon-acetone extract induced cell proliferation due to higher cytotoxic activity on cervical cancer cells and showed not toxic on normal cell. Antiproliferation activity of the extract on cancer cells may be through apoptosis induction by up regulation of pro-apoptotic Bax and initial cleaved caspase-8. Our finding demonstrated that the extract would be an effective compound and showing alternative approach for cancer treatment.

Acknowledgement

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APPENDIX B (Continued)

Conference Paper

Chothiphirat A, Sangkhathat S, Srisawat T, Kanokwiroon K, Navakanitworakul R. Cytotoxic effect of cotyledon extract of vatica diospyroides symington type ss against colorectal cancer cell line. The 20th World Congress on Clinical Nutrition (WCCN), Bangkok, Thailand. December 14, 2016. **(Poster presentation)**

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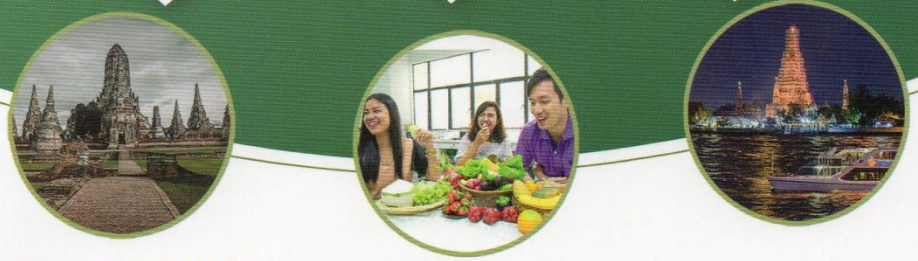

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"Traditional Medicine, Functional Food, Nutrition, Natural Health Product and Spiritual Healing: Additional Tools for Healthcare Delivery"

Cytotoxic effect of Cotyledon Extract of *Vatica diospyroides* Symington Type SS against Colorectal Cancer Cell Line

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Abstract

Vatica diospyroides Symington (VDS) is a Thai medicinal plants belonging to Dipterocarpaceae, and is called Chan-ka-pho in Thai. Flower and stem of VDS have been used as a cardiac and blood tonic treatments. Previous researches showed that the cotyledon extract of VDS was highly cytotoxic on breast cancer cell lines. In the present study, the effect of cotyledon extract of VDS type SS on growth inhibition was investigated by MTT assay in HCT116 human colorectal carcinoma cell line. The cotyledons were extracted with acetone (ACC) solution, and then HCT116 cells were incubated with various concentrations of cotyledon extract for 72 h. After treatment, the ACC extract inhibited the proliferation of HCT116 cell line in dose-dependent manner (IC_{50} of 8.18±0.18 µg/ml). Additionally, the ACC extract at half of IC_{50} was used to study the mechanism of its action using flow cytometry. We found that the ACC extract decreased the number of viable cells to 66%, and increased the number of apoptotic cells. The results demonstrated that the cotyledon extract showed a highly cytotoxic effect on HCT116 cells by inducing apoptosis. Our finding deduced that the extract could be a potential anticancer agent which can provide a solution in colorectal cancer prevention and therapy in the future.

Keywords: *Vatica diospyroides Symington, HCT116, colorectal cancer, antiproliferation, MTT, Flow cytometry*

Introduction

Colorectal cancer (CRC) is the third most common cancer cases and the fourth leading cause of cancer mortality worldwide. The global occurrence of CRC is expected to increase by 60% more than 2.2 million of incidence and 1.1 million deaths in 2030 [1]. CRC is not only public health problem of the world, but also a serious trouble in Thailand. There are 11,493 new cases and 6,848 deaths were reported in 2012. It is the fourth most common cancer incidence and mortality in Thailand [2].

Natural products and compounds derived from natural source have been reporting for donkey year as a potential source of therapeutic agents. The ancient people used plant as a medicine for disease treatment. At the present time, natural products are restored to take an interest in natural product research to alternative drug development and still use as a capital source for drug discovery process [3]. A lot of the commercially approved anticancer drugs are derived from natural product and specifically anticancer compounds that originate from plants [4].

Vatica diospyroides Symington (VDS) belongs to Dipterocarpaceae, and name in Thai is Chan-ka-pho. Flower and stem of VDS were previously used as a Thai medicinal plants for cardiac and blood tonic treatments [5]. The resveratrol were founded in ethyl acetate extract from stem of VDS, and this agent inhibits proliferation on human oral epidermoid carcinoma (KB) cell line [6]. For the research of fruit VDS extracts, the cotyledon extracts from LS type has been reported highly potential against MDA-MB-468 cell [5], and SS fruit cotyledon extract showed cytotoxicity on MCF-7 cell [7]. The phytochemicals of plans in Dipterocarpaceae were reported several biological activities including anticancer property [8].

Apoptosis is a programmed cell death with play important roles to protect against tumor. The mechanism use to eliminate abnormal or unwanted cells. Many therapeutic of cancer and chemopreventive agents seem to apoptosis induction pathway as target signaling intermediates [9]. Induction of apoptosis is

apparently the potential pathway for anticancer therapeutics development. For instance, numerous chemotherapeutic agents depress cancer cell proliferation by apoptosis induction [10].

The present study investigated the effect of cotyledon acetone extract of VDS type SS on growth inhibition of HCT116 human colorectal carcinoma cell line by MTT assay. The inhibitory effect of the extract was monitored by inverted microscope and the type of cell death by ACC extract was determined using flow cytometry.

Materials and Methods

Cell culture conditions

Human colorectal cancer cell line HCT116 was kindly provided by Assoc. Prof. Dr. Surasak Sangkhathat, Department of Surgery, Faculty of Medicine, Prince of Songkla University. Cells were cultured in *Dulbecco's Modified Eagle's medium* (DMEM) medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, streptomycin (100 µg/mL) and penicillin (100 U/mL). HCT116 cells were maintained under humidified air with 5% CO_2, in 37 °C incubator.

Plant extract preparation

The cotyledon acetone (ACC) extract was kindly provided by Asst. Prof. Dr. Theera Srisawat, Prince of Songkla University (Suratthani campus). The cotyledons were separated from fruit, and were extracted with acetone ($(CH_3)_2CO$) and protocol which described previously [11]. The crude extract was dissolved with dimethyl suloxide (DMSO) before analysis.

Cytotoxicity activity assay of ACC extract and inhibitory effect observations

Cytotoxic activity of ACC extract was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The crude extract was diluted to various concentration including 5, 10, 20, 40 and 80 µg/mL with DMEM. The HCT cells were seeded in 96-well plates with cells density at 5×10^3 cells per well (150 µL of cell suspension with DMEM), and cultured overnight. Cells were treated with extracts for 72 hours. After incubation, cells were washed with 100 µL of phosphate buffer saline (1xPBS), before treated cells were detected with 100 µL of 0.5 mg/mL MTT solution and incubated for 30 minutes. At the end of MTT reduction the solution was discarded and the crystal formazan was dissolved with DMSO. Thirty minutes later, the plate was measured live cells using multi-well plate reader at 570 nm and 650 nm. The cytotoxic results which observed in the half maximal inhibitory concentrations (IC_{50} value) were calculated by percentage of inhibition graph. Mention to National Cancer Institute guides mention that extracts with IC_{50} value less than 30 µg/mL were classify in active ability [12]. For observation of inhibitory effect, cells were monitored by inverted microscope and photographed using CellSens software.

Evaluation of apoptosis by AnnexinV-FITC/PI staining and flow cytometry

The apoptotic cell populations were identified from viable cells by AnnexinV-FITC/PI staining and flow cytometry. The cells were plated in 6-well plates with density in each well at 1.5×10^5 cells. After cultured overnight, cells were treated with ACC extracts at 0, half IC_{50}, IC_{50} and 2-dose IC_{50}. The culture duration was 48 hours. Plates were removed the old medium and cells were washed with 1xPBS buffer. The cells were harvested with 300 µL of 0.25% trypsin-EDTA. After centrifugation, pellet cells were washed and diluted to 1×10^6 cells with 1xPBS. The cells were resuspended with 1x binding buffer (0.1 M Hepes, 0.1 M NaOH pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) and stained with 5 µL of Annexin V-FITC and Propidium iodide (PI). The solutions were incubated for 15 minutes at room temperature in the dark condition. Before analyzed using flow cytometer, 400 µL of 1x binding buffer was added into each tube. Apoptotic cells were analyzed and quantitated by FACSCalibur flow cytometer (Becton Dickinson Biosciences [BDB], San Jose, CA) with 5000 events of single cell. The data were analysis by WinMDI version 2.9 software.

Results and discussion

This research determined the cytotoxicity of crude extract of VDS fruit type SS on human colorectal carcinoma cell line (HCT116). The acetone cotyledon extract (ACC) has highly cytotoxic effect against HCT116 cell with lower 50% inhibitory concentration value (IC_{50}) at 8.18±0.18 µg/ml (Fig. 1). Previous screening of *V. diospyroides* fruit extracts including acetone cotyledon on breast cancer cell line (MDA-MB-468 cell) showed the greatly cytotoxic activity with IC_{50} = 1.6 µg/ml [11]. The difference in IC_{50} value might be due to different

cell line. The criteria of cytotoxic activity of crude extracts that suggested by the American National Cancer Institute (NCI) was an IC_{50} values must be less than 30 µg/ml [12]. The IC_{50} value of ACC extract was assumed that this extract was showed inhibitory effect on cancer cells proliferation. In addition, cells were monitored by inverted microscope for inhibitory observation. Cells were untreated with extract that used as a control and cells were treated with DMSO in DMEM used as a vehicle. The cell populations in both condition were approximately 100% confluence and morphology in control were normal (Figure 2A and 2B). Cells that treated with ACC extract at various concentrations were reduced in descending order by dose (Figure 2C-2G). These results were conformed to cytotoxic screening by MTT assay. In the highest concentration, cells were obviously seen changed morphology into contract cells (Figure 2G).

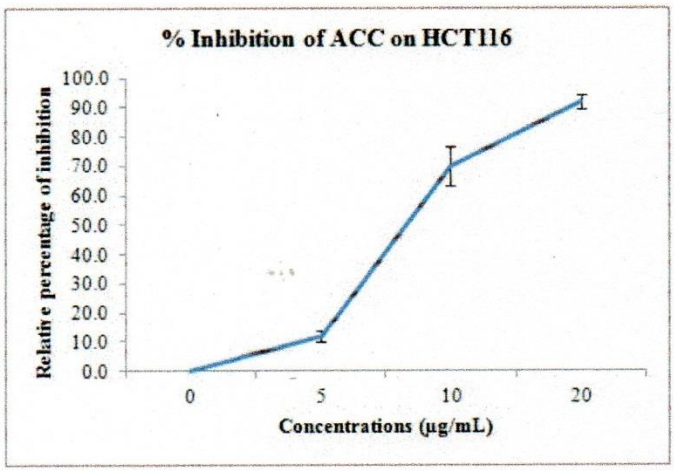

Figure 1 Inhibition activity of ACC extract at 0, 5, 10, and 20 µg/ml against HCT116 cell line.

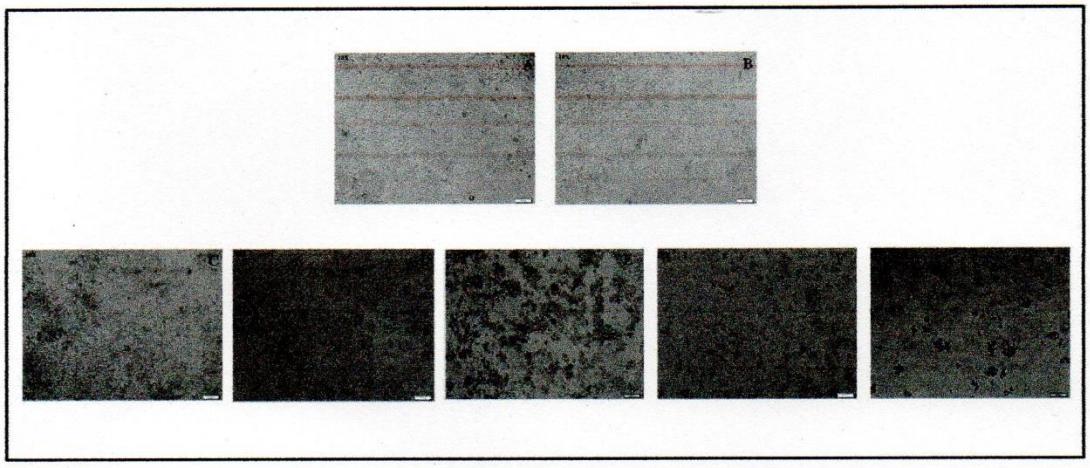

Figure 2 The morphology of HCT116 cells were untreated (control) (A), vehicle (DMSO) (B), cells treated with ACC extract at 5, (C), 10 (D), 20 (E), 40 (F), and 80 µg/ml (G) by inverted phase contrast microscopy at 10X of magnification.

Apoptosis induction of ACC extract on HCT116 cell was performed by staining of Annexin V-FITC/PI fluorescence dyes and analyzed with flow cytometer. The single cell of HCT116 was detected and separated in each quadrant of dot plot which specific characteristics of dyes. The most population of control or untreated cells was contained in lower-left quadrant (Figure 3A). This position was excluded with both Annexin V-FITC and PI, identified as viable cells. In early apoptosis stage, the phosphatidylserine of plasma membrane were reversed to outer membrane that can bind with Annexin V-FITC. Then the membranes of damaged cells in late apoptosis stage are permeable to PI [13]. The population of cell that treated with ACC extract at half of IC_{50} was decrease in viable cells and increased in under-right and upper-right quadrant that identified as early apoptotic and late apoptotic cells, respectively (Figure 3B). However, the increasing of concentration of extract as IC_{50} and 2-dose IC_{50} were induced apoptosis less than IC_{50}. The results were estimated that ACC extract induced apoptosis on HCT116 cells in independent-dose effect. This study demonstrated that the ACC extract showed a highly cytotoxic effect on HCT116 cells by inducing apoptosis. These results deduced that the extract could be a potential anticancer agent which can supported in colorectal cancer prevention and therapy in the future.

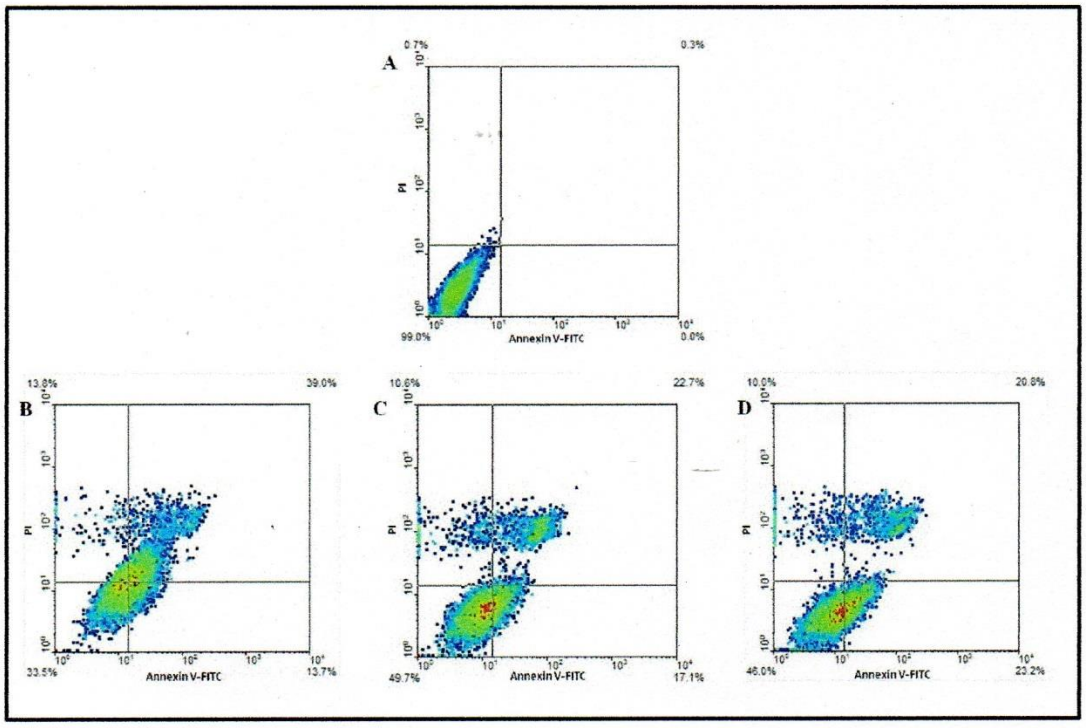

Figure 3 Dot plots from flow cytometry analysis by AnnexinV-FITC/PI staining of HCT116 cells untreated (A) and treated with ACC extract at half- IC_{50} (B), IC_{50} (C) and 2-dose IC_{50} (D) for 48 hours. Viable cells were positioned in the lower-left quadrant, the early apoptotic cells stained by annexin V and unstained by propidium iodide in the lower-right quadrant, late apoptotic cells (stained with both dyes) were positioned in the upper-right quadrant, and dead cell were presented in the upper-left quadrant.

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The researchers are appreciative to the grant from Faculty of Medicine, Prince of Songkla University for providing the financial support for this work. We also would like to thank Assoc. Prof. Dr. Surasak Sangkhathat for providing the cell lines and Asst. Prof. Dr. Theera Srisawat for providing the crude extract.

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Abstract

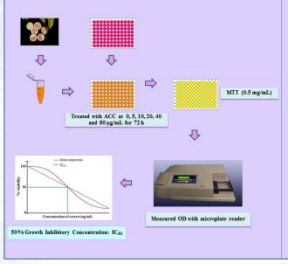
Vatica diospyroides Symington (VDS) is a Thai medicinal plants belonging to Dipterocarpaceae, and is called Chan-ka-pho in Thai. Flower and stem of VDS have been used as a cardiac and blood tonic treatments. Previous researches showed that the cotyledon extract of VDS was highly cytotoxic on breast cancer cell lines. In the present study, the effect of cotyledon extract of VDS type SS on growth inhibition was investigated by MTT assay in HCT116 human colorectal carcinoma cell line. The cotyledons were extracted with acetone solution, and then HCT116 cells were incubated with various concentrations of cotyledon extract for 72 h. After treatment, the extract inhibited the proliferation of HCT116 cell line in dose-dependent manner (IC_{50} of 8.18±0.18 µg/ml). Additionally, the extract at half of IC_{50} was used to study the mechanism of its action using flow cytometer. We found that the extract decreased the number of viable cells to 66%, and increased the number of apoptotic cells. The results demonstrated that the cotyledon extract showed a highly cytotoxic effect on HCT116 cells by inducing apoptosis. Our finding deduced that the extract could be a potential anticancer agent which can provide a solution in colorectal cancer prevention and therapy in the future.

Introduction

Colorectal cancer (CRC) is the fourth most common cancer incidence and mortality in Thailand. A lot of the commercially approved anticancer drugs are derived from natural products. *Vatica diospyroides* Symington (VDS) belongs to Dipterocarpaceae, and name in Thai is Chan-ka-pho. The previous study reported that resveratrol tetramer has been found in ethyl acetate extract from stem of VDS, and this agent inhibited proliferation of oral epidermoid carcinoma (KB) cell line. The cotyledon extracts from LS and SS type have been reported to be highly potential against MDA-MB-468 breast cancer cells, and cotyledon extract type SS showed cytotoxicity on MCF-7 breast cancer cells. The present study aims to study the cytotoxic effect of cotyledon extract of VDS type SS on HCT116 cell line using MTT assay, and the growth inhibition of the cells will be observed and photographed under inverted microscope. Meanwhile, the death mode of the cells treated with the extract will be investigated by FACs analysis.

Materials and Methods

MTT assay



Flow cytometry

Results and Discussion

The cytotoxic activity of the extract against HCT116 cells was examined by MTT assay. After 72 h treatment, the degrees of growth inhibition as compared with control cells were 11.91, 70.11, 91.96, 100 and 100% at 5, 10, 20, 40 and 80 µg/mL, respectively. The extract was shown potential anticancer agent with very strong activity at IC_{50} = 8.18±0.18 µg/mL. The results on picture of the cells growing after treatments confirm that the extract could inhibit cancer cell proliferation in dose-dependent manner.

Apoptosis induction of the extract was investigated by Annexin V-FITC/PI staining and analyzed by FACs flow analysis. Cell was treated with the extract at half- IC_{50} for 48 h. The population of treated cell decreased the viable cells to 66% as compared with control and increased apoptotic cells to 52.7 % (Figure 3). Death mode result indicated that the extract possibly induced apoptosis on HCT116 cell line.

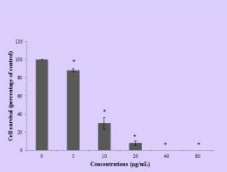


Figure 1. Antiproliferation activity of the extract at 0, 5, 10, 20, 40 and 80 µg/mL against HCT116 cell line. The results represent means ± SD from three independent experiments.

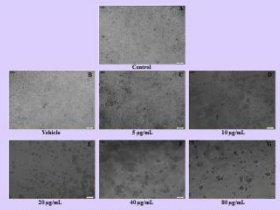


Figure 2. The morphology of HCT116 cells that were untreated (control) (A), vehicle (treated with DMSO) (B), or treated with the extract at 5, (C), 10 (D), 20 (E), 40 (F), and 80 µg/mL (G) were observed by inverted microscopy at 10X of magnification.

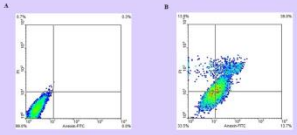


Figure 3. Dot plots of preliminary results (n=1) from flow cytometry analysis by AnnexinV-FITC/PI staining of HCT116 cells control (A) and treated with the extract at half-IC_{50} (B) for 48 hours. Viable cells were positioned in the lower-left quadrant, the early apoptotic cells stained by annexin V and unstained by propidium iodide in the lower-right quadrant, late apoptotic cells (stained with both dyes) were positioned in the upper-right quadrant, and dead cell were presented in the upper-left quadrant.

Conclusion

This study showed that the extract showed a highly cytotoxic effect on HCT116 cell with lower IC_{50} value at 8.18±0.18 µg/mL by inducing apoptosis. The extract at half-IC_{50} decreased the number of viable cells to 66%, and increased the number of apoptotic cells. This preliminary result will be carried out to confirm mode of cell death in the future study.

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Bachelor of Science (Plant biotechnology)	Prince of Songkla University	2013

Scholarship Awards during Enrolment

1) Research grant from the Post Graduate Study Grant from Faculty of Medicine, Prince of Songkla University

List of Publication and Proceedings

1) **Chothiphirat A**, Sangkhathat S, Srisawat T, Kanokwiroon K, Navakanitworakul R. Cytotoxic Effect of Cotyledon Extract of Vatica diospyroides Symington Type SS against Colorectal Cancer Cell Line. 20th World Congress on Clinical Nutrition (WCCN), Bangkok, Thailand. December 14-16, 2016. **(Poster presentation)**
2) **Chothiphirat A**, Srisawat T, Kanokwiroon K, Navakanitworakul R. *In vitro* Dose-Dependent Cytotoxicity and Apoptosis Induction of *Vatica diospyroides* Symington Type SS Fruit Extracts on Human Cervical Cancer Cell Lines. The 4th Joint Symposium BMS-BME-EU: Post-graduate Health Science and Technology Conference, Pantip Sa-nguan-chua conference center, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand. May 25-26, 2017. **(Oral presentation)**
3) **Chothiphirat A**, Nittayaboon K, Kanokwiroon K, Srisawat T, Navakanitworakul R. Anticancer Potential of Fruit Extracts from *Vatica diospyroides* Symington Type SS and Their Effect on Program Cell Death of Cervical Cancer Cell Lines **(Submitted manuscript)**.