



สำนักงานกองทุนสนับสนุนการวิจัย  
THE THAILAND RESEARCH FUND

รายงานวิจัยฉบับสมบูรณ์

เครือข่ายวิจัยนานาชาติ ” เครือข่ายวิจัยการค้นหและพัฒนาจากสารธรรมชาติ”  
(Natural product-based drug discovery and development)

โปรแกรม “การค้นห ปรับเปลี่ยนโครงสร้าง และศึกษากลไกการออกฤทธิ์ของสาร  
ธรรมชาติจากจุลินทรีย์ในทะเล และจากพืชสมุนไพรในการรักษาโรคในผู้สูงอายุ”  
(Discovery, Modification and Mechanistic studies of the Active Natural  
Products from Chinese Marine Microbes and Thai Medicinal Plants  
For treatment of Age-related Diseases)

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พ.ศ. 2564

## รายงานวิจัยฉบับสมบูรณ์

เครือข่ายวิจัยนานาชาติ ” เครือข่ายวิจัยการค้นพบและพัฒนาจากสารธรรมชาติ ”  
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Medicinal Plants for treatment of Age-related Diseases)

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### สังกัด

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

## Executive summary

Over the past two decades, growth of elderly population has rapidly increased worldwide including Thailand. Aging is primarily a change in bodily functions which is associated with a number of physiological changes. This population is generally more susceptible to multiple chronic diseases as well as non-communicable diseases (NCDs), which are a wide range of chronic illness conditions including cancer, diabetes, cardiovascular disease, hypertension, as well as Alzheimer's disease, dementia and other neurodegenerative diseases. The natural products play a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases. Therefore, in this international research network project, we have established a multi-institutional collaborative research with members from three institutes in Thailand together with the international collaborators in China and the United States. We focused on the natural product-based drug discovery and development of pharmaceutical products from natural resources including those from terrestrial tropical plants and marine microorganisms for prevention and treatment of age-related NCDs diseases. To fulfill the goal of research and to produce high quality Ph.D. graduates for sustainable development of human resources and to increase international visibility and research capability through overseas research collaboration and dissemination of advance research results, multidisciplinary research members were recruited including chemists, biologists, pharmacologist and pharmacists and the research activities consisted of 5 sub-projects research activities.

From the first sub-project, the Thai chemists had compiled 725 samples of natural, modified and synthetic compounds, while the Chinese chemists had provided more than 200 compounds from marine microorganisms with quite marked structures for evaluation of cytotoxic activity in several mammalian cancer cell lines. The non-cytotoxic compounds were further evaluated for anti-diabetic, anti-obesity and anti-Alzheimer activity. Some bioactive compounds with potent activity were selected as leads, which were further modified structurally to obtain more potent/less toxic analogues. Some of them were selected as candidates for investigating mechanisms of actions.

The second sub-project was aimed to search for anticancer activities. From our screening, a number of compounds from both Thailand and China were found to have promising anticancer activities against several types of cancers including gastric cancer, colorectal cancer and cholangiocarcinoma. The underlying anticancer mechanisms and intracellular targets of these compounds were also elucidated. The outputs from this collaboration among Thai and Chinese scientists are high impact international publications

and the highly potent anticancer candidates with potential and promising to be developed as anticancer drugs in the future. Nanoparticles of these compounds for delivery to their intracellular targets were further developed in subproject -6.

The sub-project-3 was aimed to find a new SGLT2 inhibitor from natural for diabetic treatment. Sodium glucose cotransporter (SGLT) 2 inhibition has been found to be anti-diabetic drug target for type 2 diabetes. Compounds from natural sources including plant and marine were tested for SGLT2 inhibition. Interestingly, the extracts from *Kaempferia parviflora*, and *Boesenbergia pandurata* showed an inhibitory effect on glucose transport in renal proximal tubular cells. Oral administration of the extracts of *Kaempferia parviflora*, and *Boesenbergia pandurata* to the diabetic rat -Goto Kakizaki (GK) rat reduced plasma glucose without changing plasma insulin indicating the therapeutic potential for type 2 DM treatment. In addition to the therapeutic potential application of *Boesenbergia pandurata* on diabetes, compounds from this plant panduratin A and pinostrobin showed protective effect of nephrotoxicity induced by drug including cisplatin, anti-cancer, and antibiotic drug, colistin.

Subproject-4 aimed to screen various natural compounds for PPAR $\alpha$  agonists and PPAR $\gamma$  antagonist in vitro system. We found that andrographolide and phytoestrogen diarylheptanoid from *C. comosa* (DPHD) have promising effect for suppressing PPAR $\gamma$  and activate PPAR $\alpha$ . Treatments with E2, DPHD or *C. comosa* extract in OVX rats prevented an increase in adiposity, down-regulated lipogenic genes and proteins with marked increases in the protein levels of AMPK- $\alpha$  and PPAR- $\alpha$ , supporting the use of this plant for health promotion in the post-menopausal women. In addition, DPHD also inhibited adipocyte differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) by suppressing the expression of genes involved in adipogenesis through the activation of ER and Wnt/ $\beta$  catenin signaling pathways. This finding suggests the potential role of DPHD in preventing bone marrow adiposity which is one of major factor that exacerbates osteoporosis in post-menopause.

In subproject-5, we have tested several natural product derived compounds and modified compounds for their ability to prevent neurons from damages and death caused by both oxidative stress and factors known to induce neurodegeneration. We found that several plant-derived compounds and modified compounds showed high scavenging property and can protect neurons from oxidative stress induced cell damage and death. In addition, we discovered a novel methodology for neuronal differentiation, which will be useful for further investigation of potential compounds with neuroprotective effects.

Subproject 6 aimed to develop effective novel nanocarriers containing anticancer drugs with high potency against cancer cells by using colorectal cancer (CRC), and with low toxicity to normal cells. We have developed semi-synthetic andrographolide, namely 3A.1 analog that has been investigated thoroughly for its potent and promising antitumor activity against colorectal cancer (CRC) was selected. It is a hydrophobic drug for colorectal cancer treatment. The results showed that 3A.1 was successful incorporated into self-assembled polymeric nanoparticles based on amphiphilic chitosan derivatives. These nanoparticles were formed by self-assembly in an aqueous solution and their hydrophobic part helped to solubilize the hydrophobic drug within the nanoparticles. The 3A.1-loaded NSCS nanoparticles with 40 wt% drug added prepared by a dropping method revealed the highest drug loading capacity and good physical stability in a refrigerator. Moreover, the 3A.1-loaded nanoparticles also inhibited cell migration of oral cancer HN-22 cells, suggesting it's potential to be nanocarriers for oral intestine/colon drug delivery. Furthermore, compared with the non-conjugated polymers, the folate-conjugated NSCS polymers showed much more anticancer activity and highly potent apoptotic induction, suggesting that the folate-conjugated were active-targeting drug nanocarriers for colon tumor therapy via folate mediation.

In conclusion, we have achieved the goal in establishing the international research network to collaborate conducting the project and published 37 international peer-reviewed publications. We produced Ph.D. students with outstanding intellectual abilities and a strong commitment to research. The promising drug candidates obtained from this project will lead their future research and development of such drugs.

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คณะผู้วิจัย  
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สัญญาเลขที่ IRN58W0004  
โครงการเครือข่ายวิจัยนานาชาติ “เครือข่ายวิจัยการค้นพบและพัฒนาจากสารธรรมชาติ  
(Natural product-based drug discovery and development)”

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ส่วนที่ 1 ข้อมูลโครงการ

ชื่อเครือข่ายวิจัยเครือข่ายวิจัยการค้นพบและพัฒนาจากสารธรรมชาติ

ชื่อโปรแกรมวิจัย      การค้นหา ปรับเปลี่ยนโครงสร้าง และการศึกษากลไกการออกฤทธิ์ของสารธรรมชาติ  
จากจุลินทรีย์ในทะเล และจากพืชสมุนไพรในการรักษาโรคในผู้สูงอายุ

สัญญาเลขที่              RN58W0004 ระยะเวลาโครงการ 3 ปี วันเริ่มโครงการ 1 กันยายน 2558

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4. Erik W. Dent, Associate Professor, Ph.D., University of Wisconsin-Madison, USA

### ส่วนที่ 2 รายงานการสร้างและพัฒนาเครือข่ายวิจัยนานาชาติ

1. ได้สร้างและพัฒนาเครือข่ายวิจัยนานาชาติ โดยสมาชิกร่วมวิจัยของเครือข่าย มีทั้งจากสถาบันในประเทศและต่างประเทศ กิจกรรมการวิจัยอยู่ในแนวทางของการศึกษาค้นคว้าหาตัวยาใหม่จากธรรมชาติ เพื่อการพัฒนาไปใช้เป็นยาในอนาคต โดยเน้นฤทธิ์ต่อประกอบด้วยการค้นหาสารออกฤทธิ์จากธรรมชาติ ปรับเปลี่ยนโครงสร้างทางเคมี ศึกษาฤทธิ์ทางชีววิทยาและเภสัชวิทยาเพื่อให้ได้สารที่มีฤทธิ์เด่น ใช้เป็น **candidate lead compounds** ที่มีฤทธิ์ทางยา พัฒนาระบบนำส่งยา เพื่อการพัฒนาสารเป็นผลิตภัณฑ์ที่ออกฤทธิ์ได้ดีที่เป้าหมายต่อไป การดำเนินงานวิจัยมีการรับช่วงต่อกัน ดังนี้

#### 1.1 ด้านความร่วมมือระหว่างนักวิจัย

1.1.1 กลุ่มนักวิจัยเคมี ได้ส่งสารที่แยกได้จากพืชสมุนไพรให้กลุ่มวิจัยในเครือข่ายเพื่อทดสอบฤทธิ์ทางชีวภาพ และเภสัชวิทยา เบื้องต้นดังต่อไปนี้

1.1.2. กลุ่มนักวิจัยศึกษาฤทธิ์ทางชีวภาพ และเภสัชวิทยา ได้แก่

- ฤทธิ์ cytotoxic and anticancer activities
- ฤทธิ์ anti-diabetic activity และ ฤทธิ์ต่อเซลล์ไขมัน anti-adiposity activity
- ฤทธิ์ antioxidant activity และ ฤทธิ์ป้องกันความเสื่อมของเซลล์ประสาท

1.1.3 กลุ่มนักวิจัยศึกษา ร่วมมือกับนักวิจัยสาขาเทคโนโลยีเภสัชกรรมเพื่อพัฒนาระบบนำส่งตัวยาที่ออกฤทธิ์สำคัญได้

#### 1.2 ด้านความร่วมมือระหว่างสถาบัน/องค์กร

##### ความร่วมมือระหว่างเครือข่ายในประเทศ

ได้มีความร่วมมืออย่างใกล้ชิดกับนักวิจัยในโครงการทั้งในสถาบันและต่างสถาบันดังข้อมูลผลงานวิจัยข้างต้น

โดยศ.ดร.อภิชาติ สุขสำราญ มหาวิทยาลัยรามคำแหง และผศ.ดร.รุ่งนภา แซ่เอ็งมหาวิทยาลัยบูรพา เป็นผู้

สกัดสารจากพืช และดัดแปลงโครงสร้างของสารเคมี มาใช้ในการวิจัยศึกษาหาฤทธิ์ทางชีวภาพ โดยเฉพาะฤทธิ์เกี่ยวข้องกับไขมัน

ศ.ดร.ปราณิต โอปนะโสภิต คณะเภสัชศาสตร์ มหาวิทยาลัยศิลปากร ได้นำสารที่ผ่านการศึกษาว่ามีฤทธิ์ฆ่ามะเร็งได้ดีเด่น แต่มีความเป็นพิษต่อเซลล์สูงไปพัฒนาระบบนำส่งยาด้วย chitosan-base polymeric micelles และ liposomes และติด ligand เพื่อนำส่งยาสู่เป้าหมายเซลล์มะเร็ง เพื่อลดความเป็นพิษของยาต่อเซลล์ปกติเมื่อเข้าสู่ร่างกาย

### ความร่วมมือระหว่างเครือข่ายต่างประเทศดังนี้

สาธารณรัฐประชาชนจีน เป็นการสร้างความร่วมมือใหม่กับนักวิจัยชาวจีน เน้นการค้นหารสสารจากเชื้อจุลินทรีย์ในทะเลเป็นหลัก โดยได้ร่วมมือกับ

1. Weiming Zhu, Professor, Ph.D., Vice Director of the Key Laboratory of Marine Drugs, Ocean University of China, Ministry of Education of China
2. Chunhong Dong, Professor, Ph.D., Jiaozuo University, China

ประเทศสหรัฐอเมริกา เป็นการต่อยอดความร่วมมือวิจัยที่มีอยู่ให้เข้มข้น โดยได้ร่วมมือกับ

- 1.. Professor Dr. Harry Blair, University of Pittsburgh, Department of Pathology, Section of Laboratory Medicine รัฐ Pennsylvania ประเทศสหรัฐอเมริกา
2. Assoc. Prof Dr. Erik W. Dent,. University of Wisconsin-Madison, Department of Neuroscience รัฐ Wisconsinประเทศสหรัฐอเมริกา
3. Professor Tianxin Yang University of Utah ประเทศสหรัฐอเมริกา

จากการดำเนินงานของโครงการเครือข่ายวิจัยที่ผ่าน มีความร่วมมือกันเป็นอย่างดีของนักวิจัยทั้งจากเครือข่ายภายในประเทศไทยเอง และจากต่างประเทศ การวิจัยของเครือข่ายกับต่างประเทศ ได้เน้นการสร้างความร่วมมือใหม่กับนักวิจัยจากสาธารณรัฐประชาชนจีนของกลุ่ม Prof. Dr. Zhu, WeiMing จาก School of Medicine and Pharmacy, Ocean University of China, จังหวัด Qingdao มณฑล Shandong เป็นหลัก ซึ่งมีความเชี่ยวชาญในการแยกสารเคมีจากจุลินทรีย์ในทะเล และยังมีความร่วมมือกับนักวิจัยในประเทศสหรัฐอเมริกาที่มีมาก่อน ผ่านทุนความร่วมมืออื่น เป็นการสร้างความเข้มแข็งในการวิจัยให้กับนักวิจัย นักศึกษา บุคลากร และสถาบันในประเทศ โดยในระยะเวลา 3 ปี Output –Outcome ถึงปัจจุบัน โครงการมีผลงานวิจัยตีพิมพ์ในวารสารนานาชาติรวมทั้งสิ้น 37 ชิ้น ตามเอกสารแนบ list of Publications มีบัณฑิตระดับปริญญาเอกที่เป็นผลผลิตโดยตรงจากโครงการ 2 คนและที่มีส่วนร่วมอีก 9 คน ซึ่งกลุ่มนักศึกษาทั้งหมดในกลุ่มวิจัยมีกิจกรรมทางวิชาการและวิจัยร่วมกันตลอดระยะเวลาของโครงการ

### 1.3 กิจกรรมที่สนับสนุนการพัฒนาความร่วมมือ และงานวิจัยของเครือข่าย

1. จัดประชุมกลุ่มวิจัยประจำ (ทุก 2 เดือน) เพื่อติดตามความก้าวหน้าในการดำเนินงานกับเครือข่ายกลุ่มวิจัยในประเทศ

| 2. กิจกรรมของสมาชิกในเครือข่ายที่สนับสนุนการพัฒนาความร่วมมือ และงานวิจัยของเครือข่ายมีดังนี้ |                                                                                                                                                                                                                                                                                                                                                                                                 |
|----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 20-24<br>กรกฎาคม<br>2559                                                                     | มีการจัดประชุมสรุปผลการดำเนินงานและวางแผนความร่วมมือในโครงการวิจัยกับเครือข่ายนักวิจัยในต่างประเทศ กลุ่มนักวิจัยชาวจีนโดยการนำของ Prof. Weiming Zhu และทีมนักวิจัยอีก 4 ท่านจาก Ocean University of China, Ministry of Education of China ได้เดินทางมาประเทศไทย และไปเยี่ยมชมสถาบันในเครือข่าย ได้แก่ คณะเภสัชศาสตร์ มหาวิทยาลัยศิลปากร จังหวัดนครปฐม                                           |
| 16-20<br>ตุลาคม<br>2559                                                                      | สมาชิกกลุ่มวิจัยเข้าร่วมประชุม The 6th China-Thailand Joint Workshop on Natural Products and Drug Discovery ที่โรงแรม Aonang Cliff Beach Resort จังหวัดกระบี่ จัดโดยฝ่ายวิชาการ สกว. ได้พบปะกับนักวิจัยทางเคมีสมุนไพรของประเทศ และประเทศจีนจากมหาวิทยาลัยต่างๆ รวมถึง Prof. Weiming Zhu และสมาชิกของกลุ่ม ได้นำเสนอผลงานทั้งในรูปแบบของการนำเสนอปากเปล่าและแบบโปสเตอร์                          |
| 1 ตุลาคม<br>2559                                                                             | น.ส. สมฤดี เรียบร้อย นักศึกษาปริญญาเอก คปก. หลักสูตรสรีรวิทยา ได้เดินทางกลับจากการวิจัยต่อยอดงานวิทยานิพนธ์ที่เกี่ยวข้องกับผลของสารจากสมุนไพรต่อการลุกลามของเซลล์มะเร็งกับ Assoc. Prof. Erik W. Dent, Department of Neuroscience, University of Wisconsin-Madison, รัฐ Wisconsin ประเทศสหรัฐอเมริกา โดย เป็นเวลา 9 เดือน                                                                        |
| 31 ตุลาคม<br>2559                                                                            | นส. นริรัตน์ สุจริต นักศึกษาปริญญาเอกทุนโครงการพัฒนากำลังคนทางด้านวิทยาศาสตร์ หลักสูตรพิษวิทยา ได้เดินทางกลับจากไปทำงานวิจัยกับ Professor Harry Blair, University of Pittsburgh, Department of Pathology, Section of Laboratory Medicine รัฐ Pennsylvania ประเทศสหรัฐอเมริกา โดยต่อยอดงานที่เกี่ยวข้องกับผลของสารจากสมุนไพรต่อเซลล์ไขมันเป็นเวลา 1 ปี                                           |
| 4 สิงหาคม<br>2560                                                                            | จัดประชุมเครือข่ายวิจัยและสัมมนาทางวิชาการในประเทศ ณ มหาวิทยาลัยศิลปากร จังหวัดนครปฐม เพื่อให้สมาชิกกลุ่มวิจัยได้นำเสนอผลงานวิจัย และประชุมหารือเกี่ยวกับความก้าวหน้าและแผนการทำงานร่วมกันของเครือข่ายวิจัย                                                                                                                                                                                     |
| เมษายน<br>2560                                                                               | ผศ.ดร. วิชชุดา แสงสว่าง เข้าร่วมประชุมและนำเสนอผลงานวิจัยในการประชุมระดับนานาชาติ EB meeting ณ เมือง Chicago, รัฐ Illinois ประเทศสหรัฐอเมริกา และได้เดินทางต่อ ไปหาหรือความร่วมมือและปฏิบัติงานวิจัยกับนักวิจัยในเครือข่าย Assoc. Prof. Erik W. Dent, Department of Neuroscience, University of Wisconsin-Madison, รัฐ Wisconsin ประเทศสหรัฐอเมริกาเป็นเวลา 2 เดือน (28 เมษายน -7 กรกฎาคม 2560) |
| 5-9<br>กันยายน<br>2560                                                                       | Prof. Harry Blair จาก University of Pittsburgh, Department of Pathology, Section of Laboratory Medicine รัฐ Pennsylvania ประเทศสหรัฐอเมริกา เดินทางมาประเทศไทยเพื่อติดตามความก้าวหน้าของงานวิจัยที่ทำร่วมกัน ให้สัมมนาพิเศษ ณ ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์มหาวิทยาลัยมหิดล                                                                                                                   |

| 2. กิจกรรมของสมาชิกในเครือข่ายที่สนับสนุนการพัฒนาความร่วมมือ และงานวิจัยของเครือข่ายมีดังนี้ |                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 20-24<br>ตุลาคม<br>2560                                                                      | กลุ่มนักวิจัยไทยได้เดินทางไปประเทศจีน เพื่อเยี่ยมเยียน นำเสนอความก้าวหน้าของงานวิจัยในโครงการ ประชุมปรึกษาหารือวางแผนงานวิจัยร่วมกัน และทำความรู้จักกับสถาบัน และเครือข่ายวิจัยของ Prof. Dr. Zhu, WeiMing และคณะที่ School of Medicine and Pharmacy, Ocean University of China ซึ่งเป็น The Key Laboratory of Marine Drugs, Ocean University of China, Ministry of Education of China                                                          |
| 6-8<br>ธันวาคม<br>2560                                                                       | น.ส. สมฤดี เรียบร้อย นักศึกษาในกลุ่มวิจัยนำเสนอผลงานวิจัยที่ได้จากโครงการในที่ประชุมระดับชาติ เรื่อง An andrographolide analogue suppresses the proliferation and Wnt/ $\beta$ -catenin signaling pathway in colorectal cancer cells ที่งานประชุม The 45 <sup>th</sup> Physiology Society of Thailand Annual Meeting 2017, 6-8 December 2017, Khonkaen University Thailand และได้รับรางวัล Excellent Oral Presentation for Ph.D. Student Award |
| 6-8<br>ธันวาคม<br>2560                                                                       | น.ส.นริรัตน์ สุจริต นักศึกษาในกลุ่มวิจัยนำเสนอผลงานวิจัยที่ได้จากโครงการในที่ประชุมระดับชาติ เรื่อง Modulating effect of Wan-Chak motluk in adipose tissues of ovariectomized (OVX) rats ที่งานประชุม The 45 <sup>th</sup> Physiology Society of Thailand Annual Meeting 2017, 6-8 December 2017, Khonkaen University Thailand                                                                                                                 |
| 24<br>มกราคม<br>2561                                                                         | Professor Katherine Kalil, Ph.D., Department of Neuroscience, University of Madison-wisconsin, USA เดินทางมาให้สัมมนาพิเศษเรื่อง The role of the microtubule associated protein tau in regulating the growth cone cytoskeleton พร้อมกับให้คำปรึกษางานวิจัยแก่นักวิจัย                                                                                                                                                                          |
| 24 มีนาคม<br>2561                                                                            | Prof. Erik W. Dent จาก Department of Neuroscience, University of Wisconsin-Madison รัฐ Wisconsin ประเทศสหรัฐอเมริกา กำหนดการเดินทางมาประเทศไทยวันที่ 24 มีนาคม 2561 เพื่อติดตามความก้าวหน้าของงานวิจัยที่ทำร่วมกัน ให้สัมมนาพิเศษณภาควิชาสรีรวิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดลและประชุมหารือความร่วมมือ                                                                                                                                    |
| 27-28<br>มีนาคม<br>2561                                                                      | นายจิรทัศน์ กันโสม นักศึกษาในกลุ่มวิจัยนำเสนอผลงานวิจัยที่ได้จากโครงการในที่ประชุมระดับนานาชาติ เรื่อง Effect of semi-synthetic andrographolide analogue-loaded polymeric micelles on HN22 cell migration ที่งานประชุม The 10th Walailak Research National Conference, 27-28 March 2018, Walailak University Thailand.                                                                                                                         |
| 14-18<br>เมษายน<br>2561                                                                      | รศ.ดร. อาทิตย์ ไชยร้องเตื้อ ได้เข้าร่วมประชุมวิชาการนานาชาติ Annual meeting of American Association for Cancer Research (AACR) ณ เมือง Chicago รัฐ Illinois และได้เดินทางไปติดตามความก้าวหน้าของนักศึกษาและสร้างความร่วมมืองานวิจัยด้านโรคมะเร็งกับ Assistant Professor Dr. Andrea L. Kasinski ภาควิชา Biological Sciences, Purdue University เมือง West Lafayette รัฐ Indiana ประเทศสหรัฐอเมริกา ระหว่างวันที่ 18-25 เมษายน 2561              |
| เมษายน                                                                                       | รศ. ดร.สันทาส สุดวิไล เข้าร่วมประชุมวิชาการและนำผลงานวิจัยไปนำเสนอที่งานประชุม                                                                                                                                                                                                                                                                                                                                                                 |

| 2. กิจกรรมของสมาชิกในเครือข่ายที่สนับสนุนการพัฒนาความร่วมมือ และงานวิจัยของเครือข่ายมีดังนี้ |                                                                                                                                                                                                                       |
|----------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2561                                                                                         | วิชาการนานาชาติ Experimental Biology 2018 ณ เมือง San Diego มลรัฐ California และได้เดินทางไปปรึกษางานวิจัยกับนักวิจัยในเครือข่าย Professor Tianxin Yang University of Utah ประเทศสหรัฐอเมริกาจนถึงเดือน มิถุนายน 2561 |
| 7-12<br>กรกฎาคม<br>2561                                                                      | Prof. Praneet Opanasopit มีแผนการเดินทางไปนำเสนอผลงานวิจัยที่ได้จากโครงการในที่ประชุมระดับนานาชาติ ในงานประชุม The 43rd FEBS Congress, to be held during 7–12 July 2018 ที่ Prague                                    |
| 18-20<br>ตุลาคม<br>2561                                                                      | ศ.ดร.ภาวิณี ปิยะจตุรวัฒน์ และ ศ.ดร.ปทุมรัตน์ ตูจินดา จะเข้าร่วมประชุมวิชาการและนำเสนอผลงานวิจัยในการประชุม The 7th International Conference on Natural Product (NATPRO7) ณ เมือง Gyeongju ประเทศเกาหลี                |
| 4-7<br>พฤศจิกายน<br>2561                                                                     | - ศ.ดร.ภาวิณี ปิยะจตุรวัฒน์ ศ.ดร.ปราณีต โอปนยะโสภิต และรศ.ดร.อาทิตย์ ไชยร้องเดื่อ จะเดินทางกับนักวิจัยสกว. เข้าร่วมประชุม “The 7th Chinese-Thailand Workshop on Natural Products and Drug Discovery” ณ. เมืองนานจิง   |

#### 1.4 ข้อมูลด้านจำนวนและงบประมาณ

(1) จำนวนของหน่วยงานในประเทศที่เข้าร่วมในเครือข่ายและลักษณะของการร่วมสนับสนุน

- จำนวนหน่วยงานต้นสังกัดของนักวิจัยในเครือข่ายจำนวน 3 แห่งที่เป็นทางการและ
- จำนวนหน่วยงานอื่นๆ ที่สนับสนุนเครือข่ายจำนวนไม่มี
- ลักษณะการร่วมมือหรือสนับสนุน/งบประมาณที่สนับสนุน (ถ้ามี).....

(2) จำนวนของหน่วยงานต่างประเทศที่เข้าร่วมในเครือข่ายและลักษณะของการร่วมสนับสนุน

- จำนวนหน่วยงานต้นสังกัดของนักวิจัยในเครือข่ายจำนวน 1 แห่ง (จากสาธารณรัฐประชาชนจีน-ไม่มี MOU)
- จำนวนหน่วยงานอื่นๆ ที่สนับสนุนเครือข่ายจำนวน 3 แห่งจาก USA.
- ลักษณะการร่วมมือหรือสนับสนุน/งบประมาณที่สนับสนุน (ถ้ามี).....

(3) จำนวนรวมของหน่วยงานในต่างประเทศที่เข้าร่วมในเครือข่าย....4....แห่ง

- หน่วยงานต้นสังกัดของนักวิจัยในเครือข่ายจำนวน....3....แห่ง และ
- หน่วยงานอื่นๆ ที่สนับสนุนเครือข่ายจำนวน.....แห่ง

\* อยู่ระหว่างการพัฒนาความร่วมมือเพิ่มเติม โดยเป็นอาจารย์ที่ปรึกษาร่วมในต่างประเทศของนักศึกษา

#### 3. ข้อมูลการสนับสนุนทุนผู้ช่วยวิจัยระดับปริญญาเอกและ/หรือทุนนักวิจัยหลังปริญญาเอก

☐ ได้รับทุนผู้ช่วยวิจัยระดับปริญญาเอกด้วยทุนของ

◎ โครงการ IRN จำนวน 2 ทุน เป็นเงิน 3,384,000บาท คือ

1. นายเกียรติดำรงค์ จันทรพิพัฒน์กุล ภายใต้การดูแลของ รศ.ดร.อาทิตย์ ไชยร้องเดื่อ

2. นางสาวเพ็ญใจ ทองนวลจันทร์ ภายใต้การดูแลของ รศ.ดร. สันทาส สุตวิสัย

◎ โครงการอื่นๆ ได้แก่

ก. โครงการ คปก.

1. นักศึกษา คปก. ชื่อ น.ส. สมฤดี เรียบร้อย นักศึกษาหลักสูตรสรีรวิทยา
2. นักศึกษา คปก. ชื่อ น.ส. นิตยา บุญหมื่น นักศึกษาหลักสูตรสรีรวิทยา
3. นักศึกษา คปก. ชื่อ น.ส. ศรัญญา กิจดำรงธรรม นักศึกษาหลักสูตรพิษวิทยา
4. นักศึกษา คปก. ชื่อ นางสาวศิริมา สุตวิสัย นักศึกษาหลักสูตรเทคโนโลยีเภสัชกรรม
5. นักศึกษา คปก. ชื่อ นายธีรทัศน์ กันโสม นักศึกษาหลักสูตรเทคโนโลยีเภสัชกรรม

ข. โครงการพัฒนากำลังคนด้านวิทยาศาสตร์

1. นางสาวนริรัตน์ สุจริต นักศึกษาปริญญาเอก หลักสูตรพิษวิทยา
2. นางสาวขวัญชนก อุปการะ นักศึกษาปริญญาเอก หลักสูตรพิษวิทยา
3. นายเอกพจน์ คงกล้า นักศึกษาปริญญาเอก หลักสูตรสรีรวิทยา

ค. โครงการศรีตรังทอง

1. นายจาตุรนต์ ขวัญทองดี นักศึกษาปริญญาเอก หลักสูตรสรีรวิทยา

รวมนักศึกษาได้รับทุนผู้ช่วยวิจัยระดับปริญญาเอกที่เกี่ยวข้องกับโครงการ 11 คน

☐ ได้รับทุนนักวิจัยหลังปริญญาเอกจำนวน ..... ทุน ด้วยทุนของ

○โครงการ IRN จำนวน ..... ทุน เป็นเงิน ..... บาท

○โครงการ (โปรดระบุ) ..... จำนวน ..... ทุน เป็นเงิน ..... บาท

☐ ได้รับทุนอื่น (หากมี) โปรดระบุ

4. ปัญหาและอุปสรรค/ความเห็นและข้อเสนอแนะ

ด้วยโครงการเครือข่ายวิจัยนานาชาตินี้ ระยะเวลาการรับทุน 3 ปี การสนับสนุนเงินทุนประกอบด้วย 2 ส่วน คือส่วนที่เกี่ยวข้องกับการวิจัย และส่วนที่เกี่ยวข้องกับการศึกษาของนักศึกษาระดับปริญญาเอก 2 คน ในส่วนของการวิจัย ไม่มีอุปสรรค สามารถดำเนินงานให้สำเร็จลุล่วงได้ตามเป้าหมายในเวลา 3 ปีที่ทุนกำหนด ถึงแม้ความร่วมมือกับนักวิจัยจากสาธารณรัฐประชาชนจีน จะเป็นการเริ่มต้นใหม่ ด้วยประสบการณ์วิจัย และความชำนาญ ของนักวิจัยหลักทั้ง 2 ฝ่ายเสริมกันและกัน (จีนเน้นการแยกสาร- ไทยเน้นการ screen และวิจัยต่อยอดและพัฒนา) จากความมุ่งมั่น เข้มแข็งของนักวิจัยจีนได้ทำการเพาะเลี้ยง marine microbes ซึ่งมีปริมาณน้อย

มาก สกัตแยกสาร และส่งสารมายังประเทศไทยเพื่อทำการตรวจสอบฤทธิ์ ทำการวิจัยได้อย่างต่อเนื่อง รวมถึงการส่งสารมาต่อยอดศึกษากลไกการทำงาน ทำให้ได้ผลงานร่วมกับนักวิจัยจีนเป็นจำนวนมาก

อุปสรรคหลักที่พบในโครงการ คือระยะเวลา 3 ปี ไม่เพียงพอกับการศึกษาระดับปริญญาเอกของนักศึกษาในหลักสูตรที่มี course works และการสอบประเมินคุณภาพขึ้นเป็น Ph.D.-candidate นอกจากนี้ในระยะท้ายของการวิจัยมีอุปสรรคจากการระบาดของ Virus Covid-19 มาตลอด 2 ปี มหาวิทยาลัยและคณะฯ ได้มีมาตรการจำกัดบุคคลเข้าออก การเข้าทำงาน ทำให้งานล่าช้าออกไป ประกอบกับในปัจจุบัน การตีพิมพ์ผลงานวิจัยในวารสารนานาชาติที่เป็นที่ยอมรับ จะยากขึ้นมาก มีการคัดกรอง เสนอแนะให้ทำงานเพิ่ม ซึ่งการเข้าทำงานและการส่งของล้วนมีอุปสรรค หลังจากทำงานเพิ่มแล้ว ยังถูกปฏิเสธการรับตีพิมพ์ ซึ่งจะต้องเริ่มต้นใหม่ ทุกกระบวนการล้วนใช้เวลาทั้งสิ้น ทำให้โครงการหลักที่เสร็จสิ้นแล้ว ไม่สามารถปิดโครงการได้ในเวลาที่กำหนด ต้องรอผลงานจากโครงการย่อยของนักศึกษาให้เสร็จสิ้น

โครงการวิจัยเครือข่ายนี้เป็นโครงการที่ดีมาก ทำให้สามารถดึงนักวิจัยจากสาขาที่แตกต่างกันในประเทศมารวมมือกัน ทำงานเป็นเครือข่าย รับช่วงการทำงานกันในแนวทางของการค้นหาและพัฒนาายใหม่ (Modern drug development ) ถึงแม้ผลงานวิจัยมีระดับTRL 3-4 ยังไม่ถึงจุดที่จะใช้งานได้ก็ตาม ผลงานที่ได้เป็นผลงานขั้นพื้นฐานที่สำคัญ นักวิจัยสามารถดำเนินการต่อยอดได้ต่อไปหลังจากนี้ เนื่องจากนักวิจัยหลักแต่ละท่านมีความร่วมมือกับนักวิจัยในต่างประเทศอยู่แล้ว โดยเฉพาะในประเทศสหรัฐอเมริกาผ่านนักศึกษา ทั้งจากโครงการคปก. และโครงการพัฒนากำลังคนด้านวิทยาศาสตร์ของทบวงมหาวิทยาลัย โครงการวิจัยเครือข่ายนี้ยังเปิดโอกาสให้นักวิจัยสามารถทำงานต่อยอดกับเครือข่ายวิจัยที่มีอยู่แล้ว ในต่างประเทศ เป็นการสร้างความเข้มแข็งของเครือข่ายวิจัย ต่อยอดงานวิจัยให้มีคุณภาพมากขึ้น

ในโครงการนอกจากนักศึกษาระดับปริญญาเอกที่รับทุนโดยตรงจากโครงการ ยังมีนักศึกษาระดับปริญญาเอกที่รับทุนจากโครงการอื่นอีก 9 คนเข้าร่วมวิจัย นับว่าเป็นการร่วมผลิตและพัฒนานักวิจัยรุ่นใหม่ที่มีคุณภาพได้เป็นจำนวนมาก เป็นการช่วยลดปัญหาการขาดแคลนนักวิจัยที่มีความรู้ความสามารถของประเทศ และปัจจุบันนักศึกษาเหล่านั้นได้รับการบรรจุเข้าทำงานเกือบหมดแล้ว

### ส่วนที่ 3 รายงานเนื้อหาของโครงการวิจัยภายใต้เครือข่าย

1. การดำเนินงาน ความก้าวหน้าของโครงการ .....100..... ของโครงการหลัก

- ☒ ได้ดำเนินงานตามแผนที่วางในส่วนของโครงการหลัก
- ☒ ได้ดำเนินงานล่าช้ากว่าแผนที่วางไว้ ในส่วนของโครงการย่อยของนักศึกษา
- ☐ ได้เปลี่ยนแผนงานที่วางไว้ดังนี้ .....

### 2. รายละเอียดผลการดำเนินงานของโครงการ

#### Backgrounds and the Significance of the research project:

Over the past two decades, growth of elderly population has rapidly increased



worldwide including Thailand. The increase in advance aged population has profound effects on the health care systems as the elderly has faced to various chronic diseases that require ongoing treatments and a long-term medical care. The direct costs of medical care for the elderly patients are high and become a real burden for health care systems of the country. Aging is primarily a change in bodily functions which is associated with a number of physiological changes. Thus, aging population is generally more susceptible to non-communicable diseases (NCDs), which are a wide range of chronic illness conditions including cancer, diabetes, cardiovascular disease, hypertension, as well as Alzheimer's disease, dementia and other neurodegenerative diseases.

Many natural product-derived compounds are promising sources for treatment of many diseases. Thailand is located in the tropical rainforest area that contains an amazing abundance of plant life. The exciting to scientist, particularly in non-rainforest community, is the great structural variety and the medicinal qualities of plant compounds in this rich environment. The healing power of compounds from rainforest herbs is unique. Almost half of the prescribed drugs that are used in the USA come from rainforest plants. In addition to the rainforest, the ocean is considered as the 'mother of origin of life' that contains highly ecological, chemical and biological diversity starting from microorganisms to vertebrates. Marine has been the resource of unique chemical compounds, which held great pharmaceutical prospective. Recently, a number of bioactive compounds extracted from a range of marine animals are reported as potent anti-cancers. Natural product compounds have substantially structural diversity and frequently afford new mechanisms of biological activity. Thus they are the main sources for developing new drugs by the pharmaceutical industries around the world.

Thailand and China have their own indigenous medicines with a long history of using natural products for both prevention and treatment of several diseases, especially among local people and it becomes the mainstay in health care system in some parts of the countries. With tremendous sources of natural products in Thailand and China and the collaboration between multidiscipline scientists, consisting of chemists, biologists, physiologists and pharmacists from both countries, new biologically active compounds for prevention/treatment of age-related diseases could be extensively developed, which will have a strong positive impact on Thailand health care systems and economy.

One cause of the social and economic weakness of Thailand is the severe shortage of highly qualified human resources especially in the field of science and technology as compared to our neighboring countries. Thousands of high quality Ph.D. graduates with the international standard are needed to provide academic staffs with great potential for both newly and well established universities and research institutes. Research collaborations and network formation

among Thai and overseas scientists could be a strategy to achieve this goal.

The project is focused on the natural product-based drug discovery and development of pharmaceutical product from national resources including those from marine microorganisms, and terrestrial tropical plants for prevention and treatment of age-related diseases including diabetes mellitus, obesity, cancer and neurodegenerative diseases.

**1. Objectives of the project:**

- 6.1. To establish and strengthen both national and international research networks
- 6.2. To discover and develop novel active compounds for prevention/treatment of age-related diseases from Thai and Chinese medicinal natural products
- 6.3. To produce high quality Ph.D. graduates for sustainable development of human resources
- 6.4. To increase international visibility and research capability through overseas research collaboration and dissemination of advance research results to user/practical applications

To fulfill the objectives, a multi-institutional collaborative research project from three institutes in Thailand together with international collaborators in China and the United States is formed to search for new biologically active compounds from national resources including those from marine microorganisms, and terrestrial tropical plants and development for prevention and treatment of age-related diseases. Two Ph.D. students participating in the projects are supported.

**Scope of Research**

The project is divided into 5 sub-projects. The significance and scope of each research project are described below.

## Sub-project 1: Extraction/isolation/structure elucidation of natural products

### ABSTRACT

Our research group has compiled 725 samples of natural, modified and synthetic compounds, as well as fractions and extracts from our laboratory and tabulated as a list which contains information about types of the isolated compounds, plant sources, sample types, plant parts and bioassays. In addition three more plants have been chemically investigated. The structures of forty-nine pure compounds and a mixture of  $\beta$ -sitosterol/stigmasterol were established on the basis of spectroscopic methods.

**Keywords:** Flavonoids, terpenoids, lignans, phenolics, Styryllactones, iridoids, xanthenes benzophenones

### บทคัดย่อ

คณะวิจัยได้จัดรวบรวมข้อมูล ของสารธรรมชาติ สารดัดแปลง และสารสังเคราะห์ พร้อมทั้ง รายละเอียดต่าง ๆ เกี่ยวกับพืช ส่วนของพืช ชนิดของสารที่แยกได้ รวมทั้งฤทธิ์ทางชีวภาพที่ทำการทดสอบ โดยจัดทำเป็นรายการรวมทั้งหมด 725 รายการ นอกจากนี้ยังได้ทำการศึกษาร่องรอยประกอบของพืชอีก 3 สปีชีส์ ได้แก่ ต้นกาญจนิการ์ หรือ แดง (*Santisukia pagetii*) ต้นสบ้านงาป่า (*Goniothalamus calvicarpus*) และต้นหมากเหลี่ยม (*Mallotus glomerulatus*) ซึ่งสามารถแยกสารได้ 49 สาร และสารคู่ผสมของ  $\beta$ -sitosterol/stigmasterol ที่พบในพืชทั้ง 3 สปีชีส์ และได้ทำการวิเคราะห์โครงสร้างเคมีของสารที่แยกได้ด้วยวิธีทางสเปกโทรสโกปี

**คำสำคัญ:** ฟลาโวนอยด์ เทอปีน ลิกแนน ฟีนอลิก สเตอรอล แกลโคไซด์ อิริดอย แชนโทน เบนโซฟีโนน

### 1. สกัดแยกสาร ดัดแปลงโครงสร้างและสังเคราะห์สาร เพื่อใช้เป็น compound library

นักวิจัยได้ดำเนินการวิจัยดังต่อไปนี้

The information covering the code of sample, natural (Nat) or modified (Mod) or synthetic (Syn) compound, type of compound, plant source, sample type, plant part and bioassay are tabulated (Table 1). A collection of 725 samples, including crude extracts (E), fractions (F) and pure compounds (P) are also categorized.

Table 1.1 Compound Library

| No | CODE     | Nat/Mod/Syn compound | Type of Compound      | Plant Source                 | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay         |
|----|----------|----------------------|-----------------------|------------------------------|---------------------------------------|------------|---------------|
| 1  | MUC-941  | Nat                  | Phenolic              | <i>Mallotus glomerulatus</i> | P                                     | PX         | Cytotoxic     |
| 2  | MUC-942  | Nat                  | Flavanone             | <i>Mallotus glomerulatus</i> | P                                     | PX         | Cytotoxic     |
| 3  | MUC-943  | Nat                  | Diterpenoid           | <i>Mallotus glomerulatus</i> | P                                     | PX         | Cytotoxic     |
| 4  | MUC-944  | Nat                  | Flavone               | <i>Mallotus glomerulatus</i> | P                                     | PX         | Cytotoxic     |
| 5  | MUC-1020 | Mod                  | Modified triterpenoid | <i>Gardenia collinsae</i>    | P                                     | LF+TW      | Cytotoxic     |
| 6  | MUC-1021 | Mod                  | Modified triterpenoid | <i>Gardenia collinsae</i>    | P                                     | LF+TW      | Cytotoxic     |
| 7  | MUC-1022 | Mod                  | Modified triterpenoid | <i>Gardenia collinsae</i>    | P                                     | LF+TW      | Cytotoxic     |
| 8  | MUC-1023 | Nat                  | Triterpenoid          | <i>Gardenia collinsae</i>    | P                                     | LF+TW      | Cytotoxic     |
| 9  | MUC-1028 | Mod                  | Cyclic ether          | <i>Polyalthia crassa</i>     | P                                     | LF+TW      | Cytotoxic     |
| 10 | MUC-1029 | Mod                  | Styryl lactone        | <i>Polyalthia crassa</i>     | P                                     | LF+TW      | Cytotoxic     |
| 11 | MUC-1030 | Mod                  | Styryl lactone        | <i>Polyalthia crassa</i>     | p                                     | LF+TW      | Cytotoxic     |
| 12 | MUC-1031 | Mod                  | Styryl lactone        | <i>Polyalthia crassa</i>     | p                                     | LF+TW      | Cytotoxic     |
| 13 | MUC-1032 | Nat                  | Styryl lactone        | <i>Polyalthia crassa</i>     | P                                     | LF+TW      | Cytotoxic     |
| 14 | MUC-1255 | Syn                  | Halogenated flavone   | -                            | P                                     | -          | Cytotoxic     |
| 15 | MUC-1261 | Nat                  | Lignan glycoside      | <i>Mallotus glomerulatus</i> | P                                     | LF+TW      | Cytotoxic     |
| 16 | MUC-1262 | Nat                  | Diterpenoid           | <i>Mallotus glomerulatus</i> | P                                     | LF+TW      | Cytotoxic     |
| 17 | MUC-1263 | Nat                  | Diterpenoid           | <i>Mallotus glomerulatus</i> | P                                     | LF+TW      | Cytotoxic     |
| 18 | MUC-896  | Nat                  | Flavone               | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |

| No | CODE    | Nat/Mod/Syn<br>compound | Type of Compound | Plant Source                 | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay         |
|----|---------|-------------------------|------------------|------------------------------|---------------------------------------|------------|---------------|
| 19 | MUC-897 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 20 | MUC-898 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 21 | MUC-899 | Nat                     | Flavone          | <i>Gardenia carinata</i>     | P                                     | LF+TW      | Anti-diabetic |
| 22 | MUC-900 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 23 | MUC-901 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 24 | MUC-902 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 25 | MUC-903 | Nat                     | Flavanone        | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 26 | MUC-904 | Nat                     | Flavanone        | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 27 | MUC-905 | Nat                     | Flavanone        | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 28 | MUC-906 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 29 | MUC-907 | Nat                     | Flavone          | <i>Gardenia carinata</i>     | P                                     | LF+TW      | Anti-diabetic |
| 30 | MUC-908 | Nat                     | Flavone          | <i>Gardenia carinata</i>     | P                                     | LF+TW      | Anti-diabetic |
| 31 | MUC-909 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Anti-diabetic |
| 32 | MUC-910 | Nat                     | Flavone          | <i>Mallotus glomerulatus</i> | P                                     | PX         | Anti-diabetic |
| 33 | MUC-911 | Nat                     | Flavonol         | <i>Zingiber maekongense</i>  | P                                     | RT         | Anti-diabetic |
| 34 | MUC-912 | Nat                     | Flavonol         | <i>Zingiber maekongense</i>  | P                                     | RT         | Anti-diabetic |
| 35 | MUC-913 | Nat                     | Flavone          | <i>Gardenia carinata</i>     | P                                     | LF+TW      | Anti-diabetic |
| 36 | MUC-914 | Nat                     | Flavanone        | <i>Mallotus glomerulatus</i> | P                                     | PX         | Anti-diabetic |

| No | CODE     | Nat/Mod/Syn compound | Type of Compound      | Plant Source                    | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|----|----------|----------------------|-----------------------|---------------------------------|------------------------------------|------------|---------------|
| 37 | MUC-915  | Nat                  | Flavanone             | <i>Goniotalamus calvicarpus</i> | P                                  | LF+TW      | Anti-diabetic |
| 38 | MUC-916  | Nat                  | Flavone               | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 39 | MUC-1013 | Nat                  | Diterpenoid glycoside | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 40 | MUC-1014 | Nat                  | Diterpenoid glycoside | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 41 | MUC-1015 | Mod                  | Diterpenoid glycoside | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 42 | MUC-1016 | Mod                  | Diterpenoid glycoside | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 43 | MUC-1017 | Mod                  | Diterpenoid           | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 44 | MUC-1018 | Mod                  | Diterpenoid glycoside | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 45 | MUC-1019 | Mod                  | Diterpenoid glycoside | <i>Stevia rebaudiana</i>        | P                                  | LF         | Anti-diabetic |
| 46 | MUC-1024 | Mod                  | Triterpenoid          | <i>Gardenia collinsae</i>       | P                                  | LF+TW      | Anti-diabetic |
| 47 | MUC-1025 | Mod                  | Triterpenoid          | <i>Gardenia collinsae</i>       | P                                  | LF+TW      | Anti-diabetic |
| 48 | MUC-1026 | Mod                  | Triterpenoid          | <i>Gardenia collinsae</i>       | P                                  | LF+TW      | Anti-diabetic |
| 49 | MUC-1027 | Mod                  | Triterpenoid          | <i>Gardenia collinsae</i>       | P                                  | LF+TW      | Anti-diabetic |
| 50 | MUC-1256 | Mod                  | Triterpenoid          | <i>Gardenia collinsae</i>       | P                                  | LF+TW      | Anti-diabetic |
| 51 | MUC-1257 | Mod                  | Triterpenoid          | <i>Gardenia collinsae</i>       | P                                  | LF+TW      | Anti-diabetic |
| 52 | MUC-1258 | Nat                  | Triterpenoid          | <i>Gardenia sessiliflora</i>    | P                                  | LF+TW      | Anti-diabetic |
| 53 | MUC-1259 | Nat                  | Triterpenoid          | <i>Gardenia sessiliflora</i>    | P                                  | LF+TW      | Anti-diabetic |
| 54 | MUC-1260 | Nat                  | Triterpenoid          | <i>Gardenia sessiliflora</i>    | P                                  | LF+TW      | Anti-diabetic |

| No | CODE    | Nat/Mod/Syn compound | Type of Compound | Plant Source             | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay         |
|----|---------|----------------------|------------------|--------------------------|---------------------------------------|------------|---------------|
| 55 | MUC-631 | Nat                  | Flavone          | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 56 | MUC-632 | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 57 | MUC-633 | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 58 | MUC733  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 59 | MUC734  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 60 | MUC735  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 61 | MUC736  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 62 | MUC737  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 63 | MUC738  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 64 | MUC739  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 65 | MUC740  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 66 | MUC741  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 67 | MUC742  | Nat                  | Flavone          | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 68 | MUC743  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 69 | MUC744  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 70 | MUC745  | Nat                  | Sugar            | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 71 | MUC746  | Nat                  | Phenolic         | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |
| 72 | MUC747  | Nat                  | Triterpenoid     | <i>Gardenia carinata</i> | P                                     | LF+TW      | Anti-diabetic |

| No | CODE      | Nat/Mod/Syn compound | Type of Compound       | Plant Source                | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|----|-----------|----------------------|------------------------|-----------------------------|------------------------------------|------------|---------------|
| 73 | MUC748    | Nat                  | Coumarin               | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 74 | MUC749    | Nat                  | Phenolic               | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 75 | MUC750    | Nat                  | Triterpenoid glycoside | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 76 | MUC751    | Nat                  | Triterpenoid           | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 77 | MUC752    | Nat                  | Flavone                | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 78 | MUC753    | Nat                  | Flavone                | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 79 | MUC754    | Nat                  | Flavone                | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 80 | MUC755    | Nat                  | Flavone                | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 81 | MUC756    | Nat                  | Flavone                | <i>Gardenia carinata</i>    | P                                  | LF+TW      | Anti-diabetic |
| 82 | MUC757    | Nat                  | Alkaloid               | <i>Polyalthia suberosa</i>  | P                                  | ST         | Anti-diabetic |
| 83 | MUC758    | Nat                  | Alkaloid               | <i>Polyalthia suberosa</i>  | P                                  | ST         | Anti-diabetic |
| 84 | PT-382-SS | Nat                  | Flavonol               | <i>Gardenia obtusifolia</i> | P                                  | LF+TW      | Anti-diabetic |
| 85 | PT-383-SS | Nat                  | Flavonol               | <i>Gardenia obtusifolia</i> | P                                  | LF+TW      | Anti-diabetic |
| 86 | PT-384-SS | Nat                  | Flavonol               | <i>Gardenia obtusifolia</i> | P                                  | LF+TW      | Anti-diabetic |
| 87 | PT-385-SS | Nat                  | Flavone                | <i>Gardenia collinsae</i>   | P                                  | LF+TW      | Anti-diabetic |
| 88 | PT-386-SS | Nat                  | Flavone                | <i>Gardenia obtusifolia</i> | P                                  | LF+TW      | Anti-diabetic |
| 87 | PT-387-SS | Nat                  | triterpenoid           | <i>Gardenia obtusifolia</i> | P                                  | LF+TW      | Anti-diabetic |
| 88 | PT-388-SS | Nat                  | triterpenoid           | <i>Gardenia obtusifolia</i> | P                                  | LF+TW      | Anti-diabetic |



| No  | CODE      | Nat/Mod/Syn<br>compound | Type of Compound | Plant Source                  | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|-----------|-------------------------|------------------|-------------------------------|---------------------------------------|------------|---------------|
| 89  | PT-389-SS | Nat                     | Flavone          | <i>Gardenia thailandica</i>   | P                                     | LF+TW      | Anti-diabetic |
| 90  | PT-390-SS | Nat                     | Flavone          | <i>Gardenia thailandica</i>   | P                                     | LF+TW      | Anti-diabetic |
| 91  | PT-391-SS | Nat                     | Triterpenoid     | <i>Gardenia thailandica</i>   | P                                     | LF+TW      | Anti-diabetic |
| 92  | PT-392-SS | Nat                     | Triterpenoid     | <i>Gardenia thailandica</i>   | P                                     | LF+TW      | Anti-diabetic |
| 93  | PT-393-SS | Nat                     | Alkaloid         | <i>Phyllanthus acutissima</i> | P                                     | PX         | Anti-diabetic |
| 94  | PT-394-SS | Nat                     | Alkaloid         | <i>Phyllanthus acutissima</i> | P                                     | PX         | Anti-diabetic |
| 95  | PT-395-SS | Nat                     | Triterpenoid     | <i>Phyllanthus acutissima</i> | P                                     | PX         | Anti-diabetic |
| 96  | PT-396-SS | Nat                     | Triterpenoid     | <i>Phyllanthus acutissima</i> | P                                     | PX         | Anti-diabetic |
| 97  | PT-397-SS | Nat                     | Triterpenoid     | <i>Phyllanthus acutissima</i> | P                                     | PX         | Anti-diabetic |
| 98  | PT-398-SS | Nat                     | Lignan           | <i>Phyllanthus acutissima</i> | P                                     | PX         | Anti-diabetic |
| 99  | PT-399-SS | Nat                     | Cyclic amide     | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 100 | PT-400-SS | Nat                     | Alkaloid         | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 101 | PT-401-SS | Nat                     | Conjugated amide | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 102 | PT-402-SS | Nat                     | Conjugated amide | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 103 | PT-403-SS | Nat                     | Alkaloid         | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 104 | PT-404-SS | Nat                     | Alkaloid         | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 105 | PT-405-SS | Nat                     | Alkaloid         | <i>Polyalthia suberosa</i>    | P                                     | ST         | Anti-diabetic |
| 106 | PT-406-SS | Nat                     | Styryl lactone   | <i>Polyalthia crassa</i>      | P                                     | LF+TW      | Anti-diabetic |

| No  | CODE      | Nat/Mod/Syn compound | Type of Compound   | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|-----------|----------------------|--------------------|----------------------------------|------------------------------------|------------|---------------|
| 107 | PT-407-SS | Nat                  | Alkaloid           | <i>Polyalthia crassa</i>         | P                                  | LF+TW      | Anti-diabetic |
| 108 | PT-408-SS | Nat                  | Styryl lactone     | <i>Polyalthia crassa</i>         | P                                  | LF+TW      | Anti-diabetic |
| 109 | PT-409-SS | Nat                  | Styryl lactone     | <i>Polyalthia crassa</i>         | P                                  | LF+TW      | Anti-diabetic |
| 110 | PT-410-SS | Nat                  | Diterpenoid        | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 111 | PT-411-SS | Nat                  | Diterpenoid        | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 112 | PT-412-SS | Nat                  | Diterpenoid        | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 113 | PT-413-SS | Nat                  | Diterpenoid        | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 114 | PT-414-SS | Nat                  | Flavonol glycodide | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 115 | PT-415-SS | Nat                  | Flavonol           | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 120 | PT-416-SS | Nat                  | Diterpenoid        | <i>Polyalthia sclerophylla</i>   | P                                  | LF+TW      | Anti-diabetic |
| 121 | PT-417-SS | Nat                  | Triterpenoid       | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Anti-diabetic |
| 122 | PT-418-SS | Nat                  | Lignan glycoside   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Anti-diabetic |
| 123 | PT-419-SS | Nat                  | Dipeptide          | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Anti-diabetic |
| 124 | PT-420-SS | Nat                  | Lignan glycoside   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Anti-diabetic |
| 125 | MUC-57    | Mod                  | Lignan glycoside   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic     |
| 126 | MUC-58    | Mod                  | Lignan glycoside   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic     |
| 127 | MUC-59    | Mod                  | Lignan glycoside   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic     |
| 128 | MUC-60    | Mod                  | Lignan glycoside   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic     |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay           |
|-----|---------|----------------------|------------------|-----------------------------------|------------------------------------|------------|-----------------|
| 129 | MUC-61  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 130 | MUC-62  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 131 | MUC-63  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 132 | MUC-65  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 133 | MUC-66  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 134 | MUC-67  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 135 | MUC-68  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 136 | MUC-69  | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 137 | MUC-222 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 138 | MUC-223 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 139 | MUC-225 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 140 | MUC-274 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 141 | MUC-275 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic       |
| 142 | MUC-967 | Nat                  | Flavanone        | <i>Gloniothalamus aurantiacus</i> | P                                  | LF+TW      | Neuroprotective |
| 143 | MUC-968 | Nat                  | Flavanone        | <i>Gloniothalamus aurantiacus</i> | P                                  | LF+TW      | Neuroprotective |
| 144 | MUC-969 | Nat                  | Flavone          | <i>Gardinia carinata</i>          | P                                  | LF+TW      | Neuroprotective |
| 145 | MUC-970 | Nat                  | Flavone          | <i>Gardinia carinata</i>          | P                                  | LF+TW      | Neuroprotective |
| 146 | MUC-971 | Nat                  | Flavonol         | <i>Zingiber maekongense</i>       | P                                  | RT         | Neuroprotective |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay           |
|-----|----------|----------------------|------------------|-----------------------------------|------------------------------------|------------|-----------------|
| 147 | MUC-972  | Nat                  | Flavonol         | <i>Zingiber maekongense</i>       | P                                  | RT         | Neuroprotective |
| 148 | MUC-973  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 149 | MUC-974  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 150 | MUC-975  | Nat                  | Flavanone        | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 151 | MUC-976  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 152 | MUC-977  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 153 | MUC-978  | Nat                  | Flavanone        | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 154 | MUC-979  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 155 | MUC-980  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 156 | MUC-981  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 157 | MUC-982  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 158 | MUC-983  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 159 | MUC-984  | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>      | P                                  | LF+TW      | Neuroprotective |
| 160 | MUC-1024 | Mod                  | Triterpenoid     | <i>Gardenia collinsae</i>         | P                                  | LF+TW      | Anti-diabetic   |
| 161 | MUC-1025 | Mod                  | Triterpenoid     | <i>Gardenia collinsae</i>         | P                                  | LF+TW      | Anti-diabetic   |
| 162 | MUC-1026 | Mod                  | Triterpenoid     | <i>Gardenia collinsae</i>         | P                                  | LF+TW      | Anti-diabetic   |
| 163 | MUC-1027 | Nat                  | Triterpenoid     | <i>Gardenia collinsae</i>         | P                                  | LF+TW      | Anti-diabetic   |
| 164 | MUC-1284 | Nat                  | Styryl lactone   | <i>Goniiothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic       |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|----------------------------------------|----------------------------------|---------------------------------------|------------|---------------|
| 165 | MUC-1285 | Nat                  | Styryl lactone                         | <i>Goniothalamus calvicarpus</i> | P                                     | ST         | Cytotoxic     |
| 166 | MUC-1286 | Nat                  | Flavanone                              | <i>Goniothalamus calvicarpus</i> | P                                     | ST         | Cytotoxic     |
| 167 | MUC-1405 | Nat                  | Triterpenoid                           | <i>Santisukia pagetii</i>        | P                                     | LF+TW      | Cytotoxic     |
| 168 | MUC-1406 | Nat                  | Triterpenoid                           | <i>Santisukia pagetii</i>        | P                                     | LF+TW      | Anti-diabetic |
| 169 | MUC-1407 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>        | P                                     | LF+TW      | Cytotoxic     |
| 170 | MUC-1208 | Nat                  | Iridoid glycosides                     | <i>Santisukia pagetii</i>        | P                                     | LF+TW      | Cytotoxic     |
| 171 | MUC-1020 | Mod                  | Triterpenoid                           | <i>Gardenia collinsae</i>        | P                                     | LF+TW      | Cytotoxic     |
| 172 | MUC-1021 | Mod                  | Triterpenoid                           | <i>Gardenia collinsae</i>        | P                                     | LF+TW      | Cytotoxic     |
| 173 | MUC-1022 | Mod                  | Triterpenoid                           | <i>Gardenia collinsae</i>        | P                                     | LF+TW      | Cytotoxic     |
| 174 | MUC-1023 | Nat                  | Triterpenoid                           | <i>Gardenia collinsae</i>        | P                                     | LF+TW      | Cytotoxic     |
| 175 | MUC-1028 | Mod                  | Lactone                                | <i>Polyalthia crassa</i>         | P                                     | LF+TW      | Cytotoxic     |
| 176 | MUC-1029 | Mod                  | Styryllactone                          | <i>Polyalthia crassa</i>         | P                                     | LF+TW      | Cytotoxic     |
| 177 | MUC-1030 | Mod                  | Styryllactone                          | <i>Polyalthia crassa</i>         | P                                     | LF+TW      | Cytotoxic     |
| 178 | MUC-1031 | Mod                  | Styryllactone                          | <i>Polyalthia crassa</i>         | P                                     | LF+TW      | Cytotoxic     |
| 179 | MUC-1032 | Mod                  | Styryllactone                          | <i>Polyalthia crassa</i>         | P                                     | LF+TW      | Cytotoxic     |
| 180 | MUC-1264 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i>     | P                                     | RZ         | Cytotoxic     |
| 181 | MUC-1265 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i>     | P                                     | RZ         | Cytotoxic     |
| 182 | MUC-1277 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i>     | P                                     | RZ         | Anti-diabetic |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                    | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|----------------------------------------|---------------------------------|------------------------------------|------------|---------------|
| 183 | MUC-1278 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i>    | P                                  | RZ         | Anti-diabetic |
| 184 | MUC-1279 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i>    | P                                  | RZ         | Anti-diabetic |
| 185 | MUC-1280 | Nat                  | Lignan                                 | <i>Boesenbergia thorelii</i>    | P                                  | RZ         | Anti-diabetic |
| 186 | MUC-1281 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>    | P                                  | RZ         | Anti-diabetic |
| 187 | MUC-1282 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>    | P                                  | RZ         | Anti-diabetic |
| 188 | MUC-1283 | Nat                  | Benzoic acid derivative                | <i>Boesenbergia thorelii</i>    | P                                  | RZ         | Anti-diabetic |
| 189 | MUC-1284 | Nat                  | Styryllactone                          | <i>Goniotalamus calvicarpus</i> | P                                  | ST         | Cytotoxic     |
| 190 | MUC-1285 | Nat                  | Styryllactone                          | <i>Goniotalamus calvicarpus</i> | P                                  | ST         | Cytotoxic     |
| 191 | MUC-1286 | Nat                  | Flavanone                              | <i>Goniotalamus calvicarpus</i> | P                                  | ST         | Cytotoxic     |
| 192 | MUC-1287 | Nat                  | Styryllactone                          | <i>Goniotalamus calvicarpus</i> | P                                  | ST         | Cytotoxic     |
| 193 | MUC-1303 | Nat                  | Triterpenoid                           | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 194 | MUC-1304 | Nat                  | Triterpenoid                           | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 195 | MUC-1305 | Nat                  | Diterpenoid                            | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 196 | MUC-1306 | Nat                  | Flavanone                              | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 197 | MUC-1307 | Nat                  | Diterpenoid                            | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 198 | MUC-1308 | Nat                  | Flavanone                              | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 199 | MUC-1309 | Nat                  | Flavanone                              | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |
| 200 | MUC-1310 | Nat                  | Aromatic compound                      | <i>Mallotus glomerulatus</i>    | P                                  | PX         | Anti-diabetic |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|------------------|----------------------------------|------------------------------------|------------|---------------|
| 201 | MUC-1311 | Nat                  | Diterpenoid      | <i>Mallotus glomerulatus</i>     | P                                  | PX         | Anti-diabetic |
| 202 | MUC-1312 | Nat                  | Lignan glycoside | <i>Mallotus glomerulatus</i>     | P                                  | PX         | Anti-diabetic |
| 203 | MUC-1313 | Nat                  | Flavone          | <i>Mallotus glomerulatus</i>     | P                                  | PX         | Anti-diabetic |
| 204 | MUC-1314 | Nat                  | Xanthone         | <i>Mallotus glomerulatus</i>     | P                                  | PX         | Anti-diabetic |
| 205 | MUC-1323 | Nat                  | Styryllactone    | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 206 | MUC-1324 | Nat                  | Styryllactone    | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 207 | MUC-1325 | Nat                  | Styryllactone    | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 208 | MUC-1326 | Nat                  | Styryllactone    | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 209 | MUC-1327 | Nat                  | Flavanone        | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 210 | MUC-1328 | Nat                  | Styryllactone    | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 211 | MUC-1329 | Nat                  | alkaloid         | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Anti-diabetic |
| 212 | MUC-1330 | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |
| 213 | MUC-1331 | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |
| 214 | MUC-1332 | Nat                  | Flavanone        | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |
| 215 | MUC-1333 | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |
| 216 | MUC-1334 | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |
| 217 | MUC-1335 | Nat                  | Flavanone        | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |
| 218 | MUC-1336 | Nat                  | Flavone          | <i>Gardenia sessiliflora</i>     | P                                  | LF+TW      | Cytotoxic     |

| No  | CODE     | Nat/Mod/Syn<br>compound | Type of Compound | Plant Source                 | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|----------|-------------------------|------------------|------------------------------|---------------------------------------|------------|-----------|
| 219 | MUC-1337 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 220 | MUC-1338 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 221 | MUC-1339 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 222 | MUC-1340 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 223 | MUC-1341 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 224 | MUC-1342 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 225 | MUC-1343 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 226 | MUC-1344 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 227 | MUC-1345 | Nat                     | Flavanone        | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 228 | MUC-1346 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 229 | MUC-1347 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 230 | MUC-1348 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 231 | MUC-1349 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 232 | MUC-1350 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 233 | MUC-1351 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 234 | MUC-1352 | Nat                     | Flavone          | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 235 | MUC-1353 | Nat                     | Triterpenoid     | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |
| 236 | MUC-1354 | Nat                     | Flavanone        | <i>Gardenia sessiliflora</i> | P                                     | LF+TW      | Cytotoxic |



| No  | CODE     | Nat/Mod/Syn compound | Type of Compound  | Plant Source                 | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|-------------------|------------------------------|------------------------------------|------------|---------------|
| 237 | MUC-1409 | Nat                  | Aromatic Aldehyde | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 238 | MUC-1411 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 239 | MUC-1412 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 240 | MUC-1413 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 241 | MUC-1414 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 242 | MUC-1415 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 243 | MUC-1416 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 244 | MUC-1417 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Cytotoxic     |
| 245 | MUC-1418 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 246 | MUC-1419 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 247 | MUC-1420 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 248 | MUC-1421 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 249 | MUC-1422 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 250 | MUC-1423 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 251 | MUC-1424 | Syn                  | Tertiary Amine    | -                            | P                                  | -          | Anti-diabetic |
| 252 | MUC-1491 | Nat                  | Triterpenoid      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Cytotoxic     |
| 253 | MUC-1492 | Nat                  | Triterpenoid      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Cytotoxic     |
| 254 | MUC-1493 | Nat                  | iridoid glycoside | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Cytotoxic     |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|----------------------------------------|-----------------------------------|------------------------------------|------------|---------------|
| 255 | MUC-1633 | Syn                  | Sulfide                                | -                                 | P                                  | -          | Cytotoxic     |
| 256 | MUC-1634 | Syn                  | Sulfide                                | -                                 | P                                  | -          | Cytotoxic     |
| 257 | MUC-1635 | Syn                  | Sulfide                                | -                                 | P                                  | -          | Cytotoxic     |
| 258 | MUC-1644 | Nat                  | Styryllactone                          | <i>Goniiothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic     |
| 259 | MUC-1653 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>      | P                                  | RZ         | Cytotoxic     |
| 260 | MUC-1654 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>      | P                                  | RZ         | Cytotoxic     |
| 261 | MUC-1655 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>      | P                                  | RZ         | Cytotoxic     |
| 262 | MUC-1656 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>      | P                                  | RZ         | Cytotoxic     |
| 263 | MUC-1674 | Nat                  | Triterpenoid                           | <i>Santisukia pagetii</i>         | p                                  | LF+TW      | Anti-diabetic |
| 264 | MUC-1675 | Nat                  | Triterpenoid                           | <i>Santisukia pagetii</i>         | p                                  | LF+TW      | Anti-diabetic |
| 265 | MUC-1676 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 266 | MUC-1677 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 267 | MUC-1678 | Nat                  | Monoterpenoid                          | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 268 | MUC-1679 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 269 | MUC-1680 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 270 | MUC-1681 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 271 | MUC-1682 | Nat                  | Flavonoid glycoside                    | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |
| 272 | MUC-1683 | Nat                  | Flavonoid glycoside                    | <i>Santisukia pagetii</i>         | P                                  | LF+TW      | Anti-diabetic |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|------------------|----------------------------------|------------------------------------|------------|---------------|
| 273 | MUC-1684 | Nat                  | Triterpenoid     | <i>Santisukia pagetii</i>        | P                                  | LF+TW      | Anti-diabetic |
| 274 | MUC-1685 | Nat                  | Triterpenoid     | <i>Santisukia pagetii</i>        | P                                  | LF+TW      | Anti-diabetic |
| 275 | MUC-1686 | Nat                  | Styryllactone    | <i>Goniothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic     |
| 276 | MUC-1694 | Nat                  | Styryllactone    | <i>Goniothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic     |
| 277 | MUC-1619 | Nat                  | -                | <i>Paris polyphylla</i>          | E                                  | RZ         | Cytotoxic     |
| 278 | MUC-1620 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Cytotoxic     |
| 279 | MUC-1621 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Cytotoxic     |
| 280 | MUC-1622 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Cytotoxic     |
| 281 | MUC-1623 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Cytotoxic     |
| 282 | MUC-1624 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Cytotoxic     |
| 283 | MUC-1625 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Cytotoxic     |
| 284 | MUC-1626 | Nat                  | -                | <i>Paris polyphylla</i>          | E                                  | RZ         | Anti-diabetic |
| 285 | MUC-1627 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Anti-diabetic |
| 286 | MUC-1628 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Anti-diabetic |
| 287 | MUC-1629 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Anti-diabetic |
| 288 | MUC-1630 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Anti-diabetic |
| 289 | MUC-1631 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Anti-diabetic |
| 290 | MUC-1632 | Nat                  | -                | <i>Paris polyphylla</i>          | F                                  | RZ         | Anti-diabetic |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound | Plant Source            | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|------------------|-------------------------|---------------------------------------|------------|---------------|
| 291 | MUC-1695 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 292 | MUC-1696 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 293 | MUC-1697 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 294 | MUC-1698 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 295 | MUC-1699 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 296 | MUC-1700 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 297 | MUC-1701 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 298 | MUC-1702 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 299 | MUC-1703 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Cytotoxic     |
| 300 | MUC-1704 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 301 | MUC-1705 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 302 | MUC-1706 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 303 | MUC-1707 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 304 | MUC-1708 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 305 | MUC-1709 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 306 | MUC-1710 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 307 | MUC-1711 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |
| 308 | MUC-1712 | Nat                  | -                | <i>Paris polyphylla</i> | F                                     | RZ         | Anti-diabetic |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                 | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                             |
|-----|----------|----------------------|----------------------------------------|------------------------------|------------------------------------|------------|-----------------------------------|
| 309 | MUC-1713 | Nat                  | Flavone                                | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 310 | MUC-1714 | Nat                  | Flavone                                | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 311 | MUC-1715 | Nat                  | Flavone                                | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 312 | MUC-1716 | Nat                  | Flavanone                              | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 313 | MUC-1717 | Nat                  | Flavone                                | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 314 | MUC-1718 | Nat                  | Flavanone                              | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 315 | MUC-1719 | Nat                  | Triterpenoid                           | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 316 | MUC-1720 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 317 | MUC-1721 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 318 | MUC-1722 | Nat                  | Monoterpenoid                          | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 319 | MUC-1723 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 320 | MUC-1724 | Nat                  | Flavonoid glycoside                    | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 321 | MUC-1725 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 322 | MUC-1726 | Nat                  | C <sub>6</sub> C <sub>2</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 323 | MUC-1727 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 324 | MUC-1728 | Nat                  | Aromatic aldehyde                      | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for              |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                 | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                             |
|-----|----------|----------------------|----------------------------------------|------------------------------|------------------------------------|------------|-----------------------------------|
|     |          |                      |                                        |                              |                                    |            | chemotherapy                      |
| 325 | MUC-1729 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 326 | MUC-1730 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 327 | MUC-1731 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 328 | MUC-1732 | Nat                  | lignan                                 | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Drug transporter for chemotherapy |
| 329 | MUC-1733 | Nat                  | Flavone                                | <i>Gardenia collinsae</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 330 | MUC-1734 | Nat                  | Flavone                                | <i>Gardenia thailandica</i>  | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 331 | MUC-1735 | Nat                  | Flavone                                | <i>Gardenia thailandica</i>  | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 332 | MUC-1736 | Nat                  | Triterpenoid                           | <i>Gardenia thailandica</i>  | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 333 | MUC-1737 | Nat                  | Triterpenoid                           | <i>Gardenia thailandica</i>  | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 334 | MUC-1737 | Nat                  | Flavone                                | <i>Gardenia collinsae</i>    | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 335 | MUC-1776 | Nat                  | Triterpenoid                           | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 336 | MUC-1777 | Nat                  | Triterpenoid                           | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 337 | MUC-1778 | Nat                  | Triterpenoid                           | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 338 | MUC-1779 | Nat                  | Triterpenoid                           | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |
| 339 | MUC-1780 | Nat                  | Triterpenoid                           | <i>Gardenia sessiliflora</i> | P                                  | LF+TW      | Drug transporter for chemotherapy |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                                   |
|-----|-------------|----------------------|------------------|----------------------------------|------------------------------------|------------|-----------------------------------------|
| 340 | MUC-1672    | Nat                  | Triterpenoid     | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Proliferation of cancer cells           |
| 341 | MUC-1672    | Nat                  | Triterpenoid     | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Proliferation of cancer cells           |
| 340 | MUC-1807    | Nat                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Proliferation of cancer cells           |
| 341 | MUC-1808    | Nat                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Proliferation of cancer cells           |
| 342 | MUC-535 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 343 | MUC-535 (2) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 344 | MUC-535 (3) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 345 | MUC-536 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 346 | MUC-536 (2) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 347 | MUC-536 (3) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 348 | MUC-536 (4) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 349 | MUC-536 (5) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 350 | MUC-537 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 351 | MUC-537 (2) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 352 | MUC-537 (3) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 353 | MUC-537 (4) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Mechanistic study of                    |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound     | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                                   |
|-----|-------------|----------------------|----------------------|-----------------------------------|------------------------------------|------------|-----------------------------------------|
|     |             |                      |                      |                                   |                                    |            | anti-cancer effect                      |
| 354 | MUC-538 (1) | Mod                  | Lignan glycoside     | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 355 | MUC-538 (2) | Mod                  | Lignan glycoside     | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Mechanistic study of anti-cancer effect |
| 356 | MUC-1503    | Mod                  | Flavanone derivative | <i>Boesenbergia rotunda</i>       | P                                  | RZ         | Anti-diabetic                           |
| 357 | MUC-1504    | Mod                  | Flavanone derivative | <i>Boesenbergia rotunda</i>       | P                                  | RZ         | Anti-diabetic                           |
| 358 | MUC-1505    | Mod                  | Flavanone derivative | <i>Boesenbergia rotunda</i>       | P                                  | RZ         | Anti-diabetic                           |
| 359 | MUC-1506    | Mod                  | Flavanone derivative | <i>Boesenbergia rotunda</i>       | P                                  | RZ         | Anti-diabetic                           |
| 360 | MUC-1507    | Nat                  | Flavanone derivative | <i>Boesenbergia rotunda</i>       | P                                  | RZ         | Anti-diabetic                           |
| 361 | MUC-1669    | Nat                  | Styryllactone        | <i>Goniiothalamus aurantiacus</i> | P                                  | ST         | Cytotoxic                               |
| 362 | MUC-1670    | Nat                  | Styryllactone        | <i>Goniiothalamus aurantiacus</i> | P                                  | ST         | Cytotoxic                               |
| 363 | MUC-1671    | Nat                  | Styryllactone        | <i>Goniiothalamus aurantiacus</i> | P                                  | ST         | Cytotoxic                               |
| 364 | MUC-1672    | Nat                  | triterpenoid         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Proliferation of cancer cells           |
| 365 | MUC-1673    | Nat                  | triterpenoid         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Proliferation of cancer cells           |
| 366 | MUC-1809    | Nat                  | Alkaloid             | <i>Goniiothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 367 | MUC-1810    | Nat                  | Styryllactone        | <i>Goniiothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 368 | MUC-1811    | Nat                  | Alkaloid             | <i>Goniiothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 369 | MUC-1812    | Nat                  | Alkaloid             | <i>Goniiothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |



| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                                   |
|-----|----------|----------------------|----------------------------------------|----------------------------------|------------------------------------|------------|-----------------------------------------|
| 370 | MUC-1813 | Nat                  | Lactone                                | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 371 | MUC-1814 | Nat                  | Alkaloid                               | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 372 | MUC-1815 | Nat                  | Conjugated amide                       | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 373 | MUC-1816 | Nat                  | Styryllactone                          | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 374 | MUC-1817 | Nat                  | Conjugated ester                       | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 375 | MUC-1818 | Nat                  | Styryllactone                          | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 376 | MUC-1819 | Nat                  | Styryllactone                          | <i>Goniothalamus calvicarpus</i> | P                                  | ST         | Cytotoxic                               |
| 377 | MUC-1832 | Nat                  | -                                      | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 378 | MUC-1833 | Nat                  | -                                      | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 379 | MUC-1834 | Nat                  | -                                      | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 380 | MUC-1835 | Nat                  | -                                      | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 381 | MUC-1836 | Nat                  | -                                      | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 382 | MUC-1837 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>     | P                                  | RZ         | Cytotoxic                               |
| 383 | MUC-1838 | Nat                  | Lignan                                 | <i>Boesenbergia thorelii</i>     | P                                  | RZ         | Cytotoxic                               |
| 384 | MUC-1839 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>     | P                                  | RZ         | Cytotoxic                               |
| 385 | MUC-1840 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i>     | P                                  | RZ         | Cytotoxic                               |
| 386 | MUC-1841 | Nat                  | Benzoic acid derivative                | <i>Boesenbergia thorelii</i>     | P                                  | RZ         | Cytotoxic                               |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                 | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|----------|----------------------|----------------------------------------|------------------------------|------------------------------------|------------|---------------|
| 387 | MUC-1842 | Nat                  | Lignan                                 | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Cytotoxic     |
| 388 | MUC-1843 | Nat                  | Benzaldehyde derivative                | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Cytotoxic     |
| 389 | MUC-1844 | Nat                  | Benzene derivative                     | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Cytotoxic     |
| 390 | MUC-1845 | Nat                  | Benzoic acid derivative                | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Cytotoxic     |
| 391 | MUC-1864 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Cytotoxic     |
| 392 | MUC-1865 | Nat                  | Lignan                                 | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 393 | MUC-1866 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 394 | MUC-1867 | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 395 | MUC-1868 | Nat                  | Benzoic acid derivative                | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 396 | MUC-1869 | Nat                  | Lignan                                 | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 397 | MUC-1870 | Nat                  | Benzaldehyde derivative                | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 398 | MUC-1871 | Nat                  | Benzene derivative                     | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 399 | MUC-1872 | Nat                  | Benzaldehyde derivative                | <i>Boesenbergia thorelii</i> | P                                  | RZ         | Anti-diabetic |
| 400 | MUC-1873 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Anti-oxidant  |
| 401 | MUC-1874 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Anti-oxidant  |
| 402 | MUC-1875 | Nat                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Anti-oxidant  |
| 403 | MUC-1876 | Mod                  | Iridoid glycoside                      | <i>Santisukia pagetii</i>    | P                                  | LF+TW      | Anti-oxidant  |
| 404 | MUC-1877 | Nat                  | -                                      | <i>Garcinia succifolia</i>   | E                                  | ST         | Cytotoxic     |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound    | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                                   |
|-----|----------|----------------------|---------------------|----------------------------------|------------------------------------|------------|-----------------------------------------|
| 405 | MUC-1878 | Nat                  | -                   | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic                               |
| 406 | MUC-1879 | Nat                  | -                   | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic                               |
| 407 | MUC-1881 | Mod                  | Iridoid glycoside   | <i>Santisukia pagetii</i>        | P                                  | LF+TW      | Anti-oxidant                            |
| 408 | MUC-1882 | Mod                  | Iridoid glycoside   | <i>Santisukia pagetii</i>        | P                                  | LF+TW      | Anti-oxidant                            |
| 409 | MUC-1883 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 410 | MUC-1884 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 411 | MUC-1185 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 412 | MUC-1186 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 413 | MUC-1187 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 414 | MUC-1189 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 415 | MUC-1890 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 416 | MUC-1891 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 417 | MUC-1892 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 418 | MUC-1893 | Nat                  | -                   | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 419 | MUC-1894 | Mod                  | Chalcone derivative | <i>Boesenbergia rotunda</i>      | P                                  | RZ         | Anti-diabetic                           |
| 420 | MUC-1895 | Mod                  | Chalcone derivative | <i>Boesenbergia rotunda</i>      | P                                  | RZ         | Anti-diabetic                           |

| No  | CODE     | Nat/Mod/Syn compound | Type of Compound     | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay                                   |
|-----|----------|----------------------|----------------------|----------------------------------|------------------------------------|------------|-----------------------------------------|
| 421 | MUC-1896 | Mod                  | Chalcone derivative  | <i>Boesenbergia rotunda</i>      | P                                  | RZ         | Anti-diabetic                           |
| 422 | MUC-1897 | Nat                  | Triterpenoid         | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 423 | MUC-1898 | Nat                  | Triterpenoid         | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 424 | MUC-1899 | Nat                  | Xanthone             | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 425 | MUC-1900 | Nat                  | Xanthone             | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 426 | MUC-1901 | Nat                  | Xanthone             | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 427 | MUC-1912 | Nat                  | -                    | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 428 | MUC-1913 | Nat                  | -                    | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 429 | MUC-1914 | Nat                  | -                    | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 430 | MUC-1916 | Nat                  | -                    | <i>Phyllanthus taxodiifolius</i> | F                                  | PX         | Mechanistic study of anti-cancer effect |
| 431 | MUC-1917 | Nat                  | -                    | <i>Clitoria tematea</i>          | E                                  | FL         | Anti-diabetic                           |
| 432 | MUC-1918 | Nat                  | Xanthone             | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 433 | MUC-1919 | Nat                  | Xanthone             | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 434 | MUC-1920 | Nat                  | Xanthone             | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 435 | MUC-1921 | Nat                  | Benzoic derivative   | <i>Garcinia succifolia</i>       | P                                  | ST         | Cytotoxic                               |
| 436 | MUC -102 | Nat                  | Sesquiterpenoid      | <i>Goniothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic                               |
| 437 | MUC-105  | Nat                  | Flavanone derivative | <i>Goniothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic                               |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound        | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|---------|----------------------|-------------------------|----------------------------------|---------------------------------------|------------|-----------|
| 438 | MUC-108 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 439 | MUC-111 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 440 | MUC-114 | Nat                  | Conjugated amide        | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 441 | MUC-117 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 442 | MUC-121 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 443 | MUC-124 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 444 | MUC-127 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 445 | MUC-130 | Nat                  | Styryllactone           | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 446 | MUC-133 | Nat                  | Triterpenoid            | <i>Gardenia carinata</i>         | P                                     | LF+TW      | Cytotoxic |
| 447 | MUC-136 | Nat                  | Sugar                   | <i>Gardenia carinata</i>         | P                                     | LF+TW      | Cytotoxic |
| 448 | MUC-202 | Mod                  | Lignan glycosides       | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic |
| 449 | MUC-203 | Mod                  | Lignan glycosides       | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic |
| 450 | MUC-207 | Nat                  | Xanthon                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 451 | MUC-210 | Nat                  | Xanthon                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 452 | MUC-213 | Nat                  | Triterpenoid            | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 453 | MUC-216 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 454 | MUC-219 | Nat                  | Benzophenone derivative | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 455 | MUC-256 | Nat                  | -                       | <i>Mallotus glomerulatus</i>     | E                                     | LF+TW      | Cytotoxic |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound         | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|---------|----------------------|--------------------------|-----------------------------------|------------------------------------|------------|-----------|
| 456 | MUC-257 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 457 | MUC-258 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 458 | MUC-259 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 459 | MUC-260 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 460 | MUC-261 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 461 | MUC-262 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 462 | MUC-263 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 463 | MUC-265 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 464 | MUC-266 | Nat                  | -                        | <i>Mallotus glomerulatus</i>      | F                                  | LF+TW      | Cytotoxic |
| 465 | MUC-268 | Mod                  | Styryllatone derivative  | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic |
| 466 | MUC-269 | Mod                  | Styryllatone derivative  | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic |
| 467 | MUC-270 | Mod                  | Styryllatones derivative | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic |
| 468 | MUC-276 | Mod                  | Styryllatones derivative | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic |
| 469 | MUC-277 | Nat                  | -                        | <i>Cleistanthus myrianthus</i>    | E                                  | LF+TW      | Cytotoxic |
| 470 | MUC-278 | Nat                  | -                        | <i>Cleistanthus myrianthus</i>    | F                                  | LF+TW      | Cytotoxic |
| 471 | MUC-279 | Nat                  | -                        | <i>Cleistanthus myrianthus</i>    | F                                  | LF+TW      | Cytotoxic |
| 472 | MUC-280 | Nat                  | -                        | <i>Cleistanthus myrianthus</i>    | F                                  | LF+TW      | Cytotoxic |
| 473 | MUC-281 | Nat                  | -                        | <i>Cleistanthus myrianthus</i>    | F                                  | LF+TW      | Cytotoxic |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound | Plant Source                   | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|---------|----------------------|------------------|--------------------------------|---------------------------------------|------------|-----------|
| 474 | MUC-282 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 475 | MUC-283 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 476 | MUC-284 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 477 | MUC-285 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 478 | MUC-286 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 479 | MUC-287 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 480 | MUC-288 | Nat                  | -                | <i>Cleistanthus myrianthus</i> | F                                     | LF+TW      | Cytotoxic |
| 481 | MUC-313 | Nat                  | -                | <i>Cleistanthus hirsutulus</i> | E                                     | LF+TW      | Cytotoxic |
| 482 | MUC-314 | Nat                  | -                | <i>Cleistanthus denudatus</i>  | F                                     | LF+TW      | Cytotoxic |
| 483 | MUC-315 | Nat                  | -                | <i>Cleistanthus denudatus</i>  | F                                     | LF+TW      | Cytotoxic |
| 484 | MUC-316 | Nat                  | -                | <i>Cleistanthus denudatus</i>  | F                                     | LF+TW      | Cytotoxic |
| 485 | MUC-325 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |
| 486 | MUC-326 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |
| 487 | MUC-327 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |
| 488 | MUC-328 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |
| 489 | MUC-329 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |
| 490 | MUC-330 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |
| 491 | MUC-331 | Nat                  | -                | <i>Bauhinia ordata</i>         | F                                     | PX         | Cytotoxic |

| No  | CODE           | Nat/Mod/Syn compound | Type of Compound | Plant Source                         | Extract (E)/ Fraction (F)/Pure (P) | Plant part   | Assay            |
|-----|----------------|----------------------|------------------|--------------------------------------|------------------------------------|--------------|------------------|
| 492 | MUC-332        | Nat                  | -                | <i>Bauhinia ordata</i>               | F                                  | PX           | Cytotoxic        |
| 493 | MUC-349        | Nat                  | Flavanone        | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 494 | MUC-350        | Nat                  | Styryllatone     | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 495 | MUC-351        | Nat                  | Styryllatone     | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 496 | MUC-352        | Nat                  | Styryllatones    | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 497 | MUC-353        | Nat                  | Styryllatones    | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 498 | MUC-354        | Nat                  | Styryllatone     | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 499 | MUC-355        | Nat                  | Triterpenoid     | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 500 | MUC-356        | Nat                  | Triterpenoid     | <i>Goniiothalamus calvicapus</i>     | P                                  | LF+TW        | Cytotoxic        |
| 501 | MUC-373        | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 502 | MUC-374        | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 503 | MUC375         | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 504 | MUC-376        | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 505 | MUC-377        | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 506 | <u>MUC-378</u> | <u>Nat</u>           | <u>-</u>         | <u><i>Cleistanthus denudatus</i></u> | <u>E</u>                           | <u>LF+TW</u> | <u>Cytotoxic</u> |
| 507 | MUC-379        | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 508 | MUC-380        | Nat                  | -                | <i>Cleistanthus denudatus</i>        | F                                  | LF+TW        | Cytotoxic        |
| 509 | MUC-397        | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i>     | P                                  | PX           | Cytotoxic        |



| No  | CODE        | Nat/Mod/Syn compound | Type of Compound         | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|-------------|----------------------|--------------------------|-----------------------------------|------------------------------------|------------|-----------|
| 510 | MUC-398 (1) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 511 | MUC-398 (2) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 512 | MUC-398 (3) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 513 | MUC-398 (4) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 514 | MUC-398 (5) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 515 | MUC-399 (1) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 516 | MUC-399 (2) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 517 | MUC-399 (3) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 518 | MUC-399 (4) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 519 | MUC-399 (5) | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i>  | P                                  | PX         | Cytotoxic |
| 520 | MUC-400     | Mod                  | Styryllactone derivative | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic |
| 521 | MUC-404     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |
| 522 | MUC-405     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |
| 523 | MUC-406     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |
| 524 | MUC-407     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |
| 525 | MUC-408     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |
| 526 | MUC-409     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |
| 527 | MUC-410     | Nat                  | -                        | <i>Cleistanthus hirsutulus</i>    | F                                  | LF+TW      | Cytotoxic |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|-------------|----------------------|------------------|----------------------------------|------------------------------------|------------|-----------|
| 528 | MUC-411     | Nat                  | -                | <i>Cleistanthus hirsutulus</i>   | F                                  | LF+TW      | Cytotoxic |
| 529 | MUC-412     | Nat                  | -                | <i>Cleistanthus hirsutulus</i>   | F                                  | LF+TW      | Cytotoxic |
| 530 | MUC-413     | Nat                  | -                | <i>Cleistanthus hirsutulus</i>   | F                                  | LF+TW      | Cytotoxic |
| 531 | MUC-434     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | E                                  | LF+TW      | Cytotoxic |
| 532 | MUC-435     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 533 | MUC-436     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 534 | MUC-437     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 535 | MUC-438     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 536 | MUC-439     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 537 | MUC-440     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 538 | MUC-441     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 539 | MUC-442     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 540 | MUC-443     | Nat                  | -                | <i>Gardenia sessiliflora</i>     | F                                  | LF+TW      | Cytotoxic |
| 541 | MUC-496 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 542 | MUC-496 (2) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 543 | MUC-496 (3) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 544 | MUC-507     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | E                                  | LF         | Cytotoxic |
| 545 | MUC-508     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|-------------|----------------------|------------------|----------------------------------|------------------------------------|------------|-----------|
| 546 | MUC-509     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 547 | MUC-510     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 548 | MUC-511     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 549 | MUC-512     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 550 | MUC-513     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 551 | MUC-514     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 552 | MUC-515     | Nat                  | -                | <i>Bauhinia harmsiana</i>        | F                                  | LF         | Cytotoxic |
| 553 | MUC-539     | Nat                  | Triterpenoid     | <i>Mallotus glomerulatus</i>     | P                                  | LF+TW      | Cytotoxic |
| 554 | MUC-540     | Nat                  | Flavone          | <i>Mallotus glomerulatus</i>     | P                                  | LF+TW      | Cytotoxic |
| 555 | MUC-541     | Nat                  | Xanthone         | <i>Mallotus glomerulatus</i>     | P                                  | LF+TW      | Cytotoxic |
| 556 | MUC-542     | Nat                  | Flavanone        | <i>Mallotus glomerulatus</i>     | P                                  | LF+TW      | Cytotoxic |
| 557 | MUC-543     | Nat                  | Chalcone         | <i>Mallotus glomerulatus</i>     | P                                  | LF+TW      | Cytotoxic |
| 558 | MUC-544     | Nat                  | Lignan           | <i>Polyaltha glauca</i>          | P                                  | LF+TW      | Cytotoxic |
| 559 | MUC-545     | Nat                  | Lignan           | <i>Polyaltha glauca</i>          | P                                  | LF+TW      | Cytotoxic |
| 560 | MUC-546     | Mod                  | Lignan           | <i>Polyaltha glauca</i>          | F                                  | LF+TW      | Cytotoxic |
| 561 | MUC-568 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 562 | MUC-568 (2) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 563 | MUC-569 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound         | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|-------------|----------------------|--------------------------|----------------------------------|------------------------------------|------------|-----------|
| 564 | MUC-569 (2) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 565 | MUC-570 (1) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 566 | MUC-570 (2) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 567 | MUC-571 (1) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 568 | MUC-571 (2) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 569 | MUC-572 (1) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 570 | MUC-572 (2) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 571 | MUC-573 (1) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 572 | MUC-573 (2) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 573 | MUC-584     | Mod                  | Styryllactone derivqtive | <i>Gardenia carinata</i>         | P                                  | LF+TW      | Cytotoxic |
| 574 | MUC-585 (1) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 575 | MUC-585 (2) | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 576 | MUC-586     | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 577 | MUC-587     | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 578 | MUC-599     | Nat                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 579 | MUC-600     | Mod                  | Lignan                   | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 580 | MUC-601     | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |
| 581 | MUC-602     | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay         |
|-----|-------------|----------------------|------------------|----------------------------------|------------------------------------|------------|---------------|
| 582 | MUC-603 (1) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Cytotoxic     |
| 583 | MUC-603 (2) | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                  | PX         | Drug delivery |
| 584 | MUC-604     | Nat                  | -                | <i>Garcinia succifolia</i>       | E                                  | ST         | Cytotoxic     |
| 585 | MUC-605     | Nat                  | -                | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic     |
| 586 | MUC-606     | Nat                  | -                | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic     |
| 587 | MUC-607     | Nat                  | -                | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic     |
| 588 | MUC-608     | Nat                  | -                | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic     |
| 589 | MUC-609     | Nat                  | -                | <i>Garcinia succifolia</i>       | F                                  | ST         | Cytotoxic     |
| 590 | MUC-625     | Nat                  | -                | <i>Nauclea orientalis</i>        | E                                  | LF+TW      | Anti-diabetic |
| 591 | MUC-626     | Nat                  | -                | <i>Nauclea orientalis</i>        | E                                  | LF+TW      | Anti-diabetic |
| 592 | MUC-627     | Nat                  | -                | <i>Nauclea orientalis</i>        | E                                  | LF+TW      | Anti-diabetic |
| 593 | MUC-651     | Nat                  | Styryllactone    | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |
| 594 | MUC-652     | Nat                  | Alkaloid         | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |
| 595 | MUC-653     | Nat                  | Alkaloid         | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |
| 596 | MUC-654     | Nat                  | Conjugated amide | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |
| 597 | MUC-655     | Nat                  | Flavanone        | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |
| 598 | MUC-656     | Nat                  | Styryllactone    | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |
| 599 | MUC-657     | Nat                  | Styryllactone    | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic     |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound        | Plant Source                     | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|---------|----------------------|-------------------------|----------------------------------|------------------------------------|------------|-----------|
| 600 | MUC-658 | Nat                  | Styryllactone           | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 601 | MUC-659 | Nat                  | Styryllactone           | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 602 | MUC-660 | Nat                  | Styryllactone           | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 603 | MUC-661 | Nat                  | Triterpenoid            | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 604 | MUC-662 | Nat                  | Triterpenoid            | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 605 | MUC-663 | Nat                  | Styryllactone           | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 606 | MUC-664 | Nat                  | Alkaloid                | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 607 | MUC-665 | Nat                  | Alkaloid                | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 608 | MUC-666 | Nat                  | Conjugated amide        | <i>Goniiothalamus calvicapus</i> | P                                  | LF+TW      | Cytotoxic |
| 609 | MUC-667 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 610 | MUC-668 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 611 | MUC-669 | Nat                  | Triterpenoid            | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 612 | MUC-670 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 613 | MUC-671 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 614 | MUC-672 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 615 | MUC-673 | Nat                  | Benzophenone derivative | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 616 | MUC-674 | Nat                  | triterpenoid            | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |
| 617 | MUC-675 | Nat                  | Xanthone                | <i>Garcinia succifolia</i>       | P                                  | LF+TW      | Cytotoxic |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound         | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|---------|----------------------|--------------------------|----------------------------------|---------------------------------------|------------|-----------|
| 618 | MUC-676 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 619 | MUC-677 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 620 | MUC-678 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 621 | MUC-679 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 622 | MUC-680 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 623 | MUC-681 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 624 | MUC-682 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 625 | MUC-683 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>       | P                                     | LF+TW      | Cytotoxic |
| 626 | MUC-701 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | E                                     | LF+TW      | Cytotoxic |
| 627 | MUC-702 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | F                                     | LF+TW      | Cytotoxic |
| 628 | MUC-703 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | F                                     | LF+TW      | Cytotoxic |
| 629 | MUC-704 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | F                                     | LF+TW      | Cytotoxic |
| 630 | MUC-705 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | F                                     | LF+TW      | Cytotoxic |
| 631 | MUC-706 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | F                                     | LF+TW      | Cytotoxic |
| 632 | MUC-707 | Nat                  | -                        | <i>Memecylon geddesianum</i>     | F                                     | LF+TW      | Cytotoxic |
| 633 | MUC-722 | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic |
| 634 | MUC-723 | Mod                  | Lignan glycoside         | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic |
| 635 | MUC-724 | Mod                  | Styryllactone derivative | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |

| No  | CODE    | Nat/Mod/Syn compound | Type of Compound         | Plant Source                      | Extract (E)/ Fraction (F)/Pure (P) | Plant part | Assay             |
|-----|---------|----------------------|--------------------------|-----------------------------------|------------------------------------|------------|-------------------|
| 636 | MUC-725 | Mod                  | Styryllactone derivative | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic         |
| 637 | MUC-726 | Mod                  | Styryllactone derivative | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic         |
| 638 | MUC-727 | Mod                  | Styryllactone derivative | <i>Goniiothalamus aurantiacus</i> | P                                  | LF+TW      | Cytotoxic         |
| 639 | MUC-732 | Nat                  | Flavonol                 | <i>Zingiber maekongense</i>       | P                                  | RT         | Anti-oxidant      |
| 640 | MUC-759 | Nat                  | Styryllactone            | <i>Polyalthia crassa</i>          | P                                  | LF+TW      | Anti-inflammatory |
| 641 | MUC-760 | Nat                  | Styryllactone            | <i>Polyalthia crassa</i>          | P                                  | LF+TW      | Anti-inflammatory |
| 642 | MUC-761 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>        | P                                  | LF+TW      | Anti-inflammatory |
| 643 | MUC-762 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>        | P                                  | LF+TW      | Anti-inflammatory |
| 644 | MUC-763 | Nat                  | Xanthone                 | <i>Garcinia succifolia</i>        | P                                  | LF+TW      | Anti-inflammatory |
| 645 | MUC-764 | Nat                  | Benzophenone derivative  | <i>Garcinia succifolia</i>        | P                                  | LF+TW      | Anti-inflammatory |
| 646 | MUC-765 | Nat                  | Benzophenone derivative  | <i>Garcinia succifolia</i>        | P                                  | LF+TW      | Anti-inflammatory |
| 647 | MUC-778 | Nat                  | -                        | <i>Santisukia pagetii</i>         | E                                  | LF+TW      | Cytotoxic         |
| 648 | MUC-779 | Nat                  | -                        | <i>Santisukia pagetii</i>         | F                                  | LF+TW      | Cytotoxic         |
| 649 | MUC-780 | Nat                  | -                        | <i>Santisukia pagetii</i>         | F                                  | LF+TW      | Cytotoxic         |
| 650 | MUC-781 | Nat                  | -                        | <i>Santisukia pagetii</i>         | F                                  | LF+TW      | Cytotoxic         |
| 651 | MUC-782 | Nat                  | -                        | <i>Santisukia pagetii</i>         | F                                  | LF+TW      | Cytotoxic         |
| 652 | MUC-783 | Nat                  | -                        | <i>Santisukia pagetii</i>         | F                                  | LF+TW      | Cytotoxic         |
| 653 | MUC-784 | Nat                  | -                        | <i>Santisukia pagetii</i>         | F                                  | LF+TW      | Cytotoxic         |



| No  | CODE    | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay                   |
|-----|---------|----------------------|------------------|----------------------------------|---------------------------------------|------------|-------------------------|
| 654 | MUC-785 | Nat                  | -                | <i>Santisukia pagetii</i>        | F                                     | LF+TW      | Cytotoxic               |
| 655 | MUC-786 | Nat                  | -                | <i>Santisukia pagetii</i>        | F                                     | LF+TW      | Cytotoxic               |
| 656 | MUC-787 | Nat                  | -                | <i>Santisukia pagetii</i>        | F                                     | LF+TW      | Cytotoxic               |
| 657 | MUC-808 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | E                                     | ST         | Cytotoxic               |
| 658 | MUC-809 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 659 | MUC-810 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 660 | MUC-811 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 661 | MUC-812 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 662 | MUC-813 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 663 | MUC-814 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 664 | MUC-815 | Nat                  | -                | <i>Goniothalamus calvicarpus</i> | F                                     | ST         | Cytotoxic               |
| 665 | MUC-843 | Mod                  | Styryllactone    | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Anti-inflammatory       |
| 666 | MUC-844 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 667 | MUC-845 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 668 | MUC-846 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 669 | MUC-847 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 670 | MUC-848 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 671 | MUC-849 | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay                   |
|-----|-------------|----------------------|------------------|----------------------------------|---------------------------------------|------------|-------------------------|
| 672 | MUC-850     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 673 | MUC-851     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 674 | MUC-852     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 675 | MUC-853     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 676 | MUC-854     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 677 | MUC-856     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 678 | MUC-857     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 679 | MUC-858     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 680 | MUC-859     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 681 | MUC-860     | Mod                  | Lignan glycoside | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic in stem cells |
| 682 | MUC-862     | Nat                  | -                | <i>Curcuma sp.</i>               | E                                     | RZ         | Cytotoxic               |
| 683 | MUC-873     | Nat                  | -                | <i>Boesenbergia thorelii</i>     | E                                     | RZ         | Cytotoxic               |
| 684 | MUC-884     | Nat                  | Stryllactone     | <i>Goniotalamus calvicarpus</i>  | P                                     | ST         | Cytotoxic               |
| 685 | MUC-885     | Nat                  | Flavanone        | <i>Goniotalamus calvicarpus</i>  | P                                     | ST         | Cytotoxic               |
| 686 | MUC-886     | Nat                  | Alkaloid         | <i>Goniotalamus calvicarpus</i>  | P                                     | ST         | Cytotoxic               |
| 687 | MUC-887     | Nat                  | Stryllactone     | <i>Goniotalamus calvicarpus</i>  | P                                     | ST         | Cytotoxic               |
| 688 | MUC-888     | Nat                  | Flavanone        | <i>Goniotalamus calvicarpus</i>  | P                                     | ST         | Cytotoxic               |
| 689 | MUC-889 (1) | Mod                  | Flavanone        | <i>Goniotalamus calvicarpus</i>  | P                                     | ST         | Cytotoxic               |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound        | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay     |
|-----|-------------|----------------------|-------------------------|----------------------------------|---------------------------------------|------------|-----------|
| 690 | MUC-889 (2) | Mod                  | Stryllactone            | <i>Goniothalamus calvicarpus</i> | P                                     | ST         | Cytotoxic |
| 691 | MUC-889 (3) | Mod                  | Stryllactone            | <i>Goniothalamus calvicarpus</i> | P                                     | ST         | Cytotoxic |
| 692 | MUC-890     | Mod                  | Stryllactone            | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 693 | MUC-891     | Mod                  | Stryllactone            | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 694 | MUC-892     | Mod                  | Stryllactone            | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 695 | MUC-893     | Mod                  | Stryllactone            | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 696 | MUC-894     | Mod                  | Stryllactone            | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 697 | MUC-895     | Mod                  | Stryllactone            | <i>Goniothalamus aurantiacus</i> | P                                     | LF+TW      | Cytotoxic |
| 698 | MUC-917     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | E                                     | RZ         | Cytotoxic |
| 699 | MUC-918     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 700 | MUC-919     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 701 | MUC-920     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 702 | MUC-921     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 703 | MUC-922     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 704 | MUC-923     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 705 | MUC-924     | Nat                  | -                       | <i>Boesenbergia thorelii</i>     | F                                     | RZ         | Cytotoxic |
| 706 | MUC-941     | Nat                  | Acetophenone derivative | <i>Mallotus glomerulatus</i>     | P                                     | PX         | Cytotoxic |
| 707 | MUC-942     | Nat                  | Flavanone               | <i>Mallotus glomerulatus</i>     | P                                     | PX         | Cytotoxic |

| No  | CODE        | Nat/Mod/Syn compound | Type of Compound                       | Plant Source                     | Extract (E)/<br>Fraction (F)/Pure (P) | Plant part | Assay        |
|-----|-------------|----------------------|----------------------------------------|----------------------------------|---------------------------------------|------------|--------------|
| 708 | MUC-943     | Nat                  | Diterpenoid                            | <i>Mallotus glomerulatus</i>     | P                                     | PX         | Cytotoxic    |
| 709 | MUC-944     | Nat                  | Flavone                                | <i>Mallotus glomerulatus</i>     | P                                     | PX         | Cytotoxic    |
| 710 | MUC-945     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 711 | MUC-946     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 712 | MUC-947     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 713 | MUC-948     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 714 | MUC-949     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 715 | MUC-950     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 716 | MUC-951     | Nat                  | -                                      | <i>Curcuma sp.</i>               | F                                     | RZ         | Cytotoxic    |
| 717 | MUC-966 (1) | Nat                  | Lignan glycoside                       | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic    |
| 718 | MUC-966 (2) | Nat                  | Lignan glycoside                       | <i>Phyllanthus taxodiifolius</i> | P                                     | PX         | Cytotoxic    |
| 719 | MUC-985     | Nat                  | -                                      | <i>Zingiber junceum</i>          | E                                     | RZ         | Cytotoxic    |
| 720 | MUC-986     | Nat                  | -                                      | <i>Zingiber junceum</i>          | F                                     | RZ         | Cytotoxic    |
| 721 | MUC-987     | Nat                  | -                                      | <i>Zingiber junceum</i>          | F                                     | RZ         | Cytotoxic    |
| 722 | MUC-988     | Nat                  | -                                      | <i>Zingiber junceum</i>          | F                                     | RZ         | Cytotoxic    |
| 723 | MUC-989     | Nat                  | -                                      | <i>Zingiber junceum</i>          | F                                     | RZ         | Cytotoxic    |
| 724 | MUC-1010    | Nat                  | -                                      | <i>Paris polyphylla</i>          | E                                     | RZ         | Cytotoxic    |
| 725 | MUC-1011    | Nat                  | C <sub>6</sub> C <sub>3</sub> compound | <i>Zingiber junceum</i>          | P                                     | RZ         | Anti-oxidant |

Nat = Natural compound, Mod = Modified compound, Syn = Synthetic compound; E = extract, F = fraction, P = Pure compound; PX = Aerial parts, FL = Flowers, LF+TW = Leaves and twigs, LF = Leaves, ST = Stems, RT = Roots, RZ = Rhizomes

## 2. Isolation of bioactive compounds from leaves and twigs of *Santisukia pagetii*

*Santisukia pagetii*, known in Thai as ‘Kanchanika’ or ‘Kaedong’, is a plant in Bignoniaceae family. The plant material was collected from Kanchanaburi province of Thailand.

Dried leaves and twigs of *Santisukia pagetii* were pulverized to yield powdered material (3.1 kg.). Extraction was performed in methanol (7L x 5 times). A crude methanol extract (429.4 g) was obtained after evaporation to dryness. The methanol extract was dissolved in EtOAc: MeOH (1:1) and MeOH respectively. After evaporation to dryness, EtOAc:MeOH (1:1) fraction (277.8 g), MeOH fraction (39.3 g) and residue (54.5 g) were obtained, respectively.

The crude methanol extract and fractions were submitted for cytotoxic activity testing (in collaboration with Dr. Artit Chairoungdua) and anti-HIV-1 activity in RT assay (in collaboration with Dr. Radeekorn Akkarawongsapat). The testing results indicated that all extracts and the residue showed no significant cytotoxic activity in most of cancer cell lines (see Table 1). For anti-HIV-1 activity, the EtOAc-MeOH (1:1) extract was found to be very active in HIV-1-RT assay as shown in Table 2.

The EtOAc-MeOH (1:1) fraction (272.4 g) was further separated by vacuum chromatography, eluting hexanes, EtOAc-hexane, EtOAc, EtOAc-MeOH and finally with MeOH. All fractions were combined on the basis of TLC behavior to provide 6 fractions (F1-F6). The results indicated that fractions F2-F4 exhibited cytotoxic activity in some of cancer cell lines as shown in Table 1.2.

**Table 1.2.** Cytotoxic activities from extract and fractions of leaves and twigs of *Santisukia pagetii*

| Extracts/Fractions | ED <sub>50</sub> (μg/mL) |       |       |       |       |      |         |
|--------------------|--------------------------|-------|-------|-------|-------|------|---------|
|                    | Cell lines               |       |       |       |       |      |         |
|                    | P-388                    | KB    | HT-29 | MCF-7 | A549  | ASK  | Hek 293 |
| Ellip              | 0.37                     | 0.46  | 0.51  | 0.47  | 0.46  | 0.57 | 0.51    |
| MeOH Ext.          | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |
| EtOAc:MeOH (1:1)   | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |
| MeOH               | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |
| Residue            | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |
| F1                 | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |
| F2                 | 7.06                     | 18.30 | >20   | >20   | >20   | >20  | >20     |
| F3                 | 7.16                     | >20   | >20   | >20   | >20   | >20  | >20     |
| F4                 | 13.14                    | >20   | 15.78 | 12.68 | 19.68 | >20  | >20     |
| F5                 | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |
| F6                 | >20                      | >20   | >20   | >20   | >20   | >20  | >20     |

Results are expressed at ED<sub>50</sub> value (μg/mL); ED<sub>50</sub> <20 μg/mL is considered active.

P-388 = murine lymphocytic leukemia, KB = human nasopharyngeal carcinoma, HT-29 = human colon cancer, MCF-7 = Michigan cancer foundation, human breast cancer, A 549 = human lung cancer, ASK = cell line from rat glioma cell, Hek-293 = human kidney cell

In case of anti-HIV-1 assay, only fraction F4 was found very active as shown in Table 1.3.

**Table 1.3** Anti-HIV-1-RT activities from extract and fractions of leaves and twigs of *Santisukia pagetii*

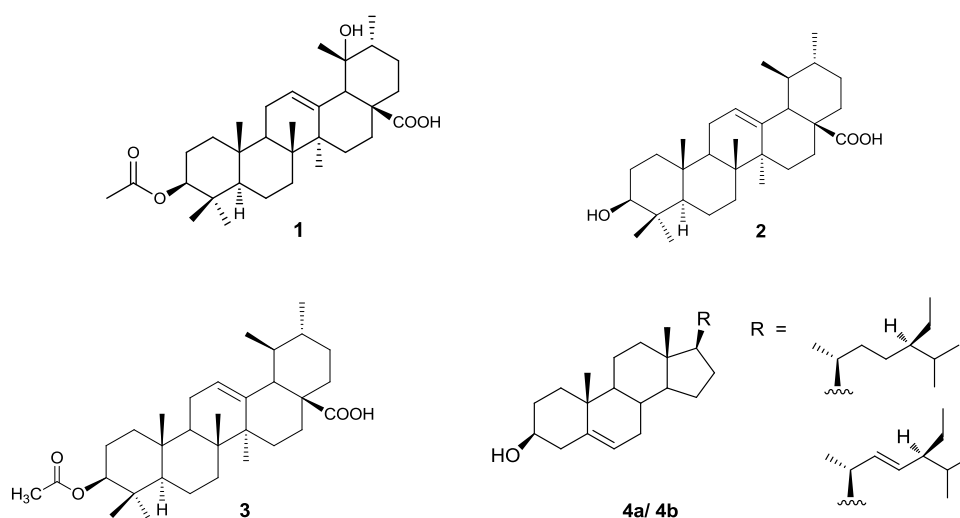
| Extract          | Anti-HIV-1-RT |          |
|------------------|---------------|----------|
|                  | % Inhibition  | Activity |
| MeOH Ext.        | 60.43         | M        |
| EtOAc-MeOH (1:1) | 72.66         | VA       |
| MeOH             | 26.49         | I        |
| Residue          | 41.57         | W        |
| F1               | 41.02         | W        |
| F2               | 43.19         | W        |
| F3               | 7.86          | I        |
| F4               | 100.10        | VA       |
| F5               | 64.40         | M        |
| F6               | 21.57         | inactive |

RT assay: activity > 70% inhibition = very active (VA); >50-70% inhibition = moderately active (M); 30-50% inhibition = weakly active (W); < 30% inhibition = inactive (I).

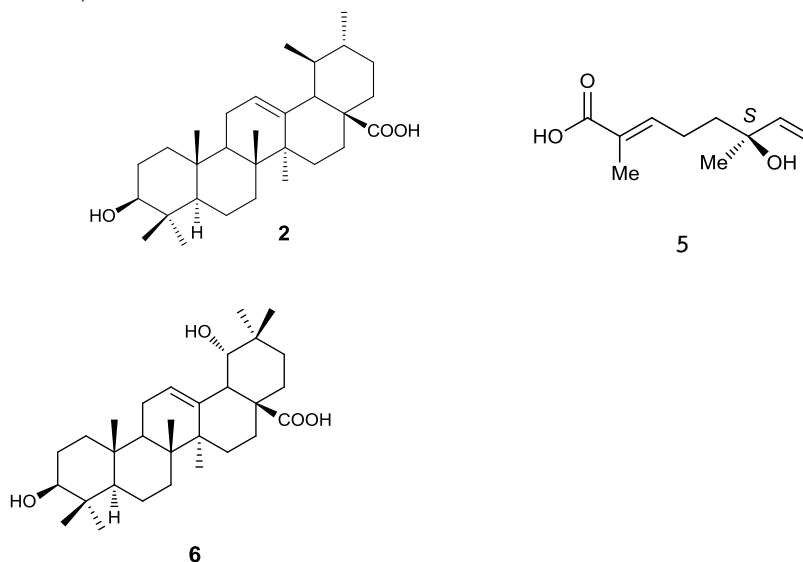
Further separation by column chromatography of each fraction was performed as follows:

Fraction F1, eluted by 0-10% EtOAc-hexane, contained mainly fat, therefore it was not further investigated.

Fraction F2, eluted by 15-25% EtOAc-hexane, was further separated by repeated column chromatography to afford triterpenoids, namely pomolic acid-3β-acetate (**1**) ursolic acid (**2**) and 3-O-acetylursolic acid (**3**) and a mixture of β-sitosterol/stigmasterol (**4a/4b**)

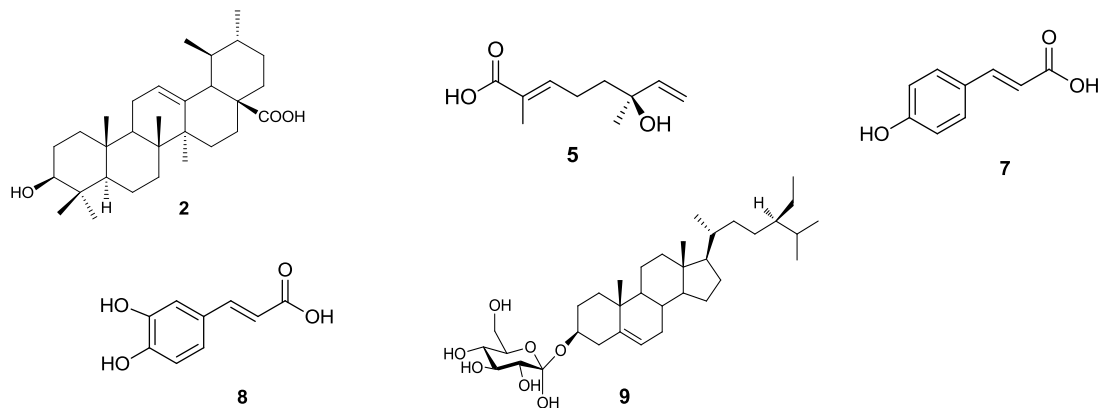


Fraction F3, eluted by 30-50% EtOAc-hexane, was further separated by repeated column chromatography to give ursolic acid (**2**) and a monoterpene, which was identified as menthiafolic acid (**5**). By comparison of the optical rotation value of **5**,  $[\alpha]_{589}^{26.6} +43.7$  (c 0.47, CHCl<sub>3</sub>) with that in the literature, Lit.  $[\alpha]_{589}^{14} 18.2$  (c 0.22, CHCl<sub>3</sub>), the absolute configuration at carbon connected to the hydroxyl group was proved to be *S*. Beside compounds **2** and **5**, another triterpenoid, siaresinolic acid (**6**) was also obtained.

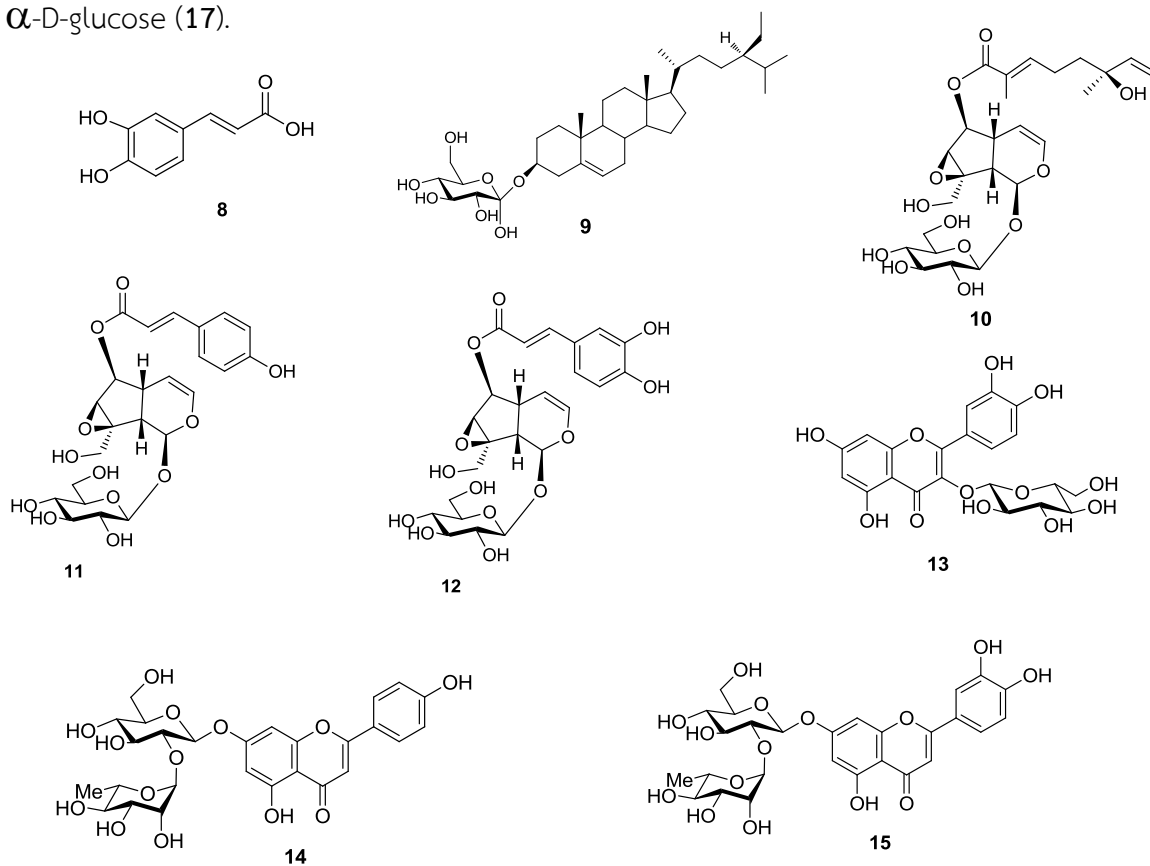


1. Yoshikawa K, Satou Y, Tokunaga Y, Tanaka M, Arihara S, Nigam S.K. Four Acylated Triterpenoid Saponins from *Albizia procera*. J Nat Prod. 1988; 61: 440-445.
2. Zou K, Tong W-Y, Liang H, Cui J-R, Tu G-Z, Zhao Y-Y, Zhang R-Y. Diastereoisomeric saponins from *Albizia julibrissin*. Carbohydrate Research. 2005; 340: 1329-1334.

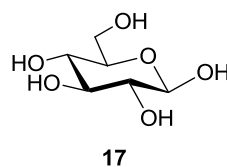
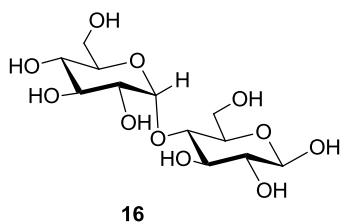
Fraction F4, eluted by 30-50% EtOAc-hexane, was further separated by repeated column chromatography to provide ursolic acid (**2**), (6*S*)-menthiafolic acid (**5**), *p*-coumaric acid (**7**), caffeic acid (**8**) and  $\beta$ -sitosterol 3-*O*- $\beta$ -D-glucopyranoside (**9**).



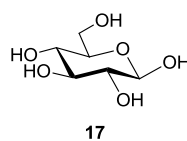
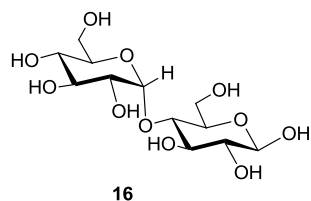
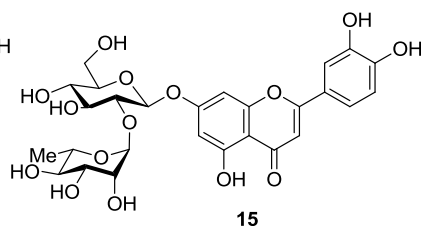
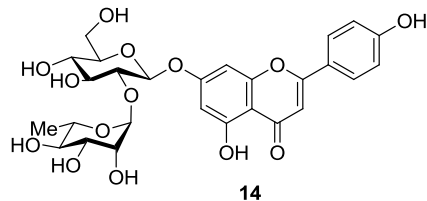
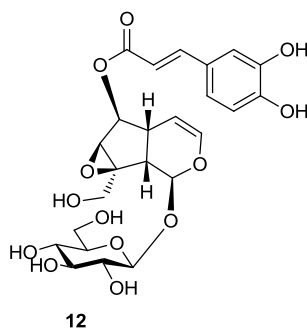
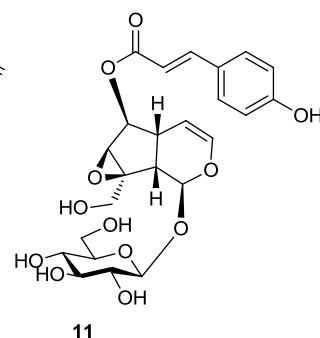
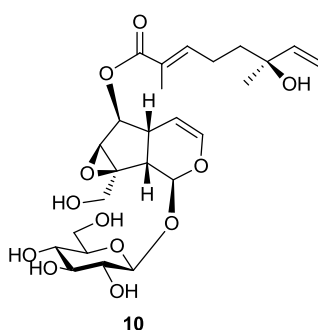
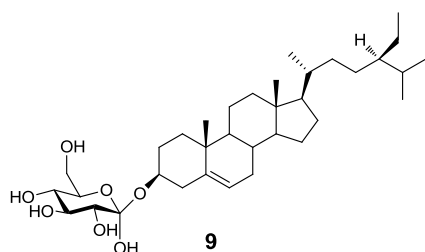
Fraction F5, eluted by 80% EtOAc-hexane-30 % MeOH-EtOAc was separated by repeated column chromatography to afford caffeic acid (**8**),  $\beta$ -sitosterol 3-*O*- $\beta$ -D-glucopyranoside (**9**), ambiguuside (**10**), specioside (**11**), verminoside (**12**), isoquercitrin (**13**), apigenin-7-*neohesperidoside* (**14**), luteolin-7-*O*-*neohesper-*terioside (**15**),  $\beta$ -D-maltose (**16**) and  $\alpha$ -D-glucose (**17**).







Fraction F6, eluted by 40% MeOH-EtOAc-100% MeOH, was separated by repeated column chromatography to give  $\beta$ -sitosterol 3-O- $\beta$ -D-glucopyranoside (**9**), ambiguoside (**10**), specioside (**11**), verminoside (**12**), apigenin-7-neohesperidoside (**14**), luteolin-7-O-neohesperioside (**15**),  $\beta$ -D-maltose (**16**) and  $\alpha$ -D-glucose (**17**).



All isolated compounds were submitted for biological testings, such as cytotoxic activity in cancer cell lines (in collaboration with Dr. Artit Chairoungdua) and anti-HIV-1 activity (in collaboration with Dr. Radeekorn Akkarawongsapat). The results indicated that no significant cytotoxic activity was observed, but some of the isolated compounds showed significant anti-HIV-1 activities in both syncytium and reverse transcriptase assays as illustrated in Table 1.4.

**Table 1.4** Anti-HIV-1 activities of pure isolated compounds from leaves and twigs of *Santisukia pagetii*

| Compound                        | Syncytium assay**              |                                |       |          | HIV-1 RT assay***                |          |                                |
|---------------------------------|--------------------------------|--------------------------------|-------|----------|----------------------------------|----------|--------------------------------|
|                                 | IC <sub>50</sub><br>( $\mu$ M) | EC <sub>50</sub><br>( $\mu$ M) | TI    | Activity | %Inhibition at<br>200 $\mu$ g/mL | Activity | IC <sub>50</sub><br>( $\mu$ M) |
| Pomolic acid 3 $\beta$ -acetate | 222.24                         | 34.29                          | 6.48  | Aa       | 63.73                            | M        | ND                             |
| Ursolic acid                    | 85.50                          | 11.35                          | 7.53  | Aa       | 84.36                            | VA       | 318.7                          |
| 3-O-Acetylursolic acid          | 39.89                          | 13.42                          | 2.97  | Aa       | 87.19                            | VA       | 210.3                          |
| Siaresinolic acid               | 254.24                         | 120.99                         | 2.1   | Aa       | 37.00                            | W        | ND                             |
| Specioside                      | >246                           | 136.97                         | >1.80 | Aa       | 12.59                            | I        | ND                             |
| Verminoside                     | 153.89                         | 124.66                         | 1.23  | Aa       | 27.34                            | I        | ND                             |
| Ambiguuside                     | >237                           | 61.21                          | 3.87  | Aa       | 3.49                             | I        | ND                             |
| Luteolin-7-O-neohesperidoside   | 40.85                          | 53.23                          | 0.77  | T        | 70.37                            | VA       | 1016.5*                        |
| Apigenin-7-O-neohesperidoside   | >762                           | >762                           | -     | Ia       | 3.70                             | I        | ND                             |
| Isoquercitrin                   | 36.77                          | 99.09                          | 0.37  | T        | 15.98                            | I        | ND                             |
| p-Coumaric acid                 | >210                           | >210                           | -     | Ia       | 60.89                            | M        | ND                             |
| Caffeic acid                    | >216                           | 123.48                         | >1.75 | Aa       | 40.90                            | W        | ND                             |
| (6S)-Menthiafolic acid          | >269                           | 201.17                         | >1.31 | Aa       | 44.16                            | W        | ND                             |

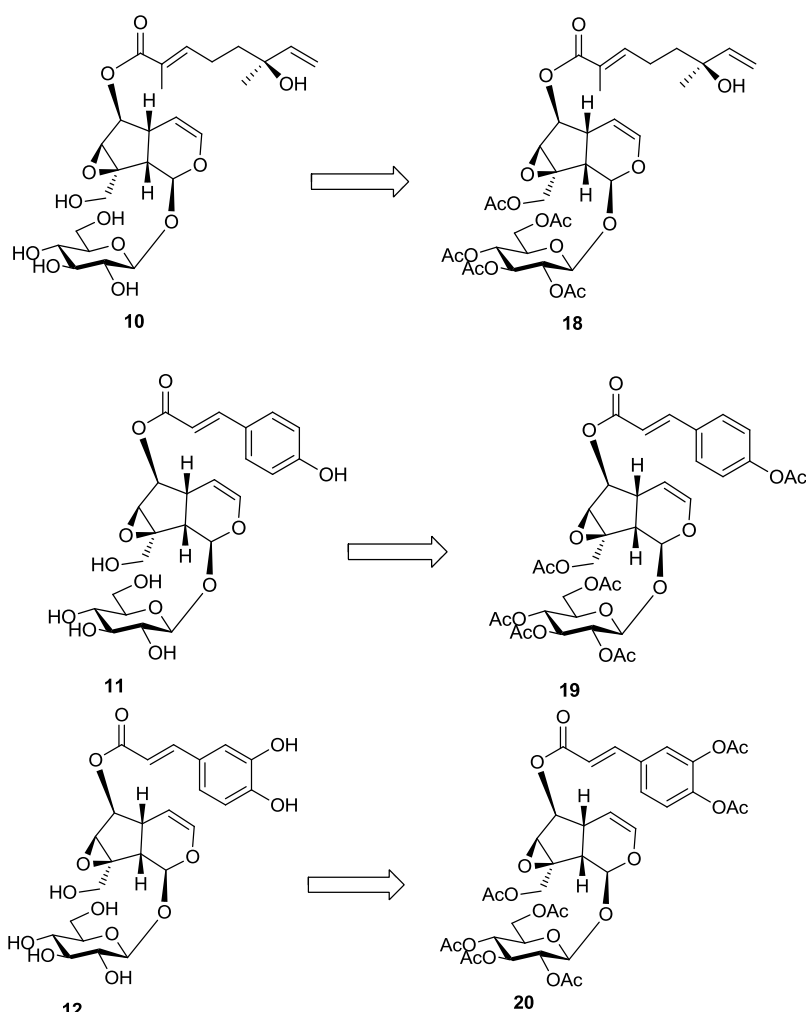
\*\* Syncytium reduction assay: EC<sub>50</sub> = dose of compound that reduced 50% syncytium formation by  $\Delta$ Tat/Rev<sup>MC99</sup> virus in 1A2 cells. AZT, averaged from four experiments, EC<sub>50</sub> 1.87 x 10<sup>-8</sup>  $\mu$ M; Aa = active (TI >1), Ia = inactive, T= toxic (IC<sub>50</sub> is less than the lowest concentration tested), TI = Therapeutic Index (TI = IC<sub>50</sub>/EC<sub>50</sub>)

\*\*\* RT assay: Compounds were prescreened at 200  $\mu$ g/mL. Only those that were very active at this concentration were furtherdetermined for IC<sub>50</sub>, the dose that inhibited 50% HIV-1 RT activity; VA= very active, M = moderately active, W = weakly active, I = inactive, ND = not determined. Positive controls, averaged from one experiments. IC<sub>50</sub> nevirapine 9.21  $\mu$ M.

A manuscript concerning this work has been accepted for publication in Natural Product Communications 2018.

### 3. Structure modification of iridoid glycosides: ambiguuside (10), specioside (11), verminoside (12)

The structures of ambiguuside (10), specioside (11), verminoside (12) were modified by conversion to their acetate derivatives 18-20 by the chemical reaction with acetic anhydride/DMAP at room temperature



The natural compounds **10-12** and modified compounds **18-20** submitted for biological testings, i.e. anti-Alzheimer's and anti-oxidant activities (in collaboration with Dr. Wichuda Sangsawang).

#### 4. Isolation of bioactive compounds from stems of *Goniothalamus calvicarpus*

*Goniothalamus calvicarpus* known in Thai as 'Sa-Ban-Nga-Pa', is a plant in Annonaceae family. The plant material was collected from Chiang Mai province of Thailand.

The dried stems of *Goniothalamus calvicarpus* were pulverized to give powdered material (6.9 kg). Extraction was performed in methanol (16L x 6 times). A crude methanol extract (319.6 g) was obtained after evaporation to dryness. The methanol extract was dissolved in EtOAc:MeOH (1:1) and MeOH respectively. After evaporation to dryness, EtOAc:MeOH (1:1) fraction (267.8 g), MeOH fraction (28.2 g) and residue (22.6 g) were obtained, respectively.

The dried extract and fractions were submitted for cytotoxic activity testing (in collaboration with Dr. Artit Chairoungdua) and anti-HIV-1 activity in RT assay (in collaboration with Dr. Radeekorn Akkarawongsapat). The testing results indicated that the EtOAc:MeOH (1:1) fraction was the most active in cytotoxic and HIV-1-RT assays as shown in Tables 1.4 and 1.5.

The EtOAc-MeOH (1:1) fraction (267.6 g) was further separated by vacuum chromatography, eluting hexanes, EtOAc-hexane, EtOAc, EtOAc-MeOH and finally with MeOH. All fractions were combined on the basis of TLC behavior to provide 6 fractions (F1-F5). The results indicated that fractions F2, F3 and F5 exhibited cytotoxic activity in some of cancer cell lines as shown in Table 1.4. In case of HIV-1 RT assay, only F2 was found to be very active (see Table 1.5).

**Table 1.5** Cytotoxic activities from extracts and fractions of the stems of *Goniothalamus calvicarpus*

| Fraction         | Cell line (ED <sub>50</sub> µg/mg) |       |       |       |      |       |         |
|------------------|------------------------------------|-------|-------|-------|------|-------|---------|
|                  | P-388                              | KB    | HT29  | MCF-7 | A549 | ASK   | Hek 293 |
| Ellipticine      | 0.39                               | 0.51  | 0.62  | 0.45  | 0.54 | 0.51  | 0.60    |
| EtOAc:MeOH (1:1) | <4                                 | 4.28  | <4    | <4    | <4   | 8.16  | <4      |
| MeOH             | <4                                 | 14.03 | 5.97  | 8.84  | 3.93 | 12.11 | 3.35    |
| Residue          | 15.16                              | >20   | >20   | >20   | >20  | >20   | 13.01   |
| F1               | >20                                | >20   | >20   | >20   | >20  | >20   | >20     |
| F2               | <4                                 | 12.74 | 11.92 | 10.80 | 5.68 | 10.83 | 10.20   |
| F3               | <4                                 | <4    | <4    | <4    | <4   | <4    | <4      |
| F4               | >20                                | >20   | >20   | >20   | >20  | >20   | >20     |
| F5               | 4                                  | 0.94  | 3.46  | 3.82  | 3.49 | 9.88  | 1.39    |

Results are expressed at ED<sub>50</sub> value (µg/mL); ED<sub>50</sub> <20 µg/mL is considered active.

P-388 = murine lymphocytic leukemia, KB = human nasopharyngeal carcinoma, HT-29 = human colon cancer, MCF-7 = Michigan cancer foundation, human breast cancer, A 549 = human lung cancer, ASK = cell line from rat glioma cell, Hek-293 = human kidney cell

**Table 1.6** Anti-HIV-1-RT activities from the extracts and fractions of the stems of *Goniothalamus calvicarpus*

| Extract/Fraction | Anti-HIV-1-RT (at 200 µg/mL) |          |
|------------------|------------------------------|----------|
|                  | % Inhibition                 | Activity |
| EtOAc:MeOH (1:1) | 94.51                        | VA       |
| MeOH             | 32.96                        | W        |
| Residue          | 39.63                        | W        |
| F1               | 11.96                        | I        |
| F2               | 97.53                        | VA       |
| F3               | 42.31                        | W        |
| F4               | -21.88                       | I        |
| F5               | 12.74                        | I        |

RT assay: VA= very active (>70% inhibition), M = moderately active (>50% to 70% inhibition), W = weakly active (30% to 50% inhibition), I = inactive (<30% inhibition)

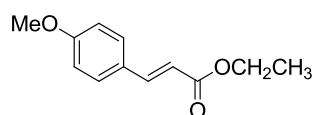
**Table 1.7** Anti-HIV 1 activities in the anti-syncytium assay from extract and fractions of the stems of *Goniothalamus calvicarpus*

| Fraction         | IC <sub>50</sub> | EC <sub>50</sub> | SI    | Activity | AZT                |                         |        |
|------------------|------------------|------------------|-------|----------|--------------------|-------------------------|--------|
|                  |                  |                  |       |          | IC <sub>50</sub>   | EC <sub>50</sub>        | SI     |
| EtOAc:MeOH (1:1) | 16.13            | <7.8             | >2.07 | A        | > 10 <sup>-8</sup> | 1.75 × 10 <sup>-9</sup> | > 5.71 |
| MeOH             | 219.38           | 44.49            | 4.93  | A        |                    |                         |        |
| Residue          | 155.33           | 29.14            | 5.33  | A        |                    |                         |        |
| F1               | 111.16           | 30.90            | 3.6   | A        |                    |                         |        |
| F2               | -                | -                | -     | T        |                    |                         |        |
| F3               | -                | -                | -     | T        |                    |                         |        |
| F4               | 233.98           | 79.62            | 2.94  | A        |                    |                         |        |
| F5               | -                | -                | -     | T        |                    |                         |        |

Syncytium reduction assay: A = active, I = inactive, T= toxic, SI = Selectivity Index (SI = IC<sub>50</sub>/EC<sub>50</sub>)

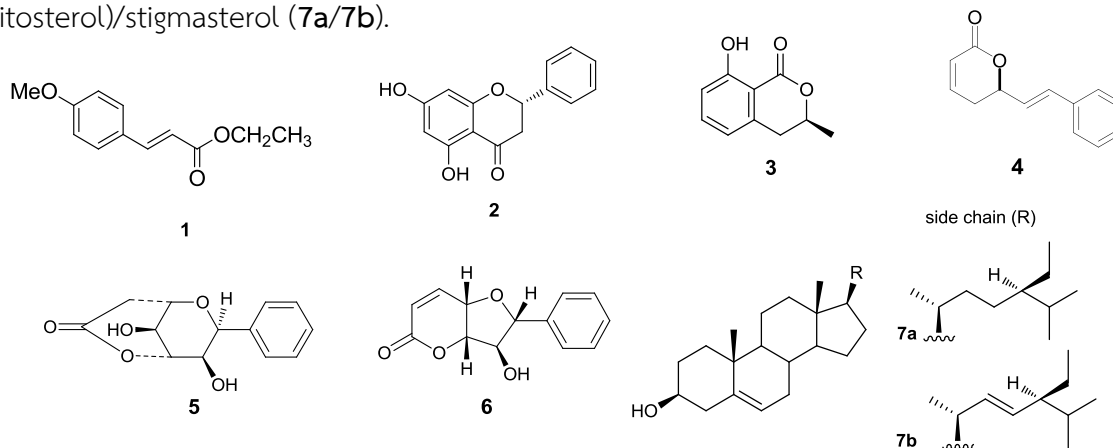
Further separation by column chromatography of each fraction was performed as follows:

Fraction F1, eluted by 10-20% EtOAc-hexanes, was further separated by repeated column chromatography to afford ethyl 4-methoxycinnamate (**1**)

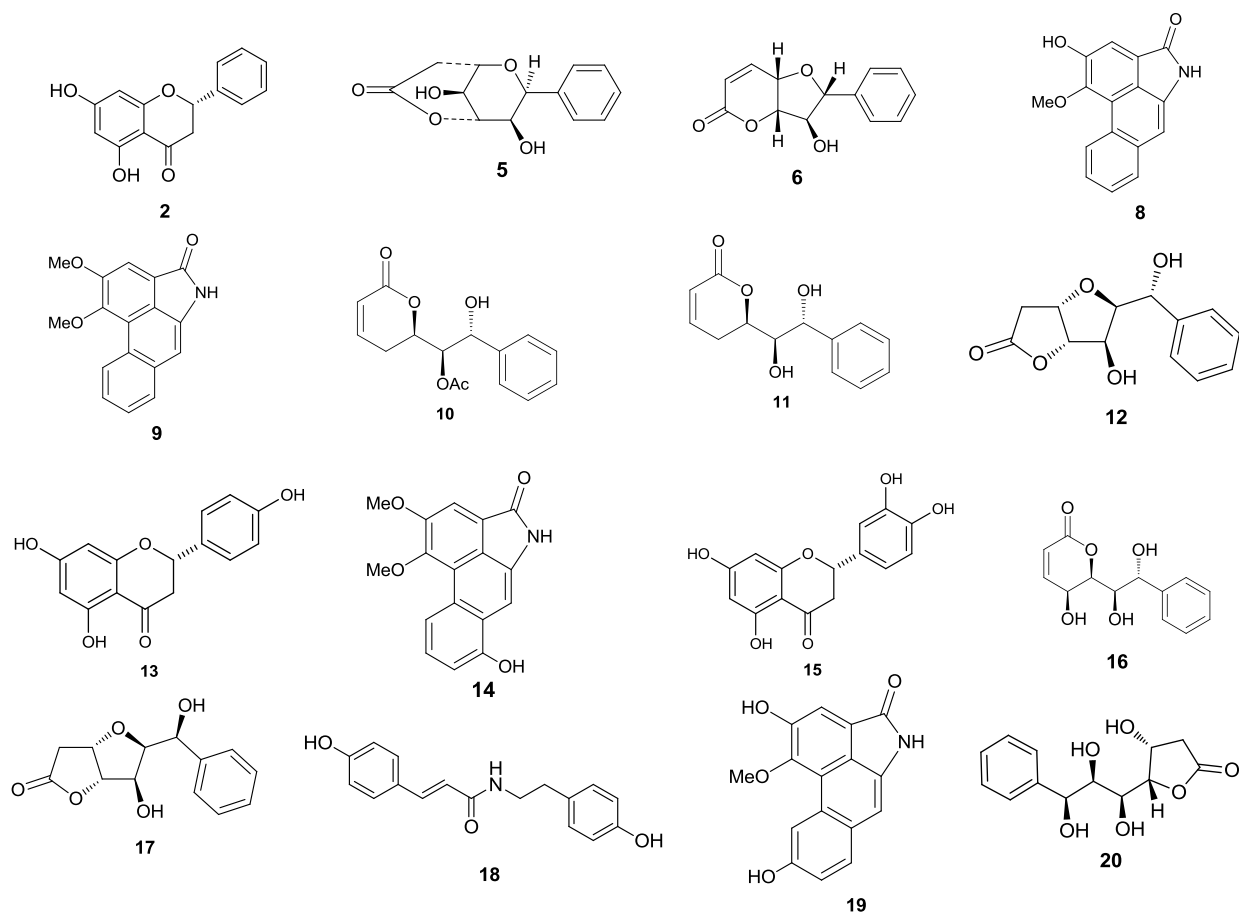


**1**

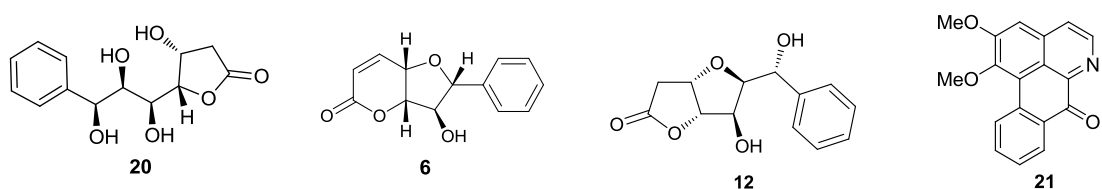
Fraction A2, eluted by 30-50% EtOAc-hexanes, was separated by repeated column chromatography to provide ethyl 4-methoxycinnamate (**1**), (–)-pinocembrin (**2**), (–)-mellein (**3**), (+)-goniothalamine (**4**), (+)-gonioppyrone (**5**), (+)-altholactone (**6**) and a mixture of β-sitosterol/stigmasterol (**7a/7b**).



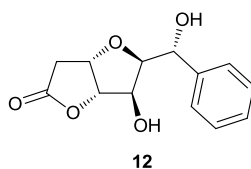
Fraction A3, eluted by 60-100% EtOAc-hexanes, was separated by repeated column chromatography to provide (–)-pinocembrin (**2**), (+)-gonioppyrone (**5**), (+)-altholactone (**6**), aristolactam AII (**8**), aristolactam BII (**9**), (+)-goniodiol-7-monoacetate (**10**), (+)-goniodiol (**11**), (+)-goniofufurone (**12**), (–)-naringenin (**13**), velutinam (**14**), (+)-eriodictyol (**15**), (+)-goniotriol (**16**), (+)-8-epi-goniofufurone (**17**), paprazine (**18**), aristolactam AIIIa (**19**) and (+)-cardiobutanolide (**20**).



Fraction A4, eluted by 5-30% MeOH-EtOAc, was further separated by repeated column chromatography to give (+)-altholactone (**6**), (+)-goniofufurone (**12**), (+)-cardiobutanolide (**20**) and lysicamine (**21**).



Fraction A5, eluted by 40-100% EtOAc, was separated by repeated column chromatography to afford (+)-goniofufurone (**12**).



All isolated compounds were submitted for biological testings, such as cytotoxic activity in cancer cell lines in collaboration with Dr. Artit Chairoungdua

**Table 1.8** Cytotoxic activities of the pure isolated compounds from the stems of *Goniotalamus calvicarpus*

| Compound                    | Cell line (ED <sub>50</sub> µg/mL) |      |       |       |       |      |         |      |
|-----------------------------|------------------------------------|------|-------|-------|-------|------|---------|------|
|                             | P-388                              | KB   | HT 29 | MCF-7 | A 549 | ASK  | Hek 293 | CL   |
| Ellipticine                 | 0.53                               | 0.63 | 0.41  | 0.62  | 0.46  | 0.70 | 0.83    | –    |
| (+)-Altholactone            | 1.73                               | 5.16 | 2.02  | 2.22  | 1.42  | 1.49 | 1.17    | –    |
| Aristolactam All            | >4                                 | 1.34 | >4    | >4    | >4    | >4   | >4      | –    |
| (–)-Pinocembrin             | >4                                 | >4   | >4    | >4    | >4    | >4   | >4      | –    |
| (+)-Eriodictyol             | >4                                 | >4   | >4    | >4    | >4    | >4   | >4      | –    |
| (+)-Goniofufurone           | >4                                 | >4   | >4    | >4    | >4    | >4   | >4      | –    |
| Ellipticine                 | 0.52                               | 0.53 | 0.63  | 0.41  | 0.62  | 0.83 | 0.46    | –    |
| (+)-Goniotalamin            | 0.34                               | 2.14 | 2.12  | 0.42  | 0.48  | 0.75 | 0.56    | –    |
| (+)-Goniodiol               | 0.73                               | 2.10 | 2.75  | 3.16  | 2.10  | 2.70 | 2.31    | –    |
| (+)-Goniotriol              | 0.29                               | >4   | 2.96  | 3.52  | >4    | >4   | 2.36    | –    |
| (+)-Goniopyrpyrone          | 0.68                               | >4   | >4    | >4    | >4    | >4   | 3.67    | –    |
| (–)-Naringenin              | 2.56                               | >4   | >4    | >4    | >4    | >4   | 3.67    | –    |
| Ellipticine                 | 0.42                               | 0.47 | 0.52  | 0.46  | 0.46  | 0.47 | –       | 0.49 |
| (+)-Goniodiol-7-monoacetate | 2.89                               | >4   | >4    | >4    | >4    | >4   | –       | >4   |
| (+)-8-epi-Goniofufurone     | >4                                 | >4   | >4    | >4    | >4    | >4   | –       | >4   |
| (+)-Cardiobutanolide        | >4                                 | >4   | >4    | >4    | >4    | >4   | –       | >4   |
| Velutinam                   | 1.97                               | 2.45 | >4    | 0.76  | >4    | >4   | –       | 1.89 |
| Aristolactam BII            | >4                                 | >4   | >4    | <0.16 | >4    | >4   | –       | >4   |
| Aristolactam Allla          | 0.71                               | 0.65 | >4    | 3.04  | 2.98  | 2.72 | –       | 0.64 |
| (–)-Mellein                 | >4                                 | >4   | >4    | >4    | >4    | >4   | –       | >4   |
| Ethyl methoxycinnamate      | >4                                 | >4   | >4    | >4    | >4    | >4   | –       | >4   |
| Lysicamine                  | 1.92                               | >4   | >4    | 3.98  | >4    | 2.15 | –       | >4   |
| Paprazine                   | >4                                 | >4   | >4    | >4    | >4    | >4   | –       | >4   |

Results are expressed at ED<sub>50</sub> value (µg/mL); ED<sub>50</sub> <4 µg/mL is considered active.

P-388 = murine lymphocytic leukemia, KB = human nasopharyngeal carcinoma, HT-29 = human colon cancer, MCF-7 = Michigan cancer foundation, human breast cancer, A 549 = human lung cancer, ASK = cell line from rat glioma cell, Hek-293 = human kidney cell transformed with adenovirus 5 DNA (normal cell control), CL = normal liver

The preparation of a manuscript of the work on *Goniotalamus calvicarpus* is in progress.

## 5. Isolation of bioactive compounds from the aerial parts of *Mallotus glomerulatus*

*Mallotus glomerulatus*, known in Thai as ‘Mak lium’, is a plant of Euphorbiaceae family. The plant material was collected from Nakhon Phanom province of Thailand.

Powdered aerial parts of *M. glomerulatus* (4.4 kg) were percolated with MeOH (5 ×24 L) at room temperature to give a crude MeOH extract (317.8 g). After sequential dissolving in

MeOH:EtOAc (1:1, 6 L), MeOH (2 L), followed by solvent removal, the MeOH:EtOAc (1:1) fraction (167.9 g), MeOH fraction (75.8 g), and the residue (72.1 g) were obtained, respectively.

The dried extract and fractions were submitted for cytotoxic activity testings (in collaboration with Dr. Artit Chairoungdua. The results from cytotoxicity testings of the crude methanol extract, the EtOAc:MeOH (1:1) and the MeOH fractions are as shown in Table 1.7.

The EtOAc-MeOH (1:1) fraction (166.9 g) was further separated by vacuum chromatography, eluting hexanes, CH<sub>2</sub>Cl<sub>2</sub>-hexanes gradient, CH<sub>2</sub>Cl<sub>2</sub> and MeOH-CH<sub>2</sub>Cl<sub>2</sub> gradient, and finally with MeOH. All fractions were combined on the basis of TLC behavior to provide 8 fractions (F1-F8). The results indicated that fractions F3-5 and F8 exhibited cytotoxic activity in some of cancer cell lines, but fractions F6 and F7 were active in all tested cancer cell lines as shown in Table 1.8.

**Table 1.9** Cytotoxic activities from extract and fractions of the aerial parts of *Mallotus glomerulatus*

| Extract/Fraction | Cell lines* (ED <sub>50</sub> (μg/mL)) |       |       |       |       |       |        |
|------------------|----------------------------------------|-------|-------|-------|-------|-------|--------|
|                  | P-388                                  | KB    | HT-29 | MCF-7 | A549  | ASK   | Hek293 |
| Ellipticine      | 0.14                                   | 0.49  | 0.64  | 0.32  | 0.44  | 0.12  | 0.43   |
| MeOH             | <0.16                                  | <0.16 | 0.5   | 0.34  | 0.74  | 2.82  | 0.44   |
| MeOH:EtOAc (1:1) | <0.16                                  | <0.16 | 0.5   | 0.27  | <0.70 | 2.76  | 0.43   |
| MeOH             | 0.32                                   | 0.46  | 2.43  | 1.09  | 3.16  | 12.79 | 1.72   |
| F <sub>1</sub>   | >20                                    | >20   | >20   | >20   | >20   | >20   | >20    |
| F <sub>2</sub>   | >20                                    | >20   | >20   | >20   | >20   | >20   | >20    |
| F <sub>3</sub>   | 7.99                                   | 13.04 | 14    | <4    | >20   | >20   | 10.86  |
| F <sub>4</sub>   | 12.45                                  | >20   | >20   | 7.07  | >20   | >20   | 19.73  |
| F <sub>5</sub>   | 10.78                                  | >20   | >20   | 7.28  | >20   | >20   | 11.63  |
| F <sub>6</sub>   | <0.16                                  | <0.16 | 0.5   | 0.19  | 0.79  | 3.01  | 0.47   |
| F <sub>7</sub>   | <0.16                                  | <0.16 | <0.16 | <0.16 | <0.16 | 0.58  | <0.16  |
| F <sub>8</sub>   | 0.45                                   | 1.67  | 3.45  | 2.38  | 4.79  | 14.31 | 2.68   |
| Ellipticine      | 0.14                                   | 0.49  | 0.64  | 0.32  | 0.44  | 0.12  | 0.43   |

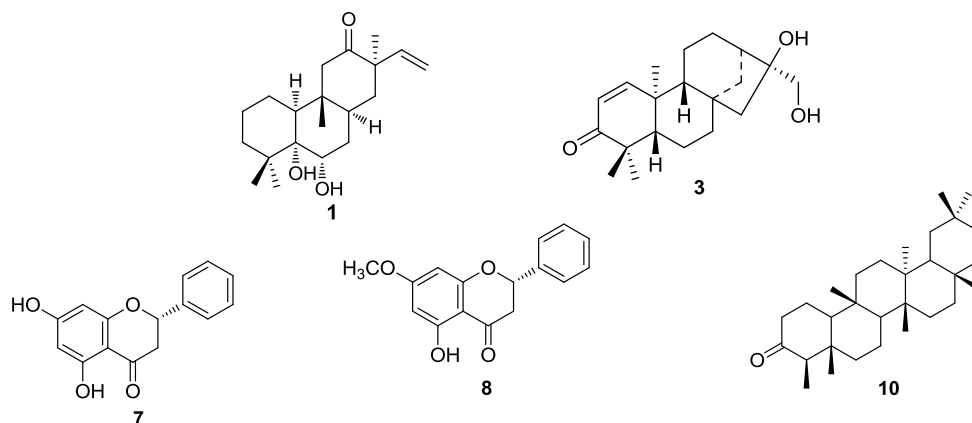
\*Results are expressed as ED<sub>50</sub> (μg/mL): ED<sub>50</sub> < 20 μg/mL is considered active. P-388 = murine lymphocytic leukemia, KB = human oral nasopharyngeal carcinoma, HT29 = human colorectal adenocarcinoma, MCF-7 = human breast cancer, A549 = human lung carcinoma, ASK = rat glioma cell, Hek293 = normal human kidney cell.

Further separation by column chromatography of each fraction was performed as follows:

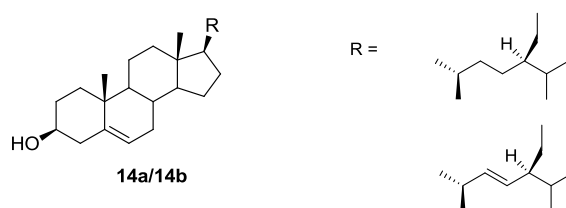


Fractions F1 (eluted by 0-25% CH<sub>2</sub>Cl<sub>2</sub>-hexanes) and F2 (eluted by 30-35% CH<sub>2</sub>Cl<sub>2</sub>-hexanes) contained mainly fat, therefore they were not further investigated.

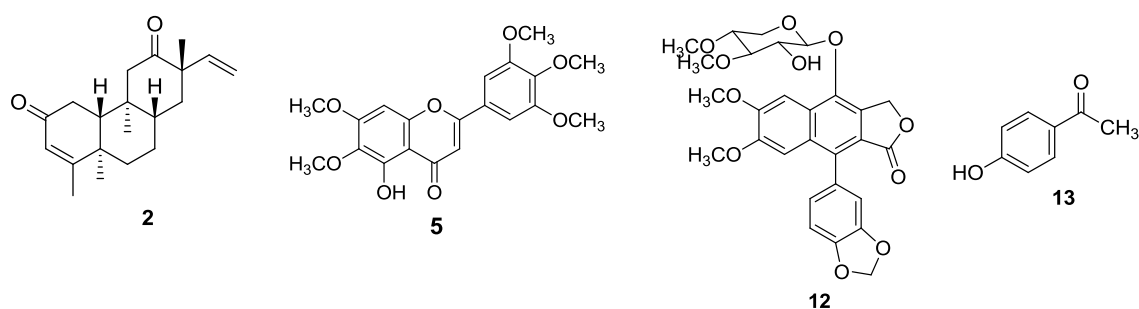
Fraction F3, eluted by 40-45% CH<sub>2</sub>Cl<sub>2</sub>-hexanes, was further separated by repeated column chromatography to give a new compound glomerulatusin (**1**), together with the known anomaluone (**3**), pinocembrin (**7**), pinostrobin (**8**), and friedelin (**10**).



Fraction F4, eluted by 50-65% CH<sub>2</sub>Cl<sub>2</sub>-hexanes, was separated by repeated column chromatography to afford a mixture of  $\beta$ -sitosterol/stigmasterol (**14a/14b**)

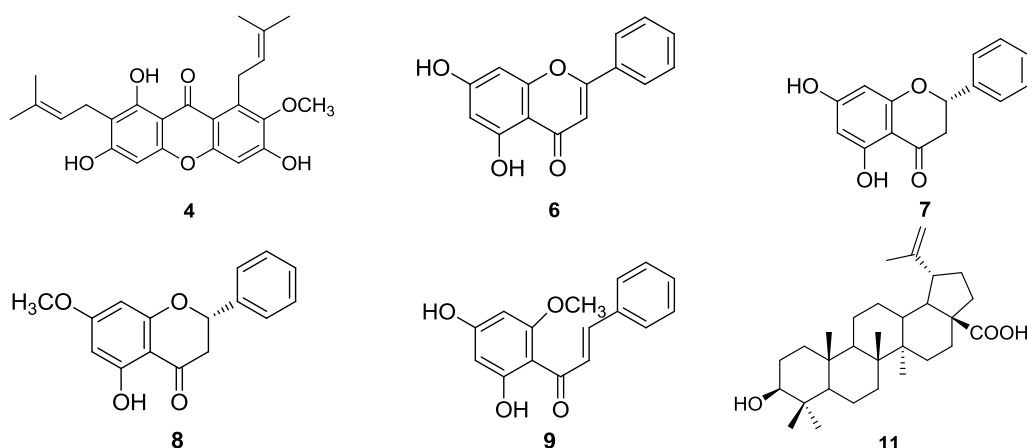


Fraction F5, eluted by 70-90% CH<sub>2</sub>Cl<sub>2</sub>-hexanes, was separated by repeated column chromatography to provide 3,6-phenanthrene-dione (**2**), 5-hydroxy-6,7,3',4',5'-pentamethoxyflavone (**5**), cleistanthin A (**12**) and 4-hydroxyacetophenone (**13**),



Fraction F6, eluted by 100% CH<sub>2</sub>Cl<sub>2</sub> and 1-5% MeOH-CH<sub>2</sub>Cl<sub>2</sub>, was further separated by repeated column chromatography to yield betulinic acid (**11**).

Fraction F7, eluted by 10-40% MeOH-CH<sub>2</sub>Cl<sub>2</sub>, was separated by repeated column chromatography to give  $\alpha$ -mangostin (**4**), 5,7-dihydroxyflavone (chrysin) (**6**), pinocembrin (**7**), pinostrobin (**8**), cardamonin (**9**) and cleistanthin A (**12**).



Fraction F8, eluted by 50-100% MeOH-CH<sub>2</sub>Cl<sub>2</sub> contained mainly sugars, therefore it was not further investigated.

All pure isolated compounds, except **6a/6b** were submitted for cytotoxic activity testings in a panel of cancer cell lines. The results are as shown in Table 1.10

**Table 1.10** Cytotoxic activities of pure compounds **1–13**, isolated from the aerial parts of *M. glomerulatus*

| Compound           | Cell lines (ED <sub>50</sub> µg/mL) <sup>a</sup> |                    |                    |                    |                    |                    |                    |
|--------------------|--------------------------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|                    | P-388                                            | KB                 | HT-29              | MCF-7              | A-549              | ASK                | HEK293             |
| <b>1</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>2</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>3</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>4</b>           | 2.29<br>(5.58 mM)                                | 1.75<br>(4.26 mM)  | 2.02<br>(4.92 mM)  | 1.92<br>(4.68 mM)  | >4                 | 2.72<br>(6.63 mM)  | 2.27<br>(5.53 mM)  |
| <b>5</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>6</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>7</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>8</b>           | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>9</b>           | 1.84<br>(6.81 mM)                                | 2.14<br>(7.92 mM)  | 3.89<br>(14.39 mM) | 2.61<br>(9.66 mM)  | 2.82<br>(10.43 mM) | >4                 | 2.56<br>(9.47 mM)  |
| <b>10</b>          | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>11</b>          | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>12</b>          | <0.05<br>(0.09 mM)                               | <0.05<br>(0.09 mM) | <0.05<br>(0.09 mM) | <0.05<br>(0.09 mM) | <0.05<br>(0.09 mM) | <0.05<br>(0.09 mM) | <0.05<br>(0.09 mM) |
| <b>13</b>          | >4                                               | >4                 | >4                 | >4                 | >4                 | >4                 | >4                 |
| <b>Ellipticine</b> | 0.46<br>(1.87 mM)                                | 0.68<br>(2.76 mM)  | 0.57<br>(2.31 mM)  | 0.51<br>(2.07 mM)  | 0.60<br>(2.44 mM)  | 0.63<br>(2.56 mM)  | 0.65<br>(2.64 mM)  |

<sup>a</sup>Cytotoxic assay: ED<sub>50</sub> < 4 µg/mL is considered active; P-388, murine lymphocytic leukemia; KB, human oral nasopharyngeal carcinoma; HT29, human colorectal adenocarcinoma; MCF-7, human breast cancer; A549, human lung carcinoma, ASK, rat glioma cell; Hek293, cell line derived from human embryonic kidney cells. Ellipticine was used as positive control.

## Sub-project 2: Cytotoxicity, Anticancer effects of natural derived compounds and their mechanism at molecular targets

### Abstract

Cancer remains the leading cause of death and becomes a significant public health burden worldwide including in Thailand and China. Chemotherapy is the gold standard for aggressive and metastasis cancer, however, the satisfactory outcome is poor because of the limitations from their side effects. Therefore, searching for novel compounds with great anticancer activities is our challenge. Herein, under the collaboration between Thai and Chinese scientists, we discover promising compounds from natural resources with anticancer activities. First, we screened 151 compounds from marine-derived actinobacteria and their analogs using sulforhodamine B assay. We found that 90 out of 151 (69.6%) exhibited cytotoxic activity. Among them, 27 compounds showed the IC<sub>50</sub> less than 10 mM against all 6 cancer cell lines. Next, we investigated the mechanisms related to the anticancer activity of compound HD ZWM 978 identified from the screening against gastric cancer cell lines, AGS, and MKN 45. HD ZWM 978 was potentially more cytotoxic than the clinically used etoposide, with IC<sub>50</sub> values at 48 h of 1.7±0.2 mM and 4.3±1.0 mM in AGS and MKN 45 cells, respectively, whereas the IC<sub>50</sub> values of etoposide were 8.6±2.4 mM and >20 mM in AGS and MKN 45 cells. Treatment with HD ZWM 978 markedly induced apoptotic cell death in AGS cells. Moreover, HD ZWM 978 significantly inhibited Topo II $\alpha$  activity leading to DNA damage as demonstrated by an increase in  $\gamma$ -H2A.X expression, the DNA damage marker, in a dose-dependent manner. The anticancer mechanism of HD ZWM 978 was further investigated in the WNT/b-catenin pathway and found that HD ZWM 978 significantly reduced the expression of b-catenin protein and WNT target gene; c-MYC, and survivin. Our results indicate that HD ZWM 978 induces gastric cancer cell apoptosis partly through induction of DNA damage-mediated by topoisomerase II $\alpha$  enzyme inhibition and inhibition of the WNT/ $\beta$ -catenin signaling pathway. In addition, we investigated the anticancer mechanism of two andrographolide analogs, analog 6 (19-triisopropyl-andrographolide) and analogs 3A.1 (19-tert-butyl-diphenylsilyl-8,17-epoxy andrographolide) on gastric cancer and colorectal cell lines, respectively. analog 6 exhibited highly cytotoxic than parent Andrographolide or the clinical drug etoposide with IC<sub>50</sub> values of 6.3±0.7 mM and 1.7±0.05 mM at 48 h for MKN 45 and AGS cells, respectively. Analogue 6 reduced the expression of Topo II enzyme, induced DNA damage, and activated PARP-1 and Caspase 3, leading to late apoptosis in AGS cells whereas treatment with the compound did not affect the expression of tumor suppressor p53. Similarly, analogs 3A.1 exhibited a significant cytotoxic effect against CRC cells and more potent than the parent compound. Mechanistically, analogs 3A.1 induced apoptotic cell death probably through inhibition of WNT/b-catenin signaling pathway-mediated DNA damages. Taken together, under the collaboration project between Thai and Chinese scientists, we able to discover the promising compounds that have the potential to be developed as novel anticancer compounds for cancer treatment.

**Keywords:** anticancer activity, medicinal plants, marine microbes, gastric cancer cells, colorectal cells

## บทคัดย่อ

โรคมะเร็งเป็นสาเหตุการตายและเป็นปัญหาทางสาธารณสุขทั่วโลก รวมถึงประเทศไทยและประเทศจีน การรักษาด้วยยาเคมีบำบัดถือเป็นวิธีการรักษามาตรฐานสำหรับมะเร็งที่มีการแพร่กระจาย แต่ผลสำเร็จของการรักษายังต่ำเนื่องจากผลข้างเคียงของการใช้ยา ดังนั้นการค้นหายาใหม่สำหรับการรักษาโรคมะเร็งจึงมีความสำคัญ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อค้นหาสารที่ออกฤทธิ์ต้านมะเร็งจากธรรมชาติ ภายใต้ความร่วมมือกับนักวิทยาศาสตร์ชาวจีน กลุ่มวิจัยได้คัดกรองสารที่ออกฤทธิ์ต้านมะเร็งจากสารที่คัดแยกได้จากแบคทีเรียใต้ทะเลและสารดัดแปลงโครงสร้างจำนวน 151 ตัว พบว่า 90 จาก 151 ตัว คิดเป็น 69.6 เปอร์เซ็นต์ ออกฤทธิ์ฆ่าเซลล์มะเร็ง ในจำนวนนี้มีสาร 27 ตัวมีฤทธิ์ดีเด่น โดยมีค่า IC<sub>50</sub> ต่ำกว่า 10  $\mu\text{M}$  ในเซลล์มะเร็งที่ใช้ทดสอบทั้งหมด 6 ชนิด กลุ่มวิจัยได้คัดเลือกสาร HD ZWM 978 มาศึกษาฤทธิ์ต้านมะเร็งและกลไกที่เกี่ยวข้องในเซลล์มะเร็งกระเพาะอาหาร 2 ชนิด คือ MKN 45 และ AGS จากการทดสอบพบว่าสาร HD ZWM 978 ออกฤทธิ์ฆ่าเซลล์มะเร็งกระเพาะอาหารได้ดีกว่า etoposide ซึ่งเป็นยาที่ใช้รักษาปัจจุบัน โดยมีค่า IC<sub>50</sub> ที่ 48 ชั่วโมง เท่ากับ  $1.7 \pm 0.2 \mu\text{M}$  และ  $4.3 \pm 1.0 \mu\text{M}$  สำหรับเซลล์ AGS และ MKN 45 ตามลำดับ ในขณะที่ค่า IC<sub>50</sub> ของ etoposide เท่ากับ  $8.6 \pm 2.4 \mu\text{M}$  และมากกว่า 20  $\mu\text{M}$  สำหรับเซลล์ AGS and MKN 45 ตามลำดับ สาร HD ZWM 978 ออกฤทธิ์เหนี่ยวนำให้เซลล์มะเร็งกระเพาะอาหารตายแบบ apoptosis การศึกษากลไกการออกฤทธิ์ในระดับโมเลกุลพบว่า สาร HD ZWM 978 ออกฤทธิ์ยับยั้งการทำงานของเอนไซม์ Topo II $\alpha$  โดยที่ความเข้มข้น 10  $\mu\text{M}$  สามารถยับยั้งฤทธิ์ของเอนไซม์ Topo II $\alpha$  ได้เกือบสมบูรณ์ นอกจากนี้ยังพบว่าสาร HD ZWM 978 สามารถเหนี่ยวนำให้เซลล์เกิดการแตกหักของสายดีเอ็นเอ ซึ่งประเมินได้จากการแสดงของตัวบ่งชี้ของการแตกหักของสายดีเอ็นเอ คือ  $\gamma$  H2A.X สาร เมื่อบ่มเซลล์ด้วยสาร HD ZWM 978 ยังพบว่าการแสดงออกของโปรตีน  $\beta$ -catenin และยีนเป้าหมายของกลไกสัญญาณ WNT/ $\beta$ -catenin คือ c-MYC, and survivin ลดลง แสดงว่าสาร HD ZWM 978 ออกฤทธิ์ยับยั้งกลไกสัญญาณ WNT/ $\beta$ -catenin ซึ่งเป็นกลไกสัญญาณที่สำคัญต่อพฤติกรรมความรุนแรงของเซลล์มะเร็ง จากผลการทดลองนี้สรุปได้ว่าสาร HD ZWM 978 เหนี่ยวนำเซลล์มะเร็งกระเพาะอาหารให้เกิดการตายแบบ apoptosis โดยยับยั้งการทำงานของเอนไซม์ Topo II $\alpha$  ซึ่งส่งผลให้เกิดการแตกหักของสายดีเอ็นเอและยังออกฤทธิ์ยับยั้งกลไกสัญญาณ WNT/ $\beta$ -catenin นอกจากนี้กลุ่มวิจัยยังได้ศึกษาฤทธิ์ต้านมะเร็งและกลไกที่เกี่ยวข้องของสารดัดแปลงโครงสร้างของ andrographolide 2 ชนิด คือ analogue 6 (19-triisopropyl-andrographolide) ในเซลล์มะเร็งกระเพาะอาหาร และ analogue 3A.1 (19-tert-butylidiphenylsilyl-8,17-epoxy andrographolide) ในเซลล์มะเร็งลำไส้ สาร analogue 6 ออกฤทธิ์ฆ่าเซลล์มะเร็งกระเพาะอาหารได้ดีกว่าสาร andrographolide และ etoposide โดยมีค่า IC<sub>50</sub> ที่ 48 ชั่วโมง เท่ากับ  $6.3 \pm 0.7 \mu\text{M}$  และ  $1.7 \pm 0.05 \mu\text{M}$  สำหรับเซลล์ AGS และ MKN 45 ตามลำดับ โดยสาร analogue 6 ออกฤทธิ์ยับยั้งเอนไซม์ Topo II ก่อให้เกิดการแตกหักของสายดีเอ็นเอ เกิดการตัดของโปรตีน PARP-1 และ Caspase 3 ซึ่งส่งผลให้เซลล์มะเร็งกระเพาะอาหารเกิดการตายแบบ apoptosis ในทำนองเดียวกัน สาร analogue 3A.1 ออกฤทธิ์ฆ่าเซลล์มะเร็งลำไส้ได้ดีกว่าสารตั้งต้น andrographolide โดยยับยั้งการทำงานของกลไกสัญญาณ WNT/ $\beta$ -catenin ส่งผลให้เกิดการตายของเซลล์มะเร็งลำไส้ใหญ่แบบ apoptosis จากการทดลองทั้งหมดแสดงให้เห็นว่า ภายใต้โครงการความร่วมมือระหว่างนักวิทยาศาสตร์ชาวไทยและจีนนี้ ส่งเสริมให้มีการค้นพบสารสำคัญที่มีศักยภาพสามารถนำไปพัฒนาต่อยอดเป็นยาต้านมะเร็งต่อไป

**คำสำคัญ:** ฤทธิ์ต้านมะเร็ง สมุนไพร เชื้อจากทะเล เซลล์มะเร็งกระเพาะอาหาร เซลล์มะเร็งลำไส้ใหญ่และทวารหนัก

## Introduction to the research problem and its significance:

Cancer is one of the most common causes of death worldwide. Based on the latest world cancer statistical data (WHO), global cancer burden rises to 14.1 million new cases in 2012 and 8.2 million cancer-related deaths occurred in 2012. Aging is one major risk factor for cancer development. It has been shown that 60% of newly diagnosed malignancies were reported in persons over 65 year-old and accounting for 70% of all cancer deaths (Berger et al., 2006). Cellular senescence play a role in oncogenic stimuli like DNA damage leading to disruption of oncogene or tumor suppressor gene expression in aging person (Campisi, 2013). Due to the increase in ageing of the global population, it predicts a substantial increase to 19.3 million new cancer cases per year by 2025 (GLOBOCAN 2012). World Health Organization has been reported that the population in their elderly years is rapidly increase in Thailand and China. In Thailand the people with more than 60 year-old of age is increase from 8.7% in 2000 to 30% in the year of 2050. Cancer is also the first leading of death in Thailand and China for many years. The mortality rate in these two countries is continuously increased. China and Thailand share the most common types of cancers. Lung cancer is the most common cancer found in Chinese population, followed by stomach, liver, esophageal and colorectal cancers (Zhao et al., 2010) whereas lung, liver and bile duct, colon, stomach, esophagus, breast and cervix cancer are prevalence in Thailand (National Cancer Institute of Thailand, 2012). Not only the above cancer types, some of rare cancer case like pancreatic cancer which has low 5-year survival rate (6.7%) is also recognized as the significant public health burden in China and Thailand. At present, chemotherapy is one of the principal method for *cancer treatment*, however, the effectiveness of most of current anticancer drugs is limited by *its adverse effect and development of drug resistance*. The toxicity to normal cells accounting for the adverse effects linked to these drugs is unavoidable. Throughout these years, natural products remain the major source of new anticancer drugs or lead compounds such as paclitaxel, docetaxel, vincristine, vinblastine, irinotecan, etoposide and bleomycin (da Rocha et al., 2001). However, the cost of these drugs is high. Thailand and china have a long history of using herbal medicine for treatment variety of diseases including cancer. In Thailand, some indigenous medicinal herbal compounds are found to be effective for cancer treatment but the sensitivity is poor. The structural modification of these compounds have showed a great improvement on the biological activities (Nateewattana et al., 2014; Nateewattana et al., 2013; Sirion et al., 2012; Wanitchakool et al., 2012). Therefore, the discovery and development of new anticancer drugs or lead compounds from natural products either from plants, marine, soil or other special microbes are our challenging subjects for Thai and China scientists. Furthermore, one current approach to overcome the complicated regulating molecular mechanisms in cancer is the development of drug targeting system (DDS). Drug delivery is designed to administer

pharmaceutical agents as an alternative choice in order to increase safety and efficacy with remained therapeutic levels. Several targeting systems have been suggested for anticancer drugs including bead delivery, controlled release, liposomal and nanoparticulate systems (Perez-Herrero and Fernandez-Medarde, 2015). The drug delivery with nanoparticles, in addition to have the capacity to transport the particular compounds to specific target cancer sites, it is also able to solve the problems of low solubility of water insoluble compounds (Puntawee et al., 2015). In this project, our group interests in many types of cancers that commonly found in Thai and Chinese population including cholangiocarcinoma, lung, colorectal cancer, breast cancer, head and neck cancer, ovarian cancer, bone cancer. The projects will cover from the candidate compound and target identification, structure optimization *in vitro* system to *in vivo* preclinical study as well as the development of drug targeting system.

#### Literature review:

Cancer is one of the most common causes of death worldwide. Based on the latest world cancer statistical data (WHO), global cancer burden rises to 14.1 million new cases in 2012 and 8.2 million cancer-related deaths occurred in 2012. Aging is one important risk factor for cancer development. It has been shown that 60% of newly diagnosed malignancies were reported in persons over 65 year-old and accounting for 70% of all cancer deaths (Berger et al., 2006). Cellular senescence play a role in oncogenic stimuli like DNA damage leading to disruption of oncogene or tumor suppressor gene expression in aging person (Campisi, 2013). World Health Organization has been reported that the population in their elderly years is rapidly increased in Thailand and China. In Thailand the people with more than 60 year-old of age is increase from 8.7% in 2000 to 30% in the year of 2050. Due to the increase in ageing of the global population, it predicts a substantial increase to 19.3 million new cancer cases per year by 2025 (GLOBOCAN 2012). At present, chemotherapy is one of the principal methods for cancer treatments; however, the effectiveness of most of current anticancer drugs is limited by *its adverse effect and development of drug resistance*. The natural products and their derivatives account for more than 50% of all drugs in clinical use of the world including for anticancer drugs. Plants and marine organisms are the important resources for the discovery of new anticancer drug. Doctetaxel and paclitaxel, two well-known examples of anticancer drugs were originally discovered from plants (da Rocha et al., 2001). In addition, anticancer drug discovery from marine natural products has been reported. Ecteinascidin-743 (ET-743/trabectedin), the first anticancer derived from *Ecteinascidia turbinata* was approved by the European Commission in July 2007. The Phase I and Phase II studies demonstrated the remarkable antitumor activity against solid tumors, in particular breast and renal carcinoma (Molinski et al., 2009). Up to date, several compounds have been extracted from marine products and shown antitumor activity that have been in

clinical trial studies. The different in chemical and biological diversity of marine organisms make them become an amazing resource for the discovery of new anticancer drug.

Drug resistance remains the main cause of cancer treatment failure. The major mechanism of drug resistance in cancer is related to the expression of multidrug resistance (MDR) proteins belonging to ATP-binding cassette (ABC) family of membrane transport ATPase (Kathawala et al., 2015). Upregulation of these membrane proteins, MDR1/ABCB1, MRP1/ABCC1 and BCRP/ABCG2 in many types of cancer have been reported to be associated with the resistance to many of recent chemotherapeutic drugs such as doxorubicin, vinblastine and methotrexate (Kathawala et al., 2015). Therefore, the identification of their inhibitors have been a major goal for cancer treatment. Indeed, many of MDR modulators have been discovered from the marine and natural compounds such as Ecteinascidin 743, Agosterol A, curcumin, nobiletin, ginsenoside Rg3, silymarin and glycyrrhetic acid (Kathawala et al., 2015; Wang et al., 2010).

Targeting cancer-related cellular signaling pathway or component has held the interest of discovery the active anticancer drugs. Aberrant activation of Wnt signaling pathway is associated with cancer development such as colon cancer, breast cancer, melanoma, head & neck cancer, non-small-cell lung cancer, gastric cancer (Barker and Clevers, 2006). Wnt/ $\beta$ -catenin signaling pathway is characterized by cytoplasmic accumulation of  $\beta$ -catenin, subsequent nuclear translocation and activates the cancer phenotype-related target gene expression (Gordon and Nusse, 2006). Therefore, wnt/ $\beta$ -catenin signaling is interesting target for anticancer drug discovery. Indeed, natural products like flavonoids (flavonone, luteolin, apigenin, genistein, kaempferol, isoquercitrin) has potential to be develop as anticancer drugs through inhibition of several proteins in wnt/ $\beta$ -catenin signaling (Amado et al., 2014)

DNA topoisomerase enzymes are also one of the promising cellular targets for developing anticancer drugs. DNA topoisomerase II (Top2) plays a critical role for ensuring the genomic integrity. It controls DNA replication, transcription and chromosome segregation (Nitiss, 2009). Alteration of its activity generates enzyme mediated DNA damage and is used as target for chemotherapeutic agents. The alteration of Top2 activity by natural products and it analogue has been report. Etoposide, a synthetic chemical compound from *Podophyllum peltatum* is used for treatment of lung and testicular tumor with a significant inhibition potency on Top2 activity (Nitiss, 2009). Recently our group reported the semi-synthetic andrographolide analogues mediated the inhibition of Top2 activity in cancer cell line (Nateewattana et al., 2013).

## Objectives

1. Screening for cytotoxic activity of natural products from Chinese Marine Microbes and Thai Medicinal Plants.

2. Identification the molecular mechanisms and molecular target of the candidate compounds.

## Results and Discussion

### Part I: Screening for cytotoxic activity of natural products from Chinese Marine Microbes and Thai Medicinal Plants.

Compounds were kindly provided by Prof. Patoomratana Tuchinda, Mahidol University and Prof. Weiming Zhu, Ocean University of China. The effect of compound on cell viability was determined by using the colorimetric SRB assay. Briefly, cancer cell lines were seeded in a 96-well plates. Cells were cultured for 24 h and then treated with various concentrations of compounds 72 h. In the control group, the cells were cultured in medium containing equivalent amount of DMSO required to dissolve the compounds. At the end of every specific duration of incubation, cell viability was determined by using the colorimetric SRB assay. As shown in Table 1 and 2, several compounds from both sources exhibited the cytotoxic effect against cancer cell lines. Interestingly, we screened 151 compounds from marine-derived actinobacteria and their analogues (all from Prof. Weiming Zhu, Ocean University of China) using sulforhodamine B assay. We found that 90 out of 151 (69.6%) exhibited cytotoxic activity. Among them, 27 compounds showed the  $IC_{50}$  less than 10  $\mu M$  against all 6 cancer cell lines. We found that 90 out of 151 (69.6%) exhibited cytotoxic activity. We therefore selected some of these compound for further mechanistic study in next experiment.

**Table 2.1.**  $IC_{50}$  values of tested compounds against cancer cell lines (Compounds were obtained from Prof. Patoomratana Tuchinda, Mahidol University)

| Compounds   | $IC_{50}$ ( $\mu M$ ) |       |       |       |       |       |         |        |
|-------------|-----------------------|-------|-------|-------|-------|-------|---------|--------|
|             | P-388                 | KB    | HT-29 | MCF-7 | A549  | ASK   | HuCCA-1 | HEK293 |
| Ellipticine | 2.07                  | 2.26  | 2.32  | 1.53  | 2.32  | 1.80  | 2.83    | 1.76   |
| MUC941      | >100                  | >100  | >100  | >100  | >100  | >100  | >100    | >100   |
| MUC942      | >100                  | 92.87 | >100  | >100  | >100  | >100  | >100    | >100   |
| MUC943      | >100                  | >100  | >100  | >100  | >100  | >100  | >100    | >100   |
| MUC944      | >100                  | >100  | >100  | >100  | >100  | >100  | >100    | >100   |
| MUC1020     | 58.73                 | 81.22 | 77.14 | 91.63 | 91.99 | 64.19 | 74.36   | 51.67  |
| MUC1021     | >100                  | >100  | >100  | >100  | >100  | 96.47 | >100    | 73.99  |
| MUC1022     | 51.93                 | 91.83 | >100  | >100  | >100  | 72.03 | >100    | 59.29  |
| MUC1023     | 54.07                 | >100  | >100  | >100  | >100  | 80.18 | >100    | 71.70  |
| MUC1028     | 68.30                 | >100  | >100  | >100  | >100  | >100  | >100    | 91.86  |



|         |       |       |      |       |       |       |      |       |
|---------|-------|-------|------|-------|-------|-------|------|-------|
| MUC1029 | 3.29  | 4.92  | 3.96 | 3.96  | 3.07  | 1.65  | 4.9  | 0.95  |
| MUC1030 | >100  | >100  | >100 | >100  | >100  | >100  | >100 | >100  |
| MUC1031 | >100  | >100  | >100 | >100  | >100  | >100  | >100 | >100  |
| MUC1032 | 5.93  | 34.91 | 9.05 | 25.23 | 8.47  | 5.41  |      | 4.72  |
| MUC1255 | 24.35 | 20.28 | >50  | 22.40 | 27.99 | 41.56 |      | 18.60 |
| MUC1261 |       | <0.1  | <0.1 | <0.1  | <0.1  | <0.1  |      |       |
| MUC1262 |       | >50   | >50  | >50   | >50   | >50   |      |       |
| MUC1263 |       | >50   | >50  | >50   | >50   | >50   |      |       |

Table 2.2 IC<sub>50</sub> values of tested compounds against cancer cell lines (Compounds were obtained from Prof. Weiming Zhu, Ocean University of China)

| Compound    | IC <sub>50</sub> (μM) |       |       |       |       |         |       |
|-------------|-----------------------|-------|-------|-------|-------|---------|-------|
|             | P-388                 | KB    | HT-29 | MCF-7 | A 549 | HEK 293 | ASK   |
| Ellipticine | 2.24                  | 2.33  | 2.43  | 2.14  | 2.55  | 2.05    | 1.65  |
| 552         | 47.58                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 553         | 16.57                 | 6.66  | 27.99 | 20.5  | 28.67 | 27.71   | 27.09 |
| 801         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 802         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 803         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 804         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 805         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 806         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 807         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 808         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 809         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 810         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 811         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 812         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 813         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 814         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 815         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 816         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 877         | 6.26                  | 7.16  | 29.78 | 19.72 | 19.32 | 20.67   | 25.64 |
| 878         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 879         | 33.18                 | 30.69 | > 50  | > 50  | 29.14 | > 50    | 32.80 |
| 888         | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |

| Compound | IC <sub>50</sub> (μM) |       |       |       |       |         |       |
|----------|-----------------------|-------|-------|-------|-------|---------|-------|
|          | P-388                 | KB    | HT-29 | MCF-7 | A 549 | HEK 293 | ASK   |
| 889      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 890      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 891      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 892      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 909      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 910      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 911      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 912      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 913      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 954      | 0.58                  | 0.81  | 0.83  | 0.65  | 0.70  | 0.63    | 0.67  |
| 960      | 2.31                  | 5.61  | 15.88 | 9.17  | 8.46  | 14.73   | 7.52  |
| 961      | 12.74                 | 34.99 | > 50  | > 50  | > 50  | > 50    | > 50  |
| 962      | 28.82                 | 5.11  | 29.61 | 33.92 | 25.37 | 24.77   | 31.91 |
| 963      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 817      | > 50                  | > 50  | > 50  | > 50  | 45.3  | > 50    | > 50  |
| 818      | > 50                  | > 50  | > 50  | > 50  | 39.2  | > 50    | 44.9  |
| 819      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 820      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 821      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 822      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 823      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 824      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 825      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 965      | 5.30                  | 5.80  | 8.26  | 7.85  | 5.53  | 5.58    | 6.99  |
| 966      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 968      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 969      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 970      | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 978      | 4.77                  | 5.06  | 6.71  | 6.06  | 0.91  | 5.81    | 3.23  |
| 1003     | 6.45                  | 7.83  | 16.73 | 9.48  | 23.08 | 8.17    | 7.36  |
| 1004     | 4.62                  | 5.57  | 6.29  | 5.19  | 4.63  | 5.35    | 4.99  |
| 1006     | 5.55                  | 5.21  | 5.29  | 5.71  | 5.21  | 5.34    | 4.84  |
| 1007     | 0.66                  | 5.10  | 6.23  | 5.29  | 5.58  | 6.69    | 5.26  |
| 1008     | 3.16                  | 3.75  | 2.39  | 2.94  | 3.83  | 4.22    | 2.79  |
| 1011     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |

| Compound | IC <sub>50</sub> (μM) |       |       |       |       |         |       |
|----------|-----------------------|-------|-------|-------|-------|---------|-------|
|          | P-388                 | KB    | HT-29 | MCF-7 | A 549 | HEK 293 | ASK   |
| 1012     | 8.64                  | 5.72  | 6.34  | 7.04  | 5.54  | 5.31    | 19.83 |
| 1014     | 40.54                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1015     | 0.85                  | 0.88  | 3.23  | 5.64  | 5.24  | 0.82    | 4.89  |
| 1016     | 18.49                 | 5.98  | 6.31  | 6.82  | 6.82  | 7.09    | 9.84  |
| 1017     | 9.61                  | 7.09  | 8.30  | 7.28  | 8.53  | 6.80    | 9.37  |
| 1020     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1021     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1024     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1041     | 6.11                  | 34.00 | 32.69 | 21.54 | 8.49  | 7.20    | 18.66 |
| 1061     | 37.58                 | > 50  | > 50  | > 50  | > 50  | 45.56   | 43.81 |
| 1062     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1065     | 31.35                 | > 50  | 44.15 | 44.24 | 10.07 | 32.89   | 15.64 |
| 1066     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1067     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1081     | 5.08                  | 21.65 | 33.02 | 20.33 | 7.80  | 27.70   | 4.84  |
| 1083     | 5.35                  | 27.61 | > 50  | 22.74 | 8.62  | > 50    | 5.13  |
| 1085     | 27.56                 | 4.47  | 39.88 | 5.29  | 11.48 | 6.26    | 32.38 |
| 1086     | 5.20                  | 2.92  | 9.38  | 4.35  | 4.58  | 4.12    | 21.70 |
| 1087     | 4.57                  | 0.62  | 6.07  | 0.71  | 2.69  | 0.78    | 5.86  |
| 1088     | 4.98                  | 0.64  | 6.13  | 0.79  | 2.98  | 0.83    | 6.14  |
| 1089     | 7.30                  | 4.86  | 28.86 | 8.69  | 6.12  | 6.13    | 7.79  |
| 1090     | > 50                  | > 50  | > 50  | 41.92 | > 50  | > 50    | > 50  |
| 1091     | > 50                  | 25.64 | > 50  | > 50  | 39.24 | > 50    | > 50  |
| 1092     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1093     | 43.31                 | 42.48 | > 50  | > 50  | 47.94 | > 50    | > 50  |
| 1094     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1095     | > 50                  | > 50  | > 50  | > 50  | 27.66 | > 50    | 36.19 |
| 1097     | > 50                  | 47.22 | > 50  | > 50  | 30.34 | 32.68   | > 50  |
| 1099     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1100     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1101     | 42.91                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1102     | > 50                  | 27.46 | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1103     | > 50                  | 30.00 | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1104     | 3.43                  | 7.38  | 8.80  | 7.73  | 8.73  | 6.21    | 7.35  |
| 1105     | 5.26                  | 5.72  | 5.36  | 5.44  | 4.76  | 9.40    | 4.92  |

| Compound | IC <sub>50</sub> (μM) |       |       |       |       |         |       |
|----------|-----------------------|-------|-------|-------|-------|---------|-------|
|          | P-388                 | KB    | HT-29 | MCF-7 | A 549 | HEK 293 | ASK   |
| 1106     | 7.06                  | 18.05 | 22.11 | 27.48 | 4.20  | 43.12   | 5.13  |
| 1107     | 3.19                  | 5.25  | 5.11  | 5.37  | 4.93  | 5.01    | 4.63  |
| 1108     | 3.71                  | 4.96  | 5.06  | 4.72  | 4.65  | 4.96    | 4.68  |
| 1109     | 1.72                  | 5.03  | 4.75  | 4.90  | 4.64  | 5.07    | 4.51  |
| 1110     | 3.62                  | 5.04  | 4.66  | 4.77  | 5.08  | 5.04    | 4.67  |
| 1113     | 4.00                  | 21.16 | 32.52 | 7.50  | 13.81 | 27.48   | 8.80  |
| 1114     | 4.66                  | 4.94  | 5.31  | 5.21  | 4.94  | 5.07    | 4.65  |
| 1115     | 4.73                  | 4.96  | 5.40  | 5.19  | 4.86  | 5.08    | 4.65  |
| 1116     | 0.99                  | 6.62  | 19.54 | 5.49  | 6.83  | 20.98   | 5.93  |
| 1117     | 3.46                  | 5.87  | 7.95  | 5.47  | 5.55  | 6.24    | 5.55  |
| 1118     | 0.83                  | 14.15 | 44.14 | 9.99  | 23.17 | 36.77   | 9.81  |
| 1119     | 5.37                  | 5.22  | 5.21  | 4.91  | 4.96  | 5.06    | 4.75  |
| 1120     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1121     | 5.30                  | 14.93 | 18.70 | 17.78 | 17.49 | 6.16    | 22.07 |
| 1122     | 5.33                  | 23.98 | 22.24 | 22.70 | 25.25 | 27.51   | 23.55 |
| 1123     | 5.32                  | 5.06  | 5.12  | 4.97  | 5.09  | 5.09    | 4.57  |
| 1124     | 4.09                  | 4.98  | 4.89  | 4.68  | 5.02  | 5.11    | 4.54  |
| 1125     | 3.50                  | 9.03  | 30.07 | 6.17  | 8.30  | 9.33    | 7.05  |
| 1126     | 5.69                  | 27.20 | 32.57 | 9.14  | 19.51 | > 50    | 18.80 |
| 1127     | 9.38                  | > 50  | > 50  | 8.93  | > 50  | > 50    | > 50  |
| 1137     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1142     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1143     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1144     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1145     | 40.24                 | > 50  | 47.32 | > 50  | > 50  | > 50    | > 50  |
| 1146     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1147     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1148     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1149     | 20.87                 | 33.78 | 41.19 | 29.96 | > 50  | 29.22   | 33.82 |
| 1150     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1151     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1152     | 31.93                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1153     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1154     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1155     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |

| Compound | IC <sub>50</sub> (μM) |       |       |       |       |         |       |
|----------|-----------------------|-------|-------|-------|-------|---------|-------|
|          | P-388                 | KB    | HT-29 | MCF-7 | A 549 | HEK 293 | ASK   |
| 1156     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1157     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1158     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1159     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1160     | 34.00                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1161     | 5.61                  | 5.50  | 5.22  | 4.64  | 20.01 | 4.97    | 5.08  |
| 1162     | 35.97                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1163     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1164     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1165     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1166     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1167     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1168     | 3.55                  | 5.03  | 5.78  | 7.16  | 4.91  | 4.61    | 5.31  |
| 1169     | 5.75                  | 7.40  | 6.01  | 5.86  | 5.59  | 3.87    | 5.70  |
| 1170     | 0.40                  | 5.29  | 5.47  | 0.99  | 0.86  | 0.57    | 0.82  |
| 1171     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1172     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1173     | 26.81                 | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1174     | > 50                  | > 50  | > 50  | > 50  | 27.95 | > 50    | > 50  |
| 1175     | 17.19                 | 28.93 | 42.31 | 31.47 | 27.97 | 21.56   | 28.26 |
| 1176     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1177     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1178     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |
| 1179     | > 50                  | > 50  | > 50  | > 50  | > 50  | > 50    | > 50  |

Cell lines: Murine lymphocytic leukemia (P-388), Human epidermoid carcinoma in the mouth (KB), Human colon cancer (HT-29), Human breast cancer (MCF-7), Human lung cancer (A-549), Rat glioma cell (ASK), Human embryonic kidney cell (HEK 293), Human breast cancer (MCF-7), Liver cancer (HepG2 and Hep3B), liver cell (Chang liver or CL), cholangiocarcinoma (HuCCA-1)

**Part II: Identification the molecular mechanisms and molecular target of the candidate compounds.**

## 2.1 Anticancer activities of Marine Actinobacterium secondary metabolite (HD ZWM

978) on gastric cancer cell lines. HD ZWM 978 was kindly provided by Prof. Weiming Zhu, Ocean University of China

2.1.1 Cytotoxic effects of HD ZWM 978 on gastric cancer cells (MKN 45 and AGS cell lines)

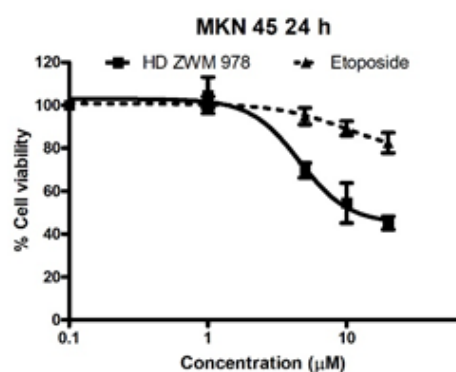
HD ZWM 978, a secondary metabolite from Marine *Actinobacterium* was investigated for its cytotoxic activities in MKN 45 gastric cancer cells at 24, 48 and 72 h using MTT assay. Etoposide, an anticancer drug, was used as a positive control. As shown in Fig. 2.1 and Table 3, the compound exhibits cytotoxic activities in a dose-related manner. The half maximal inhibitory concentration for cell survival ( $IC_{50}$  value) of HD ZWM 978 was  $14.2 \pm 1.8$ ,  $4.3 \pm 1.0$  and  $4.7 \pm 0.4$   $\mu M$  after treatment for 24, 48 and 72 h, respectively. HD ZWM 978 was more potent than the clinically used anticancer drug, etoposide, having  $IC_{50}$  of  $>20$   $\mu M$  at 24, 48 h and  $16 \pm 2.3$   $\mu M$  at 72 h.

Similarly, HD ZWM 978 was also toxic to other gastric cancer cell line (AGS cells), as shown in Fig. 2.2 and Table 2.3. At 24 h,  $IC_{50}$  values for HD ZWM 978 and etoposide are  $4.9 \pm 1.3$ , and  $19.6 \pm 0.9$   $\mu M$ , respectively. The cytotoxic activity of HD ZWM 978 was substantially increased at 48 h and 72 h with  $IC_{50}$  values of  $1.7 \pm 0.2$ , and  $2.1 \pm 0.3$   $\mu M$ , respectively. More importantly, the cytotoxicity of HD ZWM 978 was greater than the clinically used etoposide. As AGS cells were more sensitive to HD ZWM 978, it was chosen for further investigation on the mechanisms of action in details.

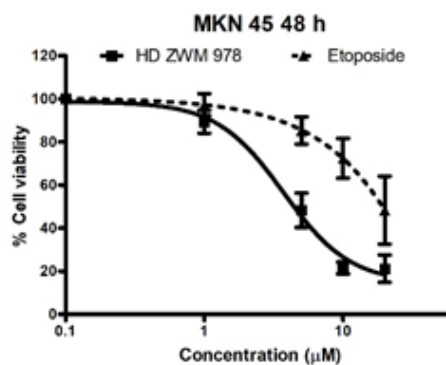
**Table 2.3**  $IC_{50}$  values of HD ZWM 978 gastric cancer cell lines Data are means  $\pm$  SEM of three independent experiments performed in triplicates.

| Cell lines | Incubation time (h) | $IC_{50}$ ( $\mu M$ ) |                |
|------------|---------------------|-----------------------|----------------|
|            |                     | Etoposide             | HD ZWM 978     |
| MKN 45     | 24                  | $>20$                 | $14.2 \pm 1.8$ |
|            | 48                  | $>20$                 | $4.3 \pm 1.0$  |
|            | 72                  | $16 \pm 2.3$          | $4.7 \pm 0.4$  |
| AGS        | 24                  | $>20$                 | $4.9 \pm 1.3$  |
|            | 48                  | $8.6 \pm 2.4$         | $1.7 \pm 0.2$  |
|            | 72                  | $2.9 \pm 0.5$         | $2.1 \pm 0.3$  |

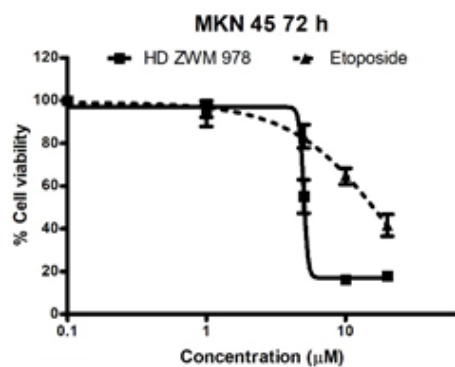
A



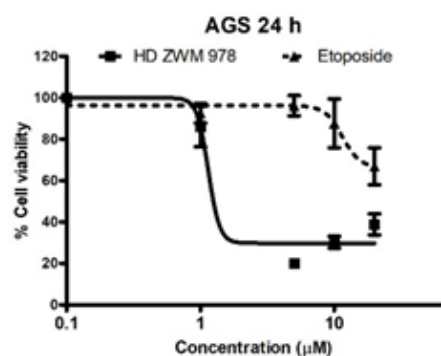
B



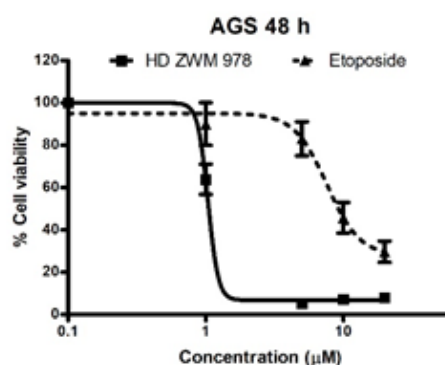
C



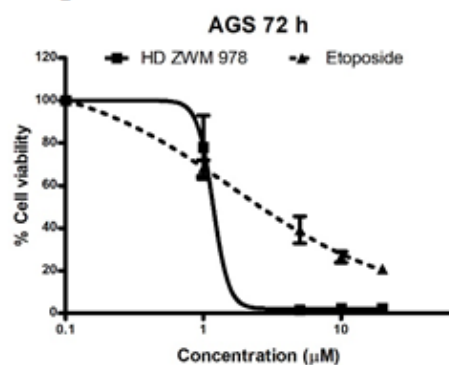
A



B



C

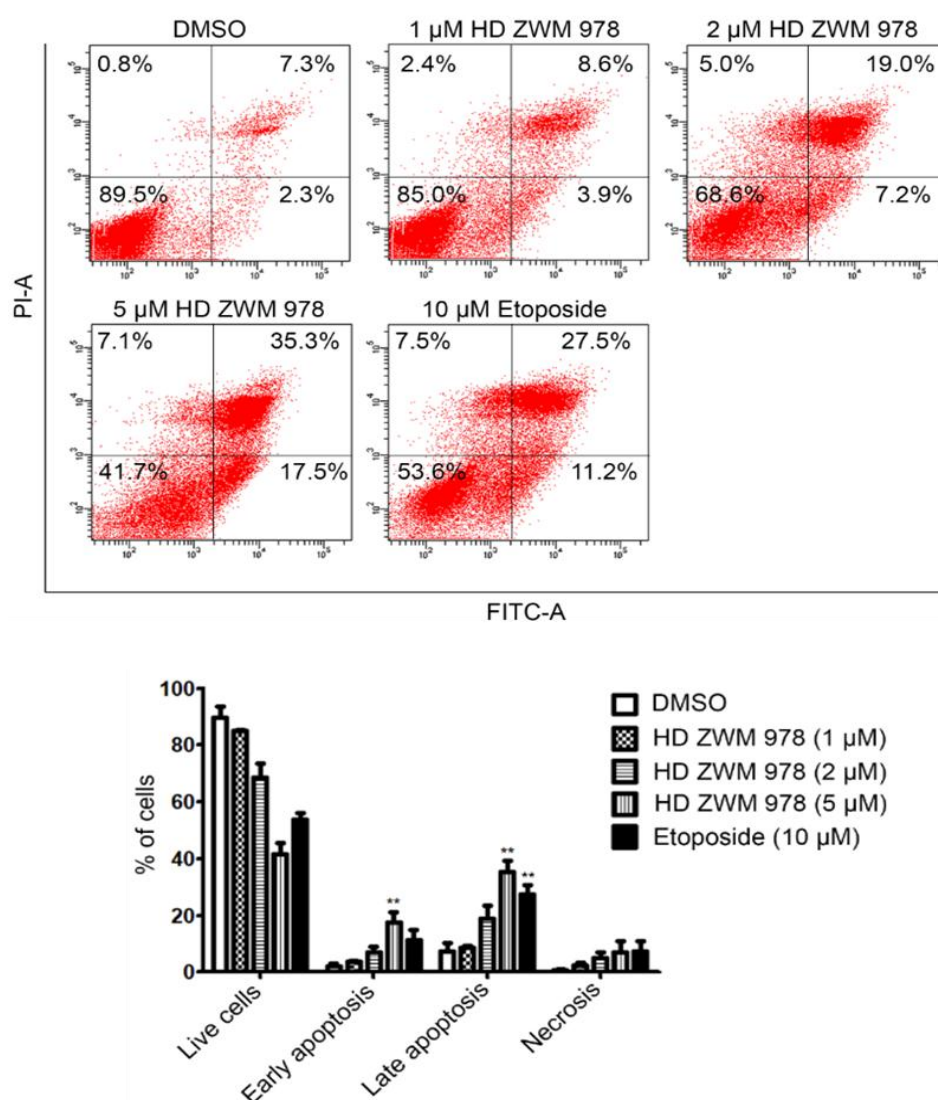


**Figure 2.1** Cytotoxic effect of HD ZWM 978 on MKN 45 cells. Cells were treated with 0.1-20  $\mu\text{M}$  of HD ZWM 978, and etoposide for 24 h (A), 48 h (B) and 72 h (C). MTT assay was used to evaluate cell viability.

**Figure 2.2** Cytotoxic effect of HD ZWM 978 on AGS cells. Cells were treated with 0.1-20  $\mu\text{M}$  of HD ZWM 978, and etoposide for 24 h (A), 48 h (B) and 72 h (C). MTT assay was used to evaluate cell viability.

### 2.1.2 Induction of cellular apoptosis by HD ZWM 978 in human gastric cancer cells

AGS cells were treated with HD ZWM 978 at 1, 2 and 5  $\mu\text{M}$  and etoposide at 10  $\mu\text{M}$  for 48 h. HD ZWM 978 induced early and late apoptosis in a dose related manner. Early apoptosis was respectively increased to  $3.9\pm0.2\%$ ,  $7.2\pm1.7\%$  and  $17.5\pm3.6\%$  by HD ZWM 978 at 1, 2 and 5  $\mu\text{M}$ , respectively, as compared to DMSO control ( $2.3\pm0.7\%$ ). Etoposide induced the early apoptosis up to  $11.2\pm3.8\%$ . In addition, HD ZWM 978 at the concentration of 1, 2 and 5  $\mu\text{M}$ , induced the late apoptosis up to  $8.6\pm0.8\%$ ,  $19.0\pm4.3\%$  and  $35.3\pm4.0\%$  as compared to DMSO control ( $7.3\pm3.0\%$ ). Likewise, etoposide at 10  $\mu\text{M}$  induced the late apoptosis significantly up to  $27.5\pm3.0\%$ . Of note HD ZWM 978 is more potent than etoposide in inducing apoptotic cell death process and it mediates cytotoxicity by causing apoptotic cell death mechanism.



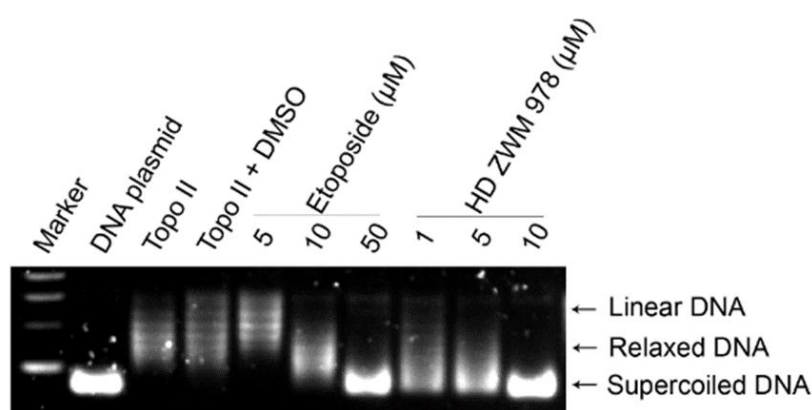
**Figure 2.3** HD ZWM 978 induces apoptosis in AGS cells. (*Upper*) Cells were treated with HD



ZWM 978 and etoposide at the indicated concentrations for 48 h, stained by Annexin V-FITC and PI, and analyzed by flow cytometry. (Lower) Percentage of cell population in each conditions. Data are means  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  significantly different from vehicle control (Turkey's multiple comparison test)

### 2.1.3 Inhibition of human topoisomerase II $\alpha$ activity by HD ZWM 978

To investigate the underlying molecular mechanisms of HD ZWM 978 induced apoptosis, its inhibition on topoisomerase II $\alpha$  enzyme activity was conducted using supercoiled plasmid DNA relaxing assay. Etoposide (a first line topoisomerase II $\alpha$  poison drug) was used as a positive control. As shown in Fig. 2.4, lane 2 represents the supercoiled pBR322 DNA without Topo II enzyme. HD ZWM 978 and Etoposide inhibited the Topo II enzyme activity in a dose-related manner. HD ZWM 978 at 10  $\mu$ M fully inhibited the topoisomerase II $\alpha$  enzyme activity. Of note, etoposide at 10  $\mu$ M partially inhibited the enzyme activities. The topoisomerase II $\alpha$  activity was fully inhibited at the concentration as high as 50  $\mu$ M. This result suggests that HD ZWM 978 was acting as a Topo II inhibitor and it is more potent than etoposide. This finding was consistent with its cytotoxic and apoptotic effects that HD ZWM 978 exhibited greater effect than etoposide.

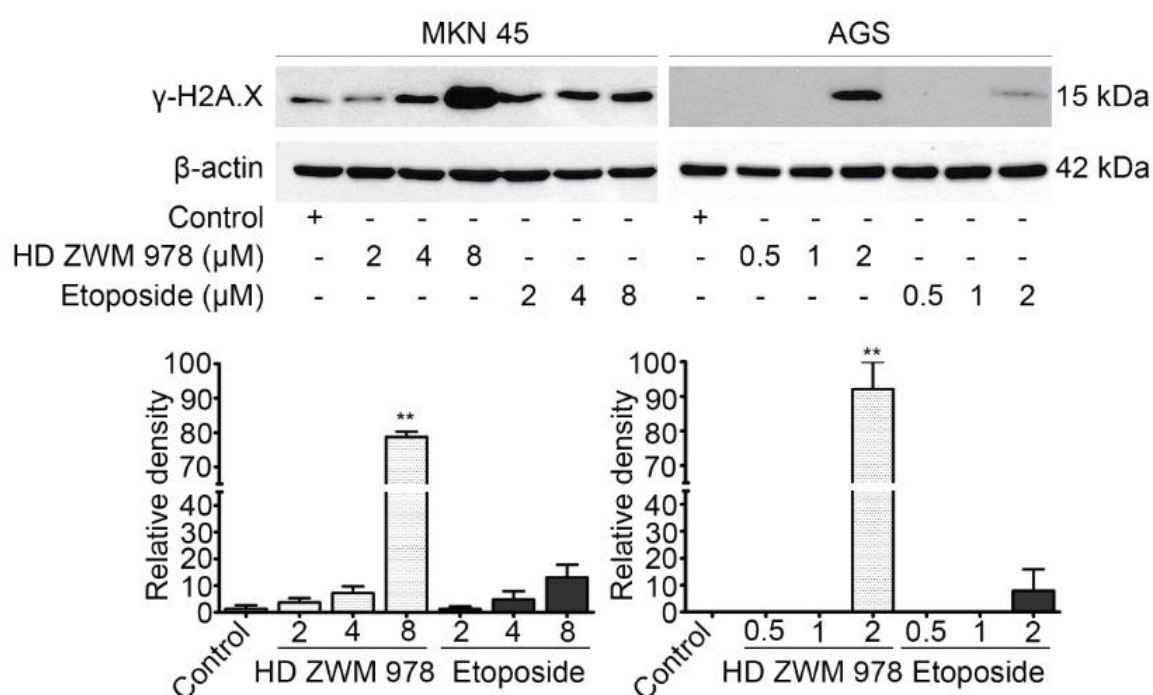


**Figure 2.4** The inhibitory effects of HD ZWM 978 and etoposide on topoisomerase II $\alpha$  enzyme activity. Supercoiled pBR322 plasmid were incubated with human topoisomerase II $\alpha$  alpha enzyme at indicated concentrations of HD ZWM 978 and etoposide at 37°C for 1 h. The samples were electrophoresed using 1% agarose gel, stained with SYBR safe dye and photographed under gel documentation machine.

### 2.1.4 Effect of HD ZWM 978 on the induction of DNA damage by in human gastric cancer cells

Inhibition of topoisomerase II $\alpha$  activity results apoptosis cell death partly through DNA damage. We therefore investigated the effect of HD ZWM 978 on induction

DNA damage in human gastric cancer cell lines. The expression of  $\gamma$ -H2A.X, DNA damage marker, was determined by western blotting after treatment with various concentrations of HD ZWM 978 and etoposide. HD ZWM 978, and etoposide at the concentrations of 2, 4 and 8  $\mu$ M markedly increased the expression of  $\gamma$ -H2A.X in a dose-related manner in MKN45 cell line (Fig.2.5). Similarly, treatment with 2  $\mu$ M of HD ZWM 978 and etoposide increased the expression of  $\gamma$ -H2A.X protein in AGS cells. Of note, HD ZWM 978 was more potent in inducing DNA damage than the clinically used etoposide in both the gastric cancer cell lines. This finding correlates with the previous results and suggests that HD ZWM 978 induces apoptosis in GC cells partially through the inhibition of topoisomerase II $\alpha$  enzyme activity.

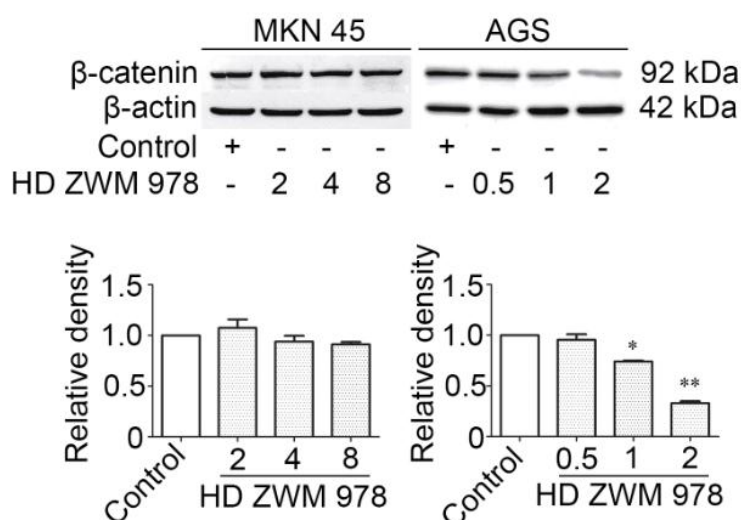


**Figure 2.5** DNA damage induced by HD ZWM 978 and etoposide in gastric cancer cell lines (MKN 45 and AGS cells). Cells were treated with HD ZWM 978 and etoposide at indicated concentrations for 24 h. Levels of  $\gamma$ -H2A.X protein were detected from total protein lysates by western blotting.  $\beta$ -actin was used as the loading control. Bar graph represents the relative calculated density, normalized with  $\beta$ -actin. \*\* $p$ <0.01 significantly different from vehicle control (Turkey's multiple comparison test).

### 2.1.5 Inhibitory effect of HD ZWM 978 on WNT/ $\beta$ -catenin signaling pathway in gastric cancer in gastric cancer cells

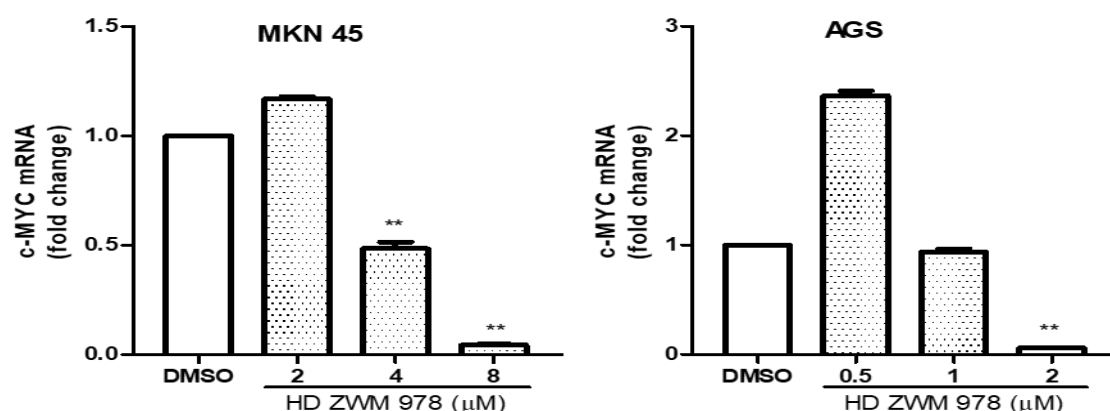
Aberrant activation of WNT/ $\beta$ -catenin signaling pathway is one of major cause of gastric cancer development and progression. To investigate whether anticancer property of

HD ZWM 978 involvement with inhibition of WNT/ $\beta$ -catenin signaling pathway, the effects of HD ZWM 978 on  $\beta$ -catenin proteins expression, transcriptional activity-mediated by  $\beta$ -catenin were investigated. The inhibitory effect of HD ZWM 978 on  $\beta$ -catenin protein expression was investigated by using western blotting in both AGS and MKN45 gastric cancer cells. As shown in Fig. 2.6, the expression of  $\beta$ -catenin protein was reduced slightly in MKN 45 cells whereas in AGS cells, treatment with 1 and 2  $\mu$ M of HD ZWM 978 significantly reduced the expression of  $\beta$ -catenin



**Figure 2.6** Effect of HD ZWM 978 on the expression of  $\beta$ -catenin protein in gastric cancer cells after treatment for 24 h. The expression of  $\beta$ -catenin was determined using western blotting.  $\beta$ -actin was used as the loading control. Three independent experiments yielded similar results. Bar graph represents the relative calculated density, normalized with  $\beta$ -actin. \* $P < 0.05$ , \*\* $p < 0.01$  significantly different from vehicle control (Turkey's multiple comparison test).

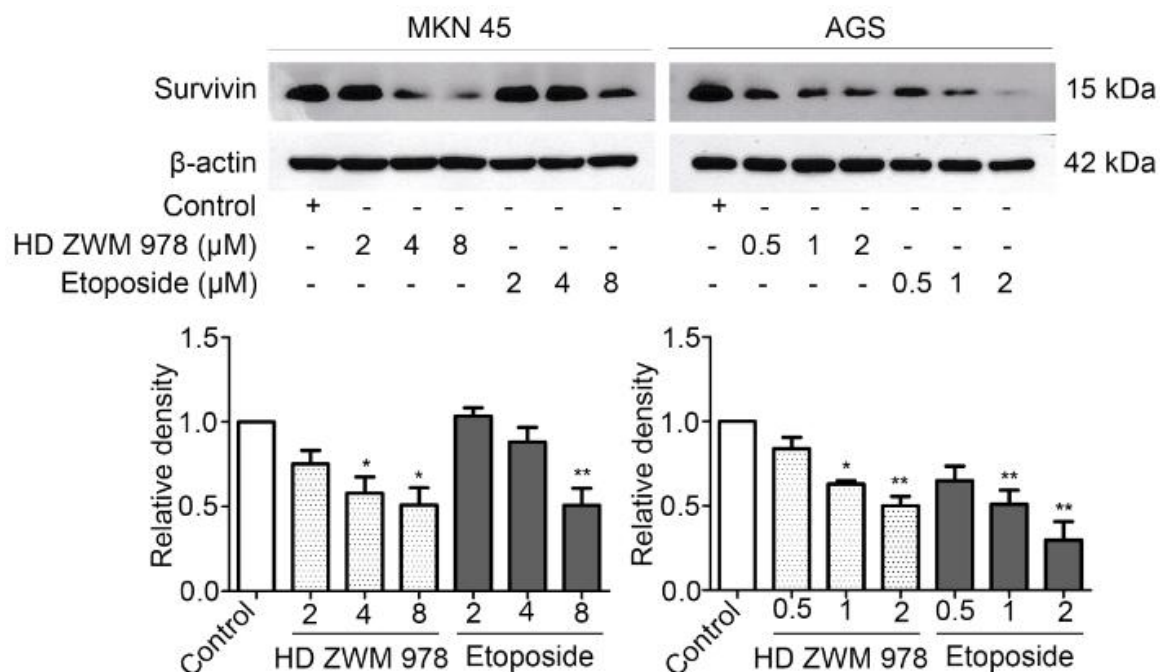
To further investigate the inhibitory effect on WNT/ $\beta$ -catenin signaling pathway by HD ZWM 978, the effects on c-MYC mRNA expression, a WNT/ $\beta$ -catenin target gene, was determined using real time quantitative (Fig. 7). The expression of c-MYC mRNA was decreased after treatment with HD ZWM 978 at 4 and 8  $\mu$ M for MKN 45 cells; and 2  $\mu$ M for AGS cells. This result suggests that HD ZWM 978 has a modulating effect on the expression of WNT responsive gene and is consistent with the previous results in which the compound inhibits the expression of key WNT signaling molecule  $\beta$ -catenin.



**Figure 2.7** Effect of HD ZWM 978 on the expression of WNT/ $\beta$ -catenin target gene. MKN45 and AGS cells were treated with indicated concentration of HD ZWM 978 for 24 hours. Total RNAs were extracted and the expression of c-MYC was analyzed by real time quantitative PCR.

#### 2.1.6 Effect on the expression of protein survivin by HD ZWM 978 in gastric cancer cells

Survivin, a member of inhibitor of apoptosis (IAP) protein family, is frequently found to be overexpressed in cancer that caused apoptosis resistance-induced by anticancer agents. To confirm the effect of tested compound on apoptosis induction, we next tested the effect of HD ZWM 978, and etoposide on the expression of survivin in gastric cancer cells (Fig. 2.4). MKN 45 cells were treated with HD ZWM 978 and etoposide at 2, 4 and 8  $\mu$ M for 24 h. HD ZWM 978, and etoposide significantly decreased survivin expression in a dose related manner. In addition, AGS cells, the more sensitive cells, were treated with lower concentrations of compounds, 0.5, 1 and 2  $\mu$ M. Similar to MKN 45 cells, treatment with either HD ZWM 978 or etoposide significantly decreased survivin expression in a dose related manner. These results indicate that HD ZWM 978 inhibits the expression of survivin and thereby promotes apoptosis in gastric cancer cells. Survivin is also a WNT/ $\beta$ -catenin target gene and inhibition of survivin expression might be a resulted from the consequence of inhibition of WNT signaling by HD ZWM 978 in both the AGS and MKN 45 gastric cancer cell lines.



**Figure 2.8** The effect of HD ZWM 978 and etoposide on the expression of survivin protein in gastric cancer cell lines; MKN 45 and AGS cells. After treatment with indicated concentrations for 24 h, total proteins were extracted and analyzed for survivin protein expression by western blotting.  $\beta$ -catenin was used as a loading control. Bar graph represents the relative calculated density, normalized with  $\beta$ -catenin. \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from vehicle control (Turkey's multiple comparison test).

**2.2 Anticancer effects of Andrographolide analogue 6 on gastric cancer cell lines.** (Andrographolide analogue 6 was kindly provided by Asst. Prof. Rungnapha Saeeng, Burapha University)

### 2.2.1 The cytotoxic effects of Andrographolide Analogue 6 on gastric cancer cell lines, MKN 45 and AGS

Andrographolide and its analogue 6 (19-triisopropyl-andrographolide) were investigated for their cytotoxic activities in MKN 45 gastric cancer cells. Both compounds exhibited cytotoxic activities in a dose- and time- related manner at 24, 48 and 72 h. The  $IC_{50}$  values of Analogue 6 were  $8.8 \pm 2.2$ ,  $6.3 \pm 0.7$  and  $5.6 \pm 0.2$   $\mu M$  at 24, 48 and 72 h, respectively (table 2.4) whereas that of the parent Andrographolide was  $>50$   $\mu M$  at 24 and 48 h and  $36.3 \pm 5.9$   $\mu M$  at 72 h. Notably analogue 6 was more cytotoxic than the parent compound.  $IC_{50}$  values of etoposide, a positive control, at 24, 48 and 72 h were  $>50$ ,  $28.5 \pm 4.4$  and  $12.6 \pm 0.2$   $\mu M$ , which was less potent than the Analogue 6. This results shows that Analogue 6 was more cytotoxic than the parent Andrographolide and etoposide. Similarly, the cytotoxic effect of Andrographolide, Analogue 6 and etoposide were also

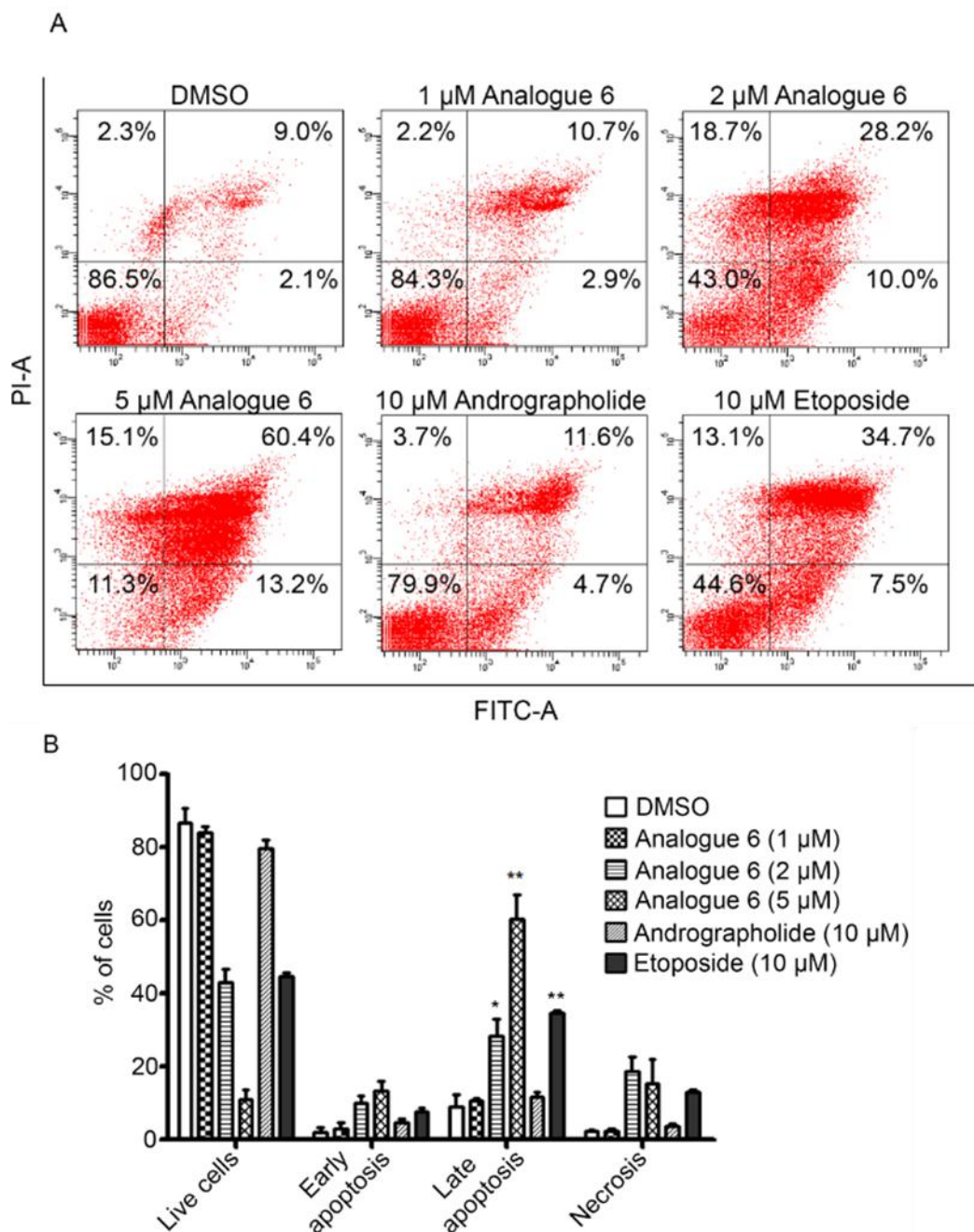
investigated in AGS cell lines. These compounds exhibited dose dependent cytotoxic effects as shown in table 2.4. At 24 h, Andrographolide, Analogue 6 and etoposide were observed to be cytotoxic with  $IC_{50}$  values of  $21.5 \pm 6$ ,  $1.9 \pm 0.09$  and  $19.6 \pm 0.9 \mu M$  respectively. The cytotoxic activities of these compounds increased at 48 hours with  $IC_{50}$  values of  $11.3 \pm 2.9$ ,  $1.7 \pm 0.05$  and  $4.0 \pm 0.5 \mu M$ . At 72 h, their cytotoxicity further increased with  $IC_{50}$  values of  $13.3 \pm 1.4$ ,  $1.8 \pm 0.1$  and  $1.9 \pm 0.3 \mu M$ . More importantly, the cytotoxicity of Analogue 6 was greater than the parent compound and clinically used etoposide. The molecular mechanism of Analogue 6 was further investigated in details.

**Table 2.4** Cytotoxicity ( $IC_{50}$  values) of Analogue 6 against gastric cancer cell lines. Data are means  $\pm$  SEM of three independent experiments performed in triplicates.

| Cell lines | Incubation time (h) | $IC_{50}$ ( $\mu M$ ) |                 |                |
|------------|---------------------|-----------------------|-----------------|----------------|
|            |                     | Etoposide             | Andrographolide | Analogue 6     |
| MKN 45     | 24                  | $19.6 \pm 0.9$        | $21.5 \pm 6.0$  | $1.9 \pm 0.09$ |
|            | 48                  | $4.08 \pm 0.5$        | $11.3 \pm 2.9$  | $1.7 \pm 0.05$ |
|            | 72                  | $1.9 \pm 0.3$         | $13.3 \pm 1.4$  | $1.8 \pm 0.1$  |
| AGS        | 24                  | >50                   | >50             | $8.8 \pm 2.2$  |
|            | 48                  | $28.5 \pm 4.4$        | >50             | $6.3 \pm 0.7$  |
|            | 72                  | $12.6 \pm 0.2$        | $36.3 \pm 5.9$  | $5.6 \pm 0.2$  |

### 2.2.2 Analysis of induction of apoptosis by Andrographolide analogue 6 in human gastric cancer cell.

AGS cells are more sensitive to Analogue 6 and was chosen for further investigation. The effects of Andrographolide and Analogue 6 on apoptosis induction in AGS human gastric cancer cells were investigated by flow cytometry (Fig. 2.9). Analogue 6 at a concentration of 1, 2 and 5  $\mu M$  induced late apoptosis up to  $10.7 \pm 0.5\%$ ,  $28.2 \pm 4.6\%$  and  $60.4 \pm 6.5\%$  as compared to DMSO control ( $9.0 \pm 3.2\%$ ), respectively. Conversely, the parent Andrographolide at 10  $\mu M$  induced up to  $11.6 \pm 1.4\%$  and etoposide (positive control) induced late apoptosis up to  $34.7 \pm 0.5\%$ . Taken together these results suggest that Analogue 6 mediated cytotoxicity is through apoptotic cell death mechanism.



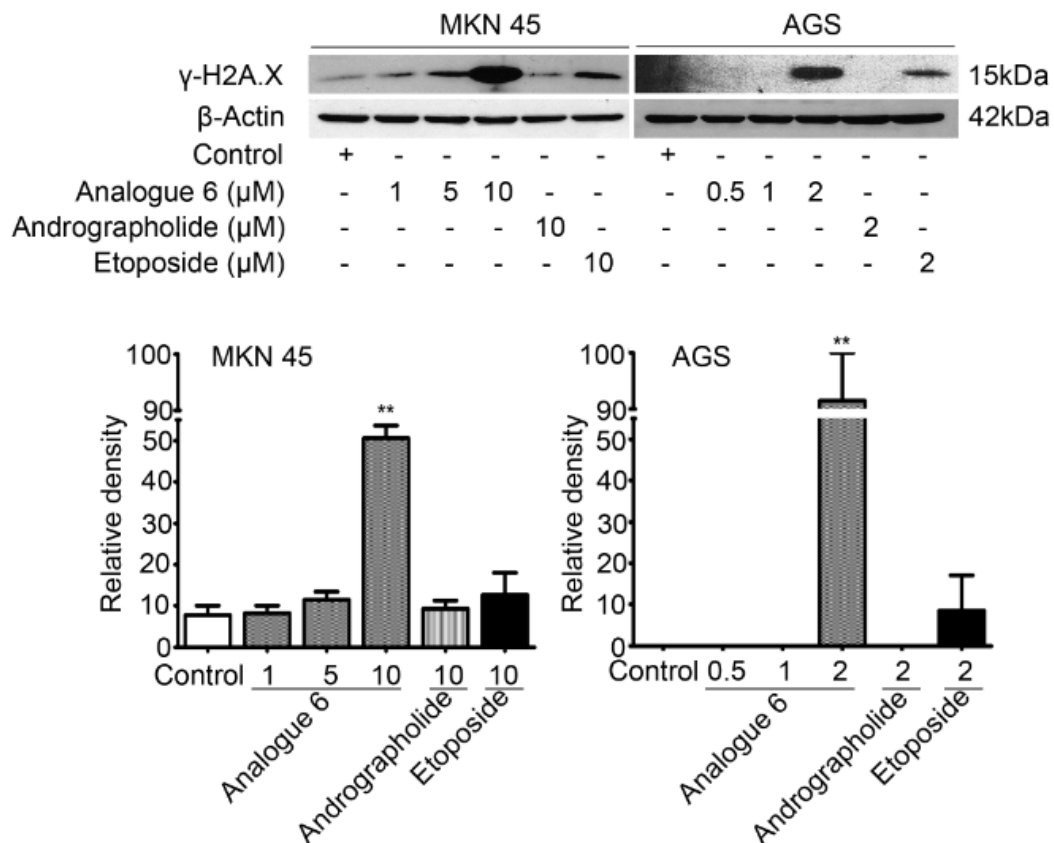
**Figure 2.9** Analogue 6 induces apoptosis in AGS cells. (A) Cells were treated with indicated concentrations of Analogue 6, Andrographolide and etoposide for 48 h. (B) Percentage of cell population in each conditions. Data represents mean  $\pm$  SEM of three independent experiments. \* $p$ <0.05 and \*\* $p$ <0.01 are significantly different from vehicle control (Turkey's multiple comparison test).

### 2.2.3 The effects of Analogue 6 on DNA damage induction and inhibition of Topoisomerase II $\alpha$ enzyme expression in gastric cancer cells

To further investigate the underlying molecular mechanism involved in the induction of apoptosis by Analogue 6, western blotting was used to detect the expression of protein involved with DNA damage response (Fig. 2.10). Analogue 6 at the concentration of

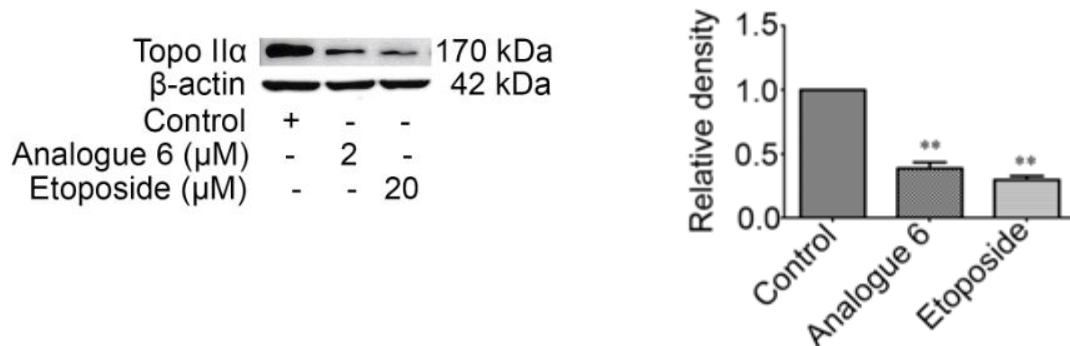


1, 2 and 10  $\mu\text{M}$  induced DNA damage in a dose related manner in MKN 45 and AGS cells demonstrated by an increase in the expression of  $\gamma\text{-H2A.X}$  protein. Notably, the parent Andrographolide and etoposide at 10  $\mu\text{M}$  were less potent than that of Analogue 6. As Analogue 6 has previously been demonstrated to inhibit human topoisomerase II $\alpha$  activity in cell-free system (Nateewattana et al., 2013). We therefore examined the inhibitory effect of Analogue 6 on topoisomerase II $\alpha$  expression in the AGS gastric cancer cells. Analogue 6 at the concentration of 2  $\mu\text{M}$  and etoposide at 20  $\mu\text{M}$  markedly decreased the expression of topoisomerase II $\alpha$  at 4 h after the treatment as compared to the DMSO control (Fig. 2.11). The expression of topoisomerase II $\alpha$  was not decreased at 6, 12 and 24 h after exposure to andrographolide analogue 6. Collectively, these results suggest that GC apoptosis-induced by Analogue 6 is, at least, through Topo II mediated DNA damage.



**Figure 2.10** DNA damage induced by andrographolide analogue 6 in gastric cancer cell lines. MKN 45 and AGS cells were treated with indicated concentrations of Andrographolide, Analogue 6 and Etoposide for 24 h. Levels of  $\gamma\text{-H2A.X}$  were detected from total protein lysates by western blotting.  $\beta\text{-actin}$  was used as the loading control. Bar graph represents the relative calculated density, normalized with  $\beta\text{-actin}$ . Data are means  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  indicates significantly different from vehicle control (Turkey's multiple comparison test).

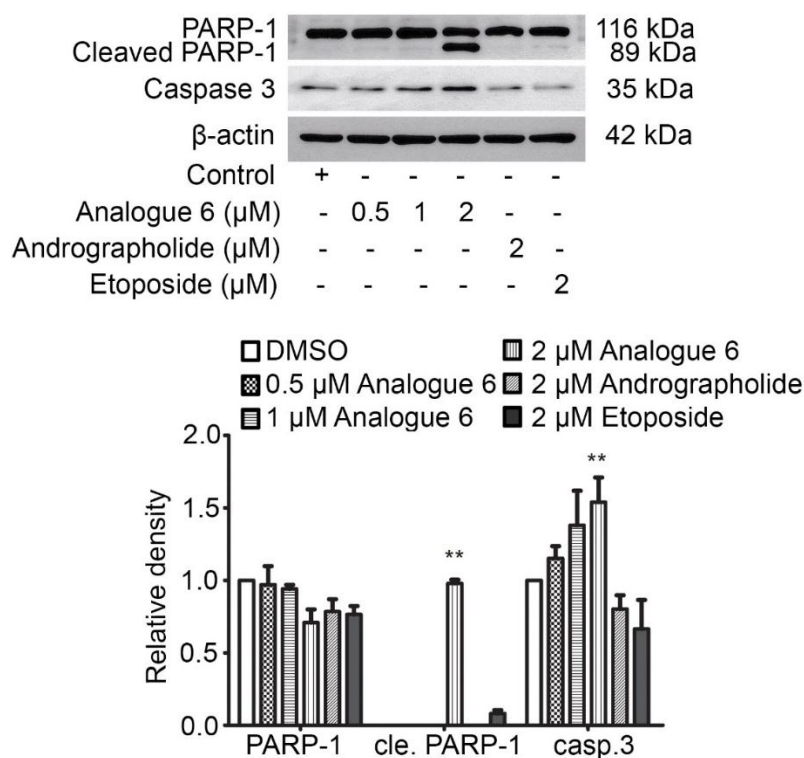




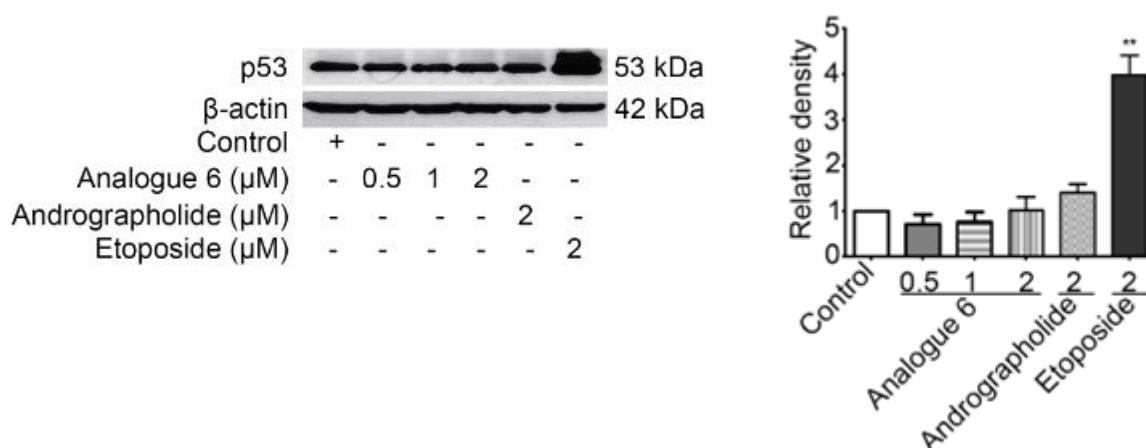
**Figure 2.11** Inhibitory effect of Andrographolide analogue 6 on Topoisomerase II $\alpha$  expression in AGS cells after 4 h of treatment.  $\beta$ -actin was used as loading control. Bar graph represents the relative calculated density, normalized with  $\beta$ -actin. Data are means  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  indicates significantly different from vehicle control (Turkey's multiple comparison test).

#### 2.2.4 The effects of Analogue 6 on proteins involved in apoptosis in human gastric cancer cells

Cleavage products of PARP-1 and caspase 3 actively participates in apoptosis pathway. We next investigated the effects of Analogue 6 on PARP-1 and caspase 3 cleavage. As shown in Fig. 2.12, treatment with 2  $\mu$ M of Analogue 6 for 24 h markedly induced PARP-1 protein cleavage. Whereas the parent andrographolide and etoposide at 2  $\mu$ M did not induced the PARP-1 cleavage. In addition, Caspase 3 protein cleavage was also increased in a dose related manner by treatment of indicated concentrations of Analogue 6. Andrographolide and etoposide were less effective in activation of caspase 3 protein. Moreover, Analogue 6 and parent andrographolide did not alter the expression of tumor suppressor protein p53 (Fig.2.13). While etoposide undoubtedly increased the expression of p53 as compared to the DMSO control. This results suggests that Analogue 6 induces AGS gastric cancer cells apoptosis via caspase 3-dependent and p53-independent pathway.



**Figure 2.12** Effects of Andrographolide analogue 6 on the proteins involved in apoptosis pathway; PARP-1, cleaved PARP-1 (cle. PARP-1) and caspase 3 in AGS cells. Western blotting was used to detect the expression of apoptotic involved proteins.  $\beta$ -actin was used as a loading control. Bar graph represents the relative calculated density, normalized with  $\beta$ -actin. The results were obtained from three independent experiments.  $**p < 0.01$  indicates significantly different from vehicle control (Turkey's multiple comparison test).



**Figure 2.13** Effects of Andrographolide analogue 6 on the proteins involved in apoptosis pathway; tumor suppressor protein p53 in AGS cells. Various concentration of Analogue 6, parent Andrographolide, and Etoposide were used for treatment of the cells for 24 h and western blotting was used to detect the expression of p53.  $\beta$ -actin was used as a loading control and the results were obtained from three independent experiments. Bar graph

represents the relative calculated density, normalized with  $\beta$ -actin. \*\* $P < 0.01$  indicates significantly different from vehicle control (Turkey's multiple comparison test).

**2.4 Anti-cancer effects of an andrographolide analogues, 3A.1 or 19-*tert*-butyldiphenylsilyl-8,17-epoxy andrographolide kindly provided by Asst. Prof. Rungnapha Saeeng, Burapha University.**

#### **2.4.1 Cytotoxic potency of analogue 3A.1.**

Cytotoxicity of analogue 3A.1 was investigated in three CRC cell lines; HT29, HCT116, and SW480. Doxorubicin was used as a positive control. Cells were treated with analogue 3A.1 at various concentrations for 24, 48 or 72 h. By using MTT viability assay, the most sensitive cell line to analogue 3A.1 is HT29 with an  $IC_{50}$  value of  $11.10 \pm 1.37 \mu M$  at 24 h of treatment, whereas those of HCT116, and SW480 cells are  $16.10 \pm 2.10$  and  $18.18 \pm 2.91 \mu M$ , respectively (Table 5).  $IC_{50}$  values of analogue 3A.1, parent andrographolide and doxorubicin in HT29 cells at various time points are demonstrated in Table 6. The results show that analogue 3A.1 decreases cellular viability in dose, and time-related manners, which its cytotoxicity is much more potent than that of the parent andrographolide ( $IC_{50} = 46.12 \pm 2.89 \mu M$  at 24 h). These results indicate a potent anti-cancer activity of analogue 3A.1 in CRC cells.

**Table 2.5** Cytotoxicity ( $IC_{50}$  values) of analogue 3A.1 against colorectal cancer cell lines. Data are means  $\pm$  SEM of three independent experiments.

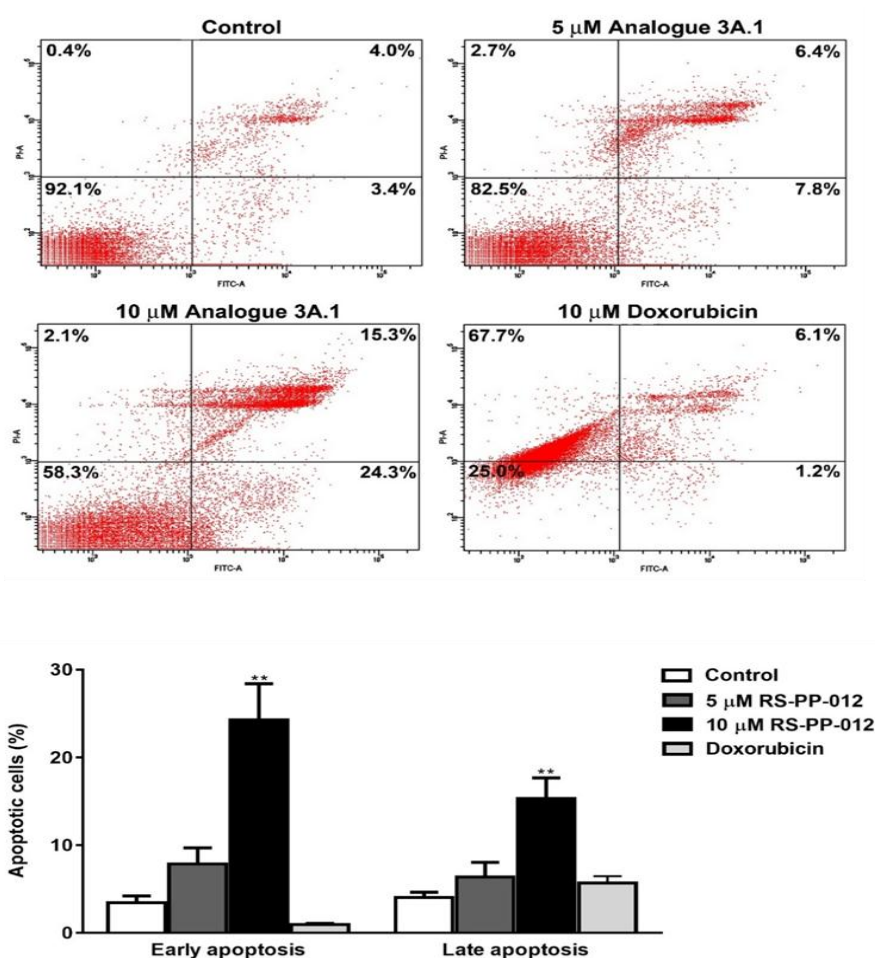
| CRC cell lines | $IC_{50}$ ( $\mu M$ ) |                 |                 |
|----------------|-----------------------|-----------------|-----------------|
|                | 24 h                  | 48 h            | 72 h            |
| HT29           | $11.10 \pm 1.37$      | $4.66 \pm 0.67$ | $3.21 \pm 0.33$ |
| HCT116         | $16.10 \pm 2.10$      | $4.78 \pm 1.22$ | $4.32 \pm 1.28$ |
| SW480          | $18.18 \pm 2.91$      | $4.97 \pm 0.99$ | $3.36 \pm 1.43$ |

**Table 2.6** Comparison the  $IC_{50}$  values ( $\mu M$ ) of analogue 3A.1 with andrographolide and doxorubicin in HT29 cells. Data are means  $\pm$  SEM from three independent experiments

| Compounds       | $IC_{50}$ ( $\mu M$ ) |                  |                  |
|-----------------|-----------------------|------------------|------------------|
|                 | 24 h                  | 48 h             | 72 h             |
| Analogue 3A.1   | $11.10 \pm 1.37$      | $4.66 \pm 0.67$  | $3.21 \pm 0.33$  |
| Andrographolide | $46.12 \pm 2.89$      | $29.89 \pm 3.53$ | $24.45 \pm 3.80$ |
| Doxorubicin     | $26.15 \pm 1.17$      | $3.83 \pm 0.65$  | $1.59 \pm 0.71$  |

### 2.4.2 Effect of analogue 3A.1 on the induction of cellular apoptosis

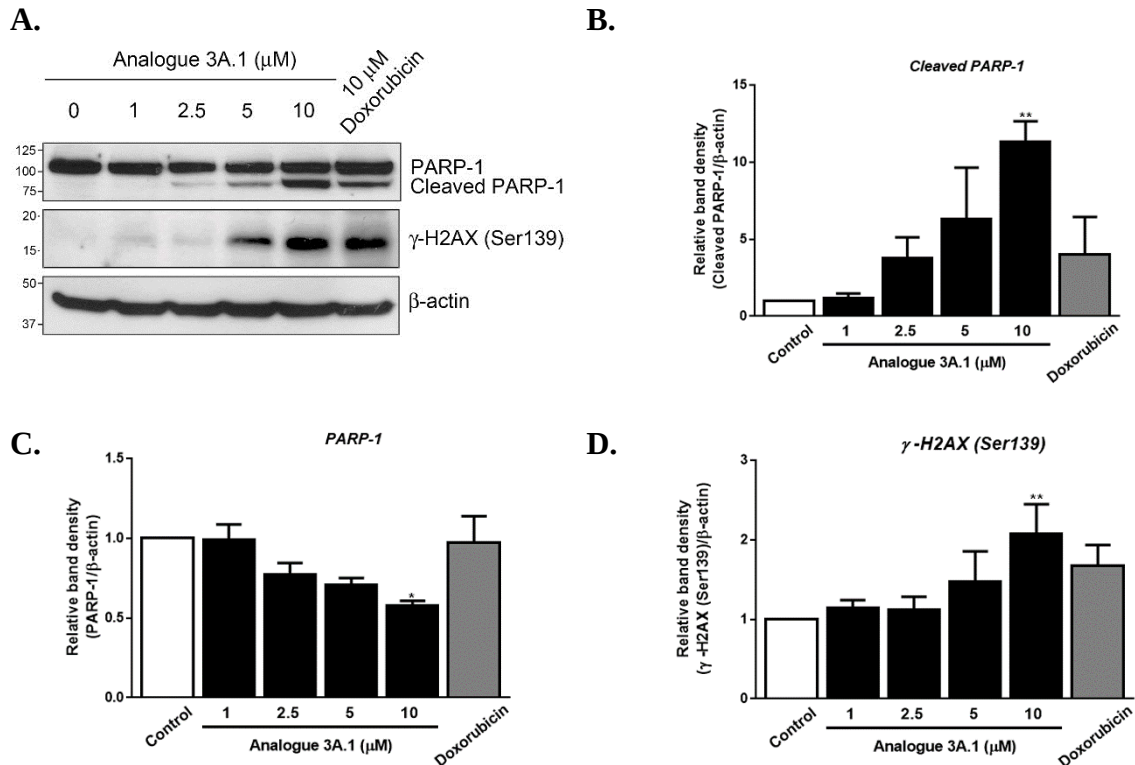
The involvement of anti-cancer effects of analogue 3A.1 with induction of apoptotic cell death was evaluated by using flow cytometry. Doxorubicin was used as a positive control. As shown in Fig. 2.14, analogue 3A.1 at 10  $\mu$ M induced apoptotic cell death significantly in HT29 cells after 24 h of treatment. Cells treated with a positive control, doxorubicin, at 10  $\mu$ M obviously induced more number of necrotic cells death. This finding suggests that anti-cancer activity of analogue 3A.1 is involved with the induction of HT29 cell death via apoptosis mechanism.



**Figure 2.14** Effect of analogue 3A.1 on HT29 cell apoptosis. Cells were treated with analogue 3A.1 (5, and 10  $\mu$ M), or doxorubicin (10  $\mu$ M), stained with Annexin V-FITC, and PI before analyzing by flow cytometry. Bar graph represents percentage of apoptotic cells in each conditions. Data are means  $\pm$  SEM (n=3). \*\*p<0.01 indicates significantly different compared with the vehicle control.

Proteins that are activated by DNA strand breaks upon the cellular apoptosis were investigated. A nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1) catalyzes

repairing process single-strand DNA breaks was examined. Corresponding to the results from flow cytometry, analogue 3A.1 at concentrations of 2.5, 5, and 10  $\mu\text{M}$  increase levels of cleaved PARP-1 in HT29-treated cells in a dose-dependent manner (Fig. 2.15 A-C). Moreover, a marker of DNA damage, the phosphorylated H2AX (ser139) or  $\gamma$ -H2AX was up-regulated at 24 h of post-treatment with analogue 3A.1 (Fig.2.15 A and D). These results indicate that analogue 3A.1 essentially induced apoptotic cell death with profound increases in markers of DNA damage.

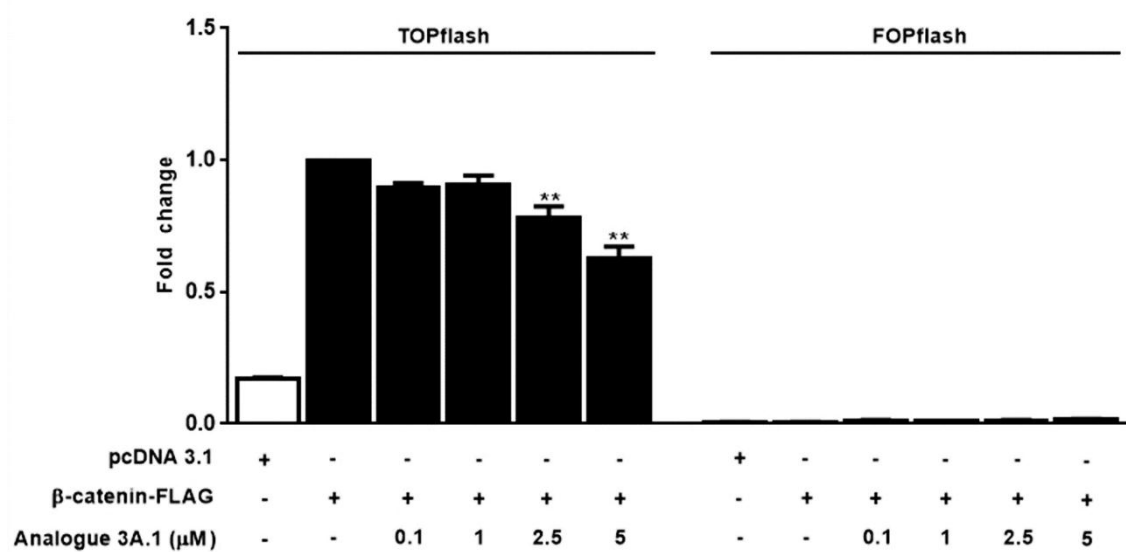


**Figure 2.15** Effect of analogue 3A.1 on the expression of proteins related to DNA damage in cell death. (A) HT29 cells were treated with various concentrations of analogue 3A.1 or doxorubicin (10  $\mu\text{M}$ ) for 24 h. Total cell lysates were extracted and subjected to western blotting with anti-PARP-1 and anti- $\gamma$ -H2AX.  $\beta$ -actin serves as a loading control. (B-D) The normalized band intensities are shown as means  $\pm$  SEM (n=3). \* $p$ <0.05 and \*\* $p$ <0.01 indicate significantly different compared with the vehicle control.

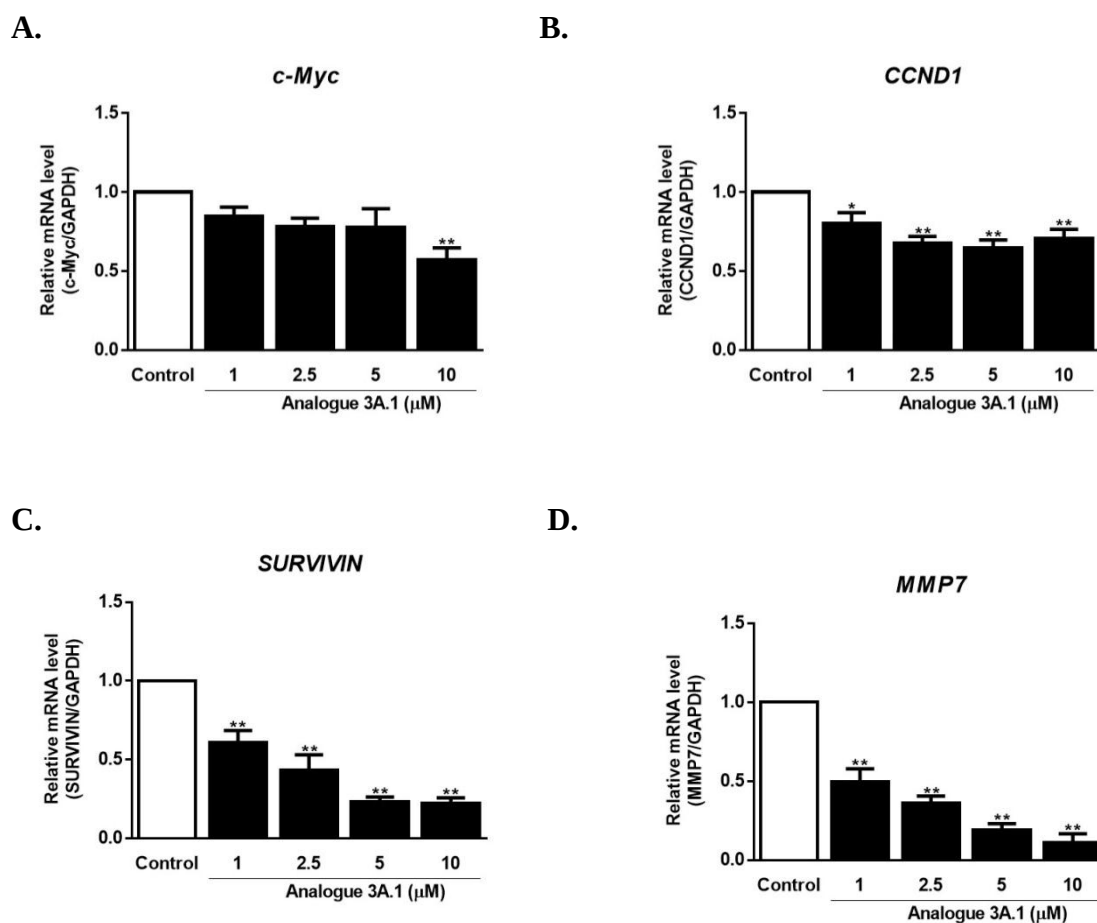
#### 2.4.3 Effect of analogue 3A.1 on the inhibition of Wnt/ $\beta$ -catenin signaling

The effect of analogue 3A.1 on the transcriptional activity and protein expression of Wnt signaling were investigated. In Fig. 2.16, the TOPflash luciferase activity induced by  $\beta$ -catenin overexpression in HEK293T cells was markedly inhibited by analogue 3A.1 as compared with control. While the luciferase activity of FOPflash, a mutant of TCF/LEF

binding site for  $\beta$ -catenin, was not affected, suggesting the specific suppressive function of analogue 3A.1 on Wnt/ $\beta$ -catenin activity. In addition, the effect on expression of Wnt-responsive genes which play the important roles in CRC progression were further determined. As shown in Fig. 2.17A-D, the mRNA expression levels of Wnt target genes, *c-Myc*, *CCDN1*, *SURVIVIN*, and *MMP7*, are decreased with increasing concentrations of analogue 3A.1 in HT29 cells. This finding indicates to the inhibitory activity of analogue 3A.1 in Wnt/ $\beta$ -catenin signaling pathway at transcriptional level.



**Figure 2.16** Effects of analogue 3A.1 on TCF/LEF reporter activity. HEK293T cells were transiently transfected with  $\beta$ -catenin-FLAG or pcDNA3.1, and TOPflash or FOPflash, and *Renilla* luciferase reporter plasmids. After transfection, cells were incubated with analogue 3A.1 for 24 h. The relative firefly luciferase activity units (RLUs) were then measured and normalized corresponding to *Renilla* luciferase activity. Bar graph is the fold change compared with  $\beta$ -catenin-FLAG-transfected cells and represented as means  $\pm$  S.E.M (n=3) (\*\* $p$ <0.01).



**Figure 2.17** Effects of analogue 3A.1 on the expressions of downstream target genes of Wnt/ $\beta$ -catenin signaling pathway. Quantitative real-time PCR showing the concentration-dependent reduction in mRNA expression of Wnt target genes: (A) *c-Myc*, (B) *CCND1*, (C) *SURVIVIN*, and (D) *MMP7* in HT29 cells after treatment with analogue 3A.1 for 24 h. The relative mRNA expression was quantified and normalized with GAPDH. Data are means  $\pm$  S.E.M (n=3). \* $p<0.05$  and \*\* $p<0.01$  compared with the vehicle control.

#### Summary:

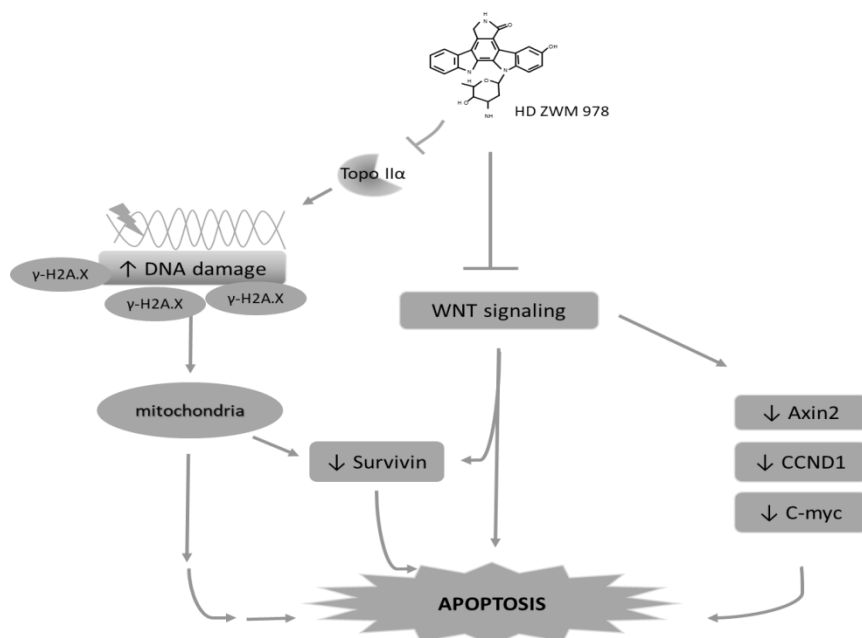
#### Part I: Screening for cytotoxic activity of natural products from Chinese Marine Microbes and Thai Medicinal Plants.

Under research corroboration through Thailand Research Fund (TRF) and Natural Science Foundation of China (NSFC), we obtained natural-derived compounds from Thai and Chinese scientist. The cytotoxic effects of these compounds were determined by using the colorimetric MTT assay against several cancer cell lines. We obtain several compounds with great cytotoxic effects against cancer cell lines and were selected for further mechanistic study in part II.

## Part II: Identification the molecular mechanisms and molecular target of the candidate compounds.

The present study was to investigate the anticancer effects of the natural derived compounds, the marine Actinobacterium secondary metabolite HD ZWM 978 and an Andrographolide analogue 6, in gastric cancer cells.

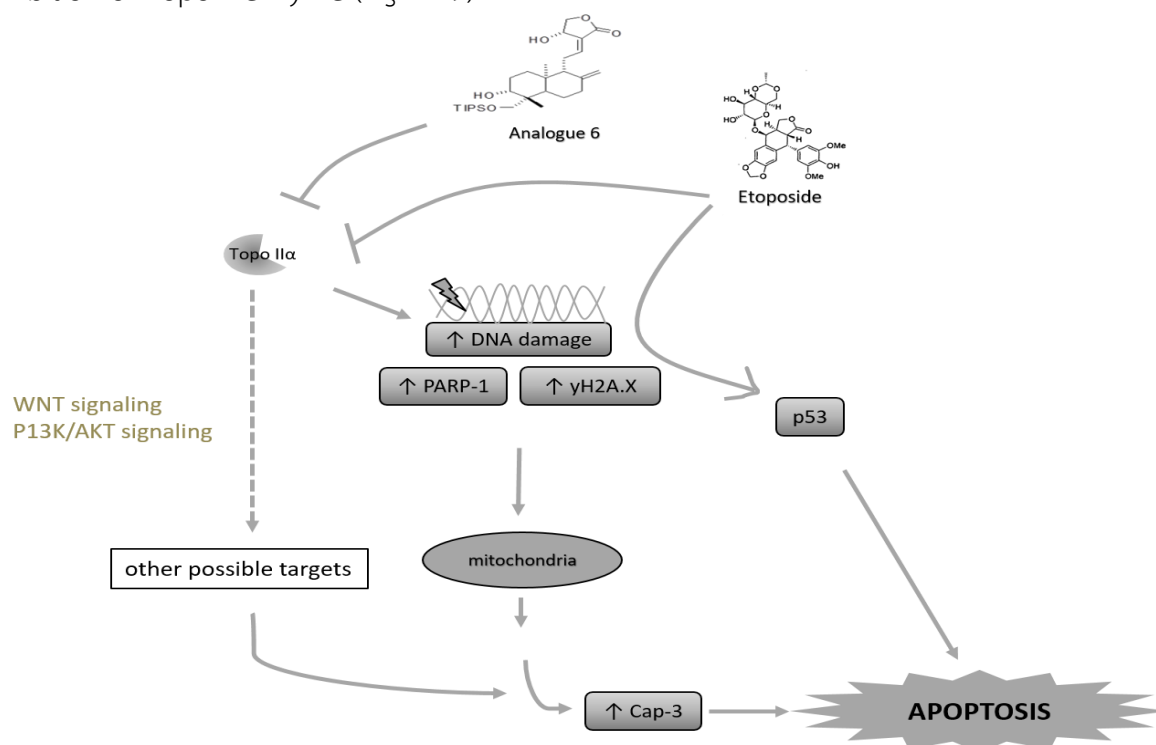
HD ZWM 978 exhibited potent dose related cytotoxic effects on both the gastric cancer cell lines. Notably, the cytotoxicity of HD ZWM 978 was more potent than the clinically used Etoposide. Mechanically study, the cytotoxic effect of HD ZWM 978 in gastric cancer cells is via induction of apoptosis. Further investigations on the underlying molecular mechanism have found that HD ZWM 978 inhibited the activity of topoisomerase II $\alpha$  enzyme concomitant with the induction of DNA damage. Aberrant activation of WNT/ $\beta$ -catenin signaling pathway is one of major cause of gastric cancer development and progression. To investigate whether anticancer property of HD ZWM 978 involvement with inhibition of WNT/ $\beta$ -catenin signaling pathway, the effects of HD ZWM 978 on  $\beta$ -catenin proteins expression, transcriptional activity-mediated by  $\beta$ -catenin were investigated. We found that WNT/ $\beta$ -catenin activity was inhibited by HD ZWM 978 treatment as demonstrated by the suppression of  $\beta$ -catenin protein and WNT/ $\beta$ -catenin target gene (survivin and c-myc) expressions. Our results indicate that HD ZWM 978 induces gastric cancer cell apoptosis partly through (i) induction of DNA damage-mediated by topoisomerase II $\alpha$  enzyme inhibition and (ii) inhibition of WNT/ $\beta$ -catenin signaling pathway (Fig. 2.18).



**Figure 2-18** Diagramm showing the probable mechanistic action of HD ZWM 978 in gastric cancer cells.

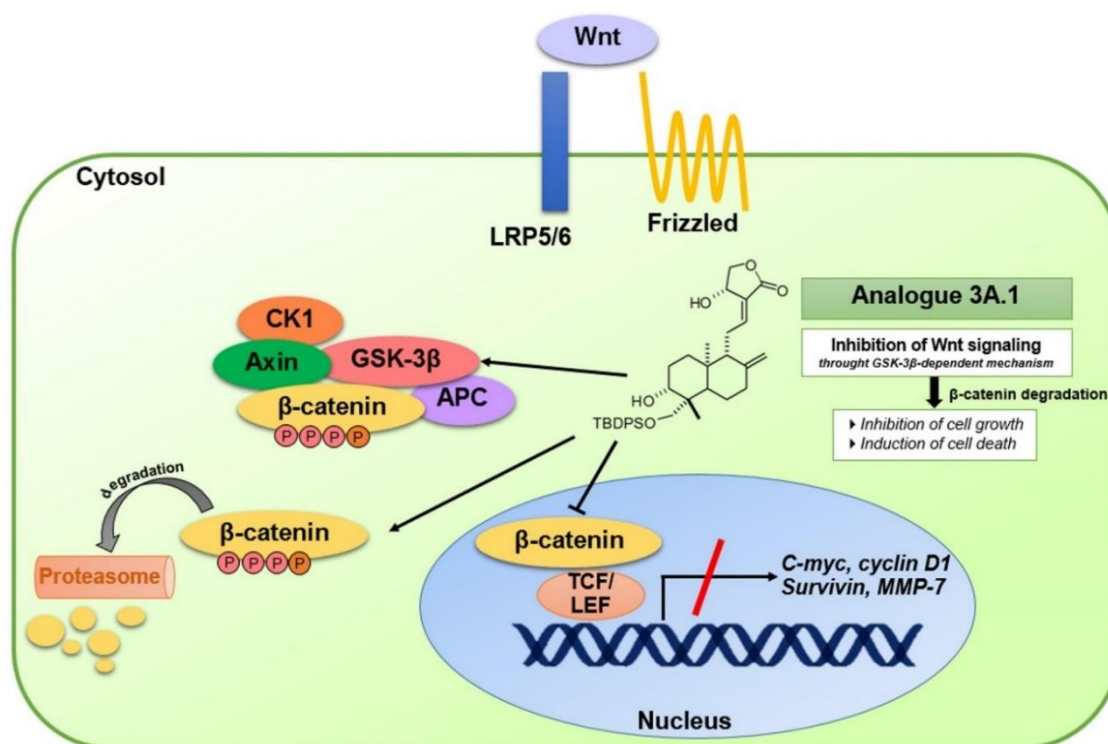


Andrographolide analogue 6 showed the cytotoxic effects against two gastric cancer cells MKN45 and AGS. Interestingly, Analogue 6 was more potent than the parent Andrographolide compound as well as the clinically used etoposide. Mechanistically, Analogue 6 induced apoptosis in AGS gastric cancer cells as demonstrated by an increase in cleaved PARP-1 and caspase 3 products after treatment. Moreover, the apoptosis promoting of Andrographolide analogue 6 is probably through induction of DNA damage-induced by an inhibition of Topo II enzyme (Fig. 2.19).



**Figure 2.19** Diagram showing the potential mechanistic pathway of Analogue 6 in gastric cancer cells

In the present study, the anticancer effects and mechanisms of andrographolide analogue 3A.1 in colorectal cancers (CRC) were investigated. Analogue 3A.1 is highly toxic in three CRC cell lines; HT29, HCT116, and SW480 and they are more potent than that of parent compound (andrographolide). Analogue 3A.1 induced cell apoptosis and increased the expression of proteins related to DNA damage; PARP-1 and  $\gamma$ -H2AX. 3. Mechanistically, this analogue inhibited Wnt/ $\beta$ -catenin signaling pathway demonstrated by suppressed TCF/LEF-mediated transcriptional activity of Wnt/ $\beta$ -catenin signaling in HEK293T cells and suppression the expressions of Wnt target genes; *c-myc*, *CCDN1*, *SURVIVIN*, and *MMP7* in HT29 cells (Fig. 2.20). Thus, this compound has potential to be developed as the chemotherapeutic agents to target malignancies harboring hyperactivated Wnt/ $\beta$ -catenin signaling.



**Figure 2.20** Schematic diagram representing the molecular mechanism of analogue 3A.1 on Wnt/β-catenin signaling pathway in HT29 cells.

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### Sub-project 3: Anti-diabetic effect of compounds

#### ABSTRACT

Kidney plays is one of the most important organ regulating blood glucose by reabsorption of filtered glucose from glomerulus via renal glucose transporters located at renal proximal tubular cells. Sodium glucose cotransporter (SGLT) 2 inhibition has been found to be anti-diabetic drug target for type 2 diabetes, all SGLT2 inhibitors have same core structure as same as phlorizin (first SGLT2 inhibitor). So, it is interesting to find a new SGLT2 inhibitor from natural for diabetic treatment. First, the inhibitory effect of 134 compounds was screened by uptake assay in human renal proximal cell line (HK-2 cell) using [ $^3$ H]-2-deoxy-d-glucose (2DG) as radioactive substrate. 3 marines (HD-ZWM-1081, HD-ZWM-1083, HD-ZWM-1084) inhibited SGLT2 in HK-2 cells with cytotoxicity effect. *Kaempferia parviflora* extract (KPE) and *Boesenbergia pandurata* extract (BPE) were found to inhibit SGLT2-mediated [ $^3$ H]-2DG uptake in HK-2 cell with IC<sub>50</sub> of 124 and 211 µg/ml, respectively. Moreover, effect of KPE and BPE on glucose transporter (GLUT) 2 inhibition was examined. KPE and BPE inhibited GLUT2-mediated [ $^3$ H]-2DG uptake with IC<sub>50</sub> of 71.59 and 190.9 µg/ml, respectively. Selectivity of hit compounds was tested by examining effects of hit compounds on SGLT1 and GLUTs inhibition in enterocytes (Caco-2 cell). KPE did not inhibit SGLT1 while BPE significant inhibited SGLT1. Both KPE and BPE had no effect on GLUTs inhibition. The effect of KPE and BPE on anti-hyperglycemia was examined in diabetic rat (Goto Kakizaki (GK) rat). Orally administration of KPE and BPE reduce plasma glucose without changing plasma insulin indicating the therapeutic potential for type 2 DM treatment. In addition to the therapeutic potential application of *Boesenbergia pandurata* on diabetes, compounds from this plant panduratin A and pinostrobin showed protective effect of nephrotoxicity induced by drug including cisplatin, anti-cancer, and antibiotic drug, colistin. The protective effect of these compounds were mediated by decrease in ROS, pro-apoptotic proteins, ERK and caspases, and mitochondria damage. Collectively, panduratin A and pinostrobin have potential protective effect on nephrotoxicity induced by cisplatin and colistin.

**Keywords:** Diabetes; Sodium glucose co-transporter 2; flavonoids; kidney injury; *Boesenbergia pandurata*

## บทคัดย่อ

ไตเป็นหนึ่งในอวัยวะที่สำคัญในการควบคุมระดับน้ำตาลกลูโคสในเลือด โดยการควบคุมการดูดกลับกลูโคสที่ผ่านการกรองจากโกลเมอรูลัสโดยอาศัยการทำงานของตัวขนส่งกลูโคสที่เซลล์ของหลอดไตส่วนต้น การยับยั้งการทำงานของตัวขนส่งตัวโคสชนิด Sodium glucose cotransporter (SGLT) 2 จึงเป็นหนึ่งในเป้าหมายในการลดระดับน้ำตาลในเลือดเพื่อรักษาโรคเบาหวานชนิดที่ 2 ยารักษาโรคเบาหวานกลุ่ม SGLT2 inhibitors นั้นมีโครงสร้างพื้นฐานมาจากสาร phlorizin ซึ่งเป็น SGLT2 inhibitor ตัวแรก ดังนั้นจึงเป็นที่น่าสนใจที่จะค้นหาสารที่มีฤทธิ์ยับยั้ง SGLT2 ที่มีโครงสร้างอื่นๆจากสารธรรมชาติ โดยเริ่มทำการทดสอบฤทธิ์ของสาร 134 ตัว ในการยับยั้ง SGLT2 โดยการวัดการขนส่ง  $[^3\text{H}]\text{-2-deoxy-d-glucose}$  (2DG) ในเซลล์หลอดไตส่วนต้นของมนุษย์ชนิด HK-2 cells ที่มีการแสดงออกของ SGLT2 จากการทดสอบพบว่าสารจากทะเล 3 ชนิด ได้แก่ HD-ZWM-1081, HD-ZWM-1083, HD-ZWM-1084 มีฤทธิ์ยับยั้งการขนส่งของ  $[^3\text{H}]\text{-2DG}$  เข้าสู่เซลล์ อย่างไรก็ตามทั้ง 3 ชนิดมีความเป็นพิษต่อเซลล์ สารสกัดจากกระชายดำ *Kaempferia parviflora* และกระชาย *Boesenbergia pandurata* มีฤทธิ์ยับยั้ง SGLT โดยการยับยั้งการขนส่งสาร  $[^3\text{H}]\text{-2DG}$  เข้าสู่เซลล์หลอดไตส่วนต้น ด้วยค่า  $\text{IC}_{50}$  ที่ 124 และ 211  $\mu\text{g/ml}$  ตามลำดับ นอกจากนี้ยังพบว่าสารสกัดทั้ง 2 มีฤทธิ์ยับยั้งตัวขนส่งกลูโคสชนิด GLUT2 ด้วยค่า  $\text{IC}_{50}$  ที่ 71.59 และ 190.9  $\mu\text{g/ml}$  ตามลำดับ เนื่องจากเซลล์ HK-2 มีการแสดงออกทั้ง SGLT1 และ SGLT2 ดังนั้นจึงทำการทดสอบว่าสารสกัดทั้งสองมีฤทธิ์ต่อ SGLT1 หรือไม่ โดยทำการวัดการขนส่ง  $[^3\text{H}]\text{-2DG}$  ในเซลล์ลำไส้ชนิด CaCo-2 ซึ่งมีการแสดงออกของ SGLT1 แต่ไม่พบการแสดงออกของ SGLT2 จากผลพบว่าสารสกัดจากกระชายดำไม่มีฤทธิ์ยับยั้งการทำงานของ SGLT1 ขณะที่สารสกัดจากกระชายมีฤทธิ์ยับยั้งการทำงานของ SGLT1 ด้วย อย่างไรก็ตามไม่พบฤทธิ์ของสารทั้งสองต่อการทำงานของ GLUT ของเซลล์ลำไส้ ฤทธิ์ลดระดับน้ำตาลในเลือดของสารสกัดกระชายดำและกระชายได้รับการทดสอบในหนูที่เป็นโรคเบาหวานชนิดที่ 2 พบว่าสารสกัดกระชายดำและกระชายมีฤทธิ์ลดระดับน้ำตาลในเลือดโดยไม่มีผลเปลี่ยนแปลงระดับฮอร์โมนอินซูลิน ดังนั้นสารสกัดกระชายดำและกระชายอาจมีศักยภาพในการรักษาโรคเบาหวานชนิดที่ 2 ได้ นอกเหนือจากฤทธิ์ด้านเบาหวานของสารสกัดจากกระชาย สารแพนดูราตินเอ และสารไฟโนสโทรบิน ซึ่งเป็นสารที่แยกได้จากสารสกัดกระชายมีฤทธิ์ลดการเกิดความเป็นพิษต่อไตจากการได้รับยาต้านมะเร็งซิสพลาตินและยาปฏิชีวนะโคลิสติน ฤทธิ์ด้านความเป็นพิษต่อไตของสารแพนดูราตินเอ และสารไฟโนสโทรบิน เกิดจากการลดการเกิด ROS โปรตีนกระตุ้นการตายของเซลล์ ได้แก่ ERK และ caspases และการลดการทำลายไมโทคอนเดรีย ดังนั้นสารทั้งสองมีศักยภาพในการพัฒนาเพื่อเป็นสารสำหรับป้องกันความเป็นพิษต่อไตจากการใช้ยาต้านมะเร็งซิสพลาตินและยาปฏิชีวนะโคลิสติน

**คำสำคัญ** โรคเบาหวาน ตัวขนส่งกลูโคส 2 ฟลาโวนอยด์ ความเป็นพิษต่อไต กระชาย

## INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorder, which is more prevalent in elderly. The causes of T2DM are resulted from the defects in insulin secretion and/or insulin action. Approximately 2.8% of the population worldwide suffers from T2DM and it may reach 5.4% by the year 2025 (Wild, 2004). The prevalence of T2DM increases with age and it affects nearly 1 in 5 individuals over the age of 65 years. Blood glucose control is

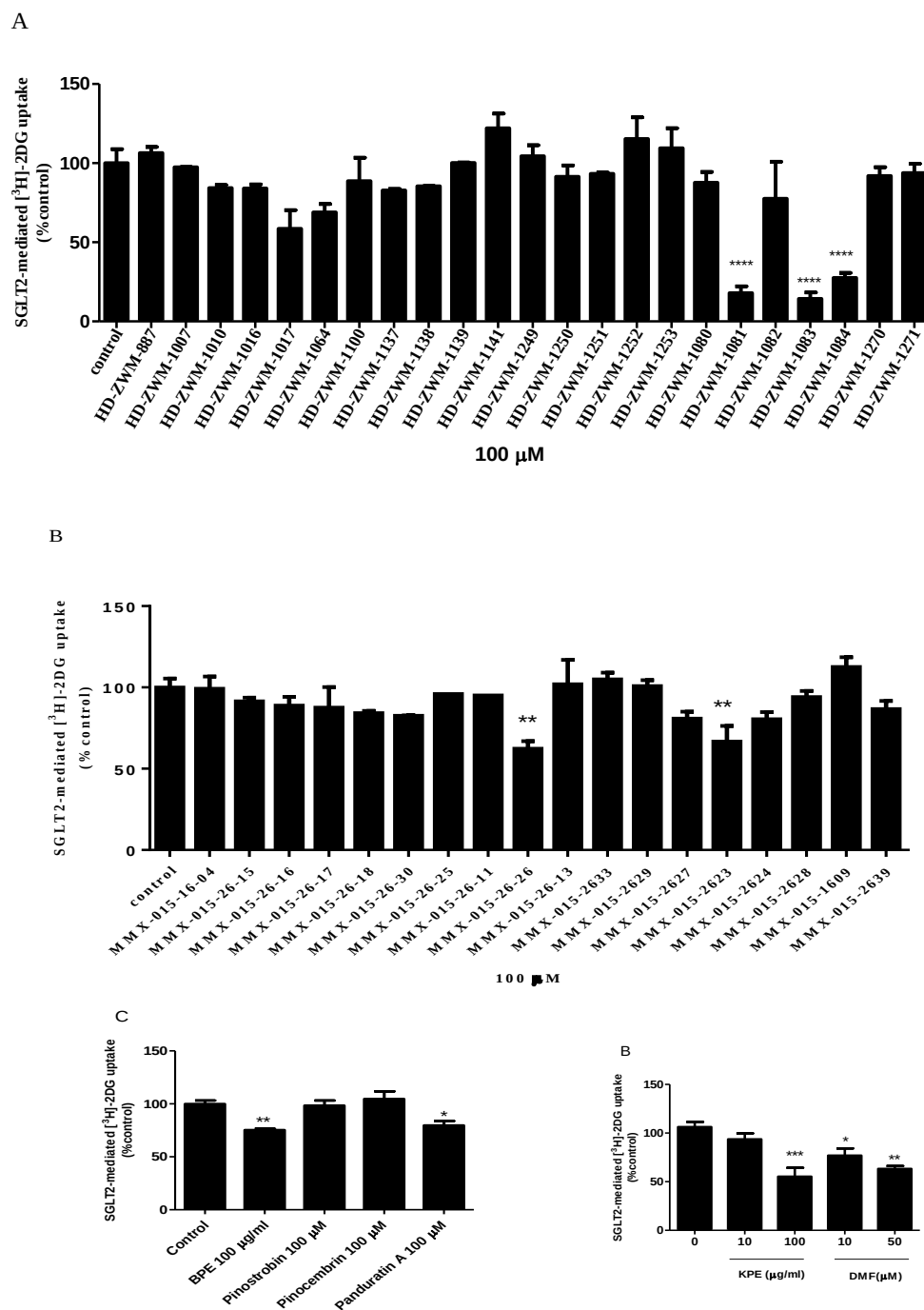
fundamental to diabetes management and is proven to reduce the risk of developing its complications. Therefore, antihyperglycemic agents are a key component of treatment strategies. A number of oral anti-diabetic agents are commercially available. However, adverse effects including hypoglycemia, weight gain and edema are frequently reported. A number of new antidiabetes drug classes have emerged over the past decade, the most recent being the sodium glucose co-transporter-2 (SGLT2) inhibitors. Inhibition of SGLT2 has generated the greatest pharmacological interest partly because SGLT2 reabsorbs the majority of glucose and also because it is expressed predominantly within the kidney and is, therefore, not expected to have off-target effects. A number of SGLT2 inhibitors have been developed based on the structure of phlorizin, a naturally occurring compound from the plant. Therefore, the natural products have high potential to show an inhibitory effect on SGLT2. Thailand and China possess a great diversity of natural materials; therefore, this research project will search for the potential compounds for treatment of type 2 diabetes from the natural sources of Thailand and China.

The scope of research work consists of three parts including 1) high-throughput screening for SGLT2 inhibitor and 2) investigation of the pharmacological effects of the potential compounds in a validated animal model of diabetes.

## RESULTS

### Screened for natural compounds inhibiting SGLT2 at renal proximal tubule

To obtain the natural compounds inhibiting SGLT2 activity, 134 natural compounds in the library which were tested for SGLT2 inhibitor. Natural compounds in the library can divide into 2 groups which are marine natural and plant compounds. The effect of natural compounds was screened by using glucose uptake assay. The human renal proximal tubule cell (HK-2 cell) was used as a model for in vitro study because this cell line highly expresses SGLT2 compared with SGLT1. HK-2 cells were seeded on 24 wells plate with 1:4 seeding ratio. The confluent cells were incubated with [ $^3$ H]-2DG in the presence of 100  $\mu$ M or  $\mu$ g/ml of natural compounds for 30 minutes. Accumulation of [ $^3$ H]-2DG was measured to assess SGLT2 activity. Effect of 134 natural compounds comprising of 87 marine natural and 47 plant natural compounds on SGLT2 was determined. We found that 3 marines (HD-ZWM-1081, HD-ZWM-1083, HD-ZWM-1084) and *Kaempferia parviflora* extract (KPE), its active compound (DMF) and *Boesenbergia pandurata* extract (BPE), and its active compound (panduratin A) were found to inhibit SGLT2-mediated [ $^3$ H]-2-DG uptake in screening experiment.



**Figure 3.1** Screen for marine natural compounds inhibiting SGLT2-mediated [ $^3\text{H}$ ]-2DG uptake in HK-2 cell. Data are shown as mean  $\pm$  SEM. One-way ANOVA \* $p$ <0.05, \*\* $p$ <0.01 compared to control.

Inhibitory potency of hit compounds on SGLT2-mediated glucose uptake in real proximal tubular cell.

To find inhibitory potency of hit compounds on SGLT2-mediated glucose uptake in human renal proximal tubular cell. Glucose uptake assay was performed using HK-2 cells.

The transport activity of SGLT2 was detected by measurement of [ $^3$ H]-2DG accumulation. Cells were incubated with HD-ZWM-1081 (0-200  $\mu$ M), HD-ZWM-1083 (0-200  $\mu$ M), HD-ZWM-1084 (0-200  $\mu$ M), KPE (0-500  $\mu$ g/ml), BPE (0-200  $\mu$ g/ml), DMF, and panduratin A (0-200  $\mu$ M). As shown in Table 1, the IC<sub>50</sub> values of HD-ZWM-1081, HD-ZWM-1083, HD-ZWM-1084, KPE, and BPE were 41.73, 75.92, 208.1  $\mu$ M, 124, and 211.6  $\mu$ g/ml, respectively. Unfortunately, IC<sub>50</sub> of DMF and panduratin A could not be found because of low magnitude of inhibition. However, DMF at dose of 50-200  $\mu$ M significantly inhibited SGLT2-mediated [ $^3$ H]-2DG uptake by ~20-30%. Inhibitory effect was not found in panduratin A treatment group.

### Inhibitory potency of hit compounds on GLUT2-mediated glucose uptake in real proximal tubular cell.

Glucose reabsorption mechanism at human renal proximal tubule at S1 segment requires 2 steps of glucose transport. The first is to uptake glucose into cell by SGLT2 activity. Then glucose pass through blood circulation by GLUT2 (5). Moreover, in high glucose condition, high expression and translocation of GLUT2 to apical membrane were observed (86). So, inhibition of GLUT2 at renal proximal tubule may aid to reduce plasma glucose concentration in T2DM. To determine whether hit compounds inhibit GLUT2-mediated [ $^3$ H]-2DG uptake in human renal proximal tubular cell, glucose uptake assay was performed. Phlorizin at dose of 200  $\mu$ M was used to inhibit SGLT2 transport function. HK-2 cells were incubated with various doses of hit compounds. Cells were incubated with HD-ZWM-1081 (0-200  $\mu$ M), HD-ZWM-1083 (0-200  $\mu$ M), HD-ZWM-1084 (0-200  $\mu$ M), KPE (0-500  $\mu$ g/ml), BPE (0-200  $\mu$ g/ml), DMF, and panduratin A (0-200  $\mu$ M). As shown in figures 4.5. – 4.6, IC<sub>50</sub> of HD-ZWM-1081, HD-ZWM-1083, HD-ZWM-1084, KPE, and BPE were 42.58, 43.35, 384.1  $\mu$ M, 71.59, 190.9  $\mu$ g/ml, and 31.56  $\mu$ M, respectively. Panduratin A significantly inhibited GLUT2-mediated [ $^3$ H]-2DG uptake at doses of 50-200  $\mu$ M.

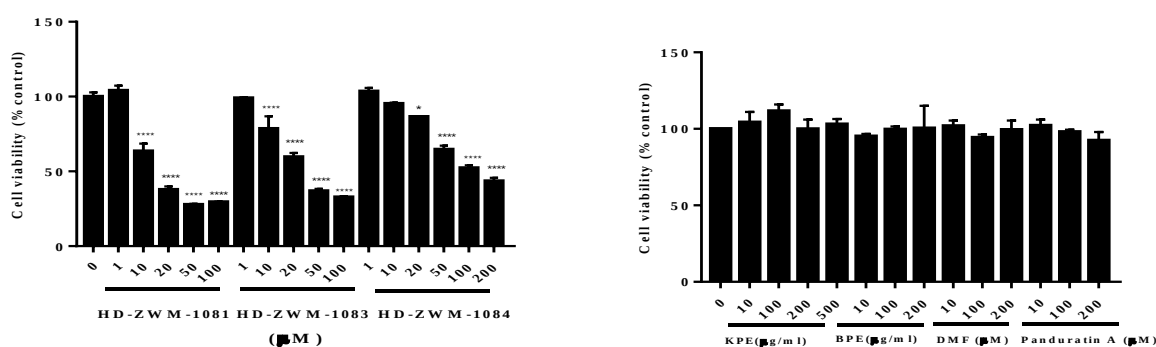
**Table 3.1** IC<sub>50</sub> of hit compounds in HK-2 cells

| Compounds                  | Half maximal inhibitory concentration (IC <sub>50</sub> ) |       |
|----------------------------|-----------------------------------------------------------|-------|
|                            | SGLT2                                                     | GLUT2 |
| 1. KPE ( $\mu$ g/ml)       | 124                                                       | 71.59 |
| 2. BPE ( $\mu$ g/ml)       | 211.6                                                     | 190.9 |
| 3. DMF ( $\mu$ M)          | -                                                         | 31.56 |
| 4. Panduratin A ( $\mu$ M) | -                                                         | -     |
| 5. HD-ZWM-1081 ( $\mu$ M)  | 41.73                                                     | 42.58 |
| 6. HD-ZWM-1083 ( $\mu$ M)  | 75.92                                                     | 43.35 |
| 7. HD-ZWM-1084 ( $\mu$ M)  | 208.1                                                     | 384.1 |



### Cell viability of hit compounds

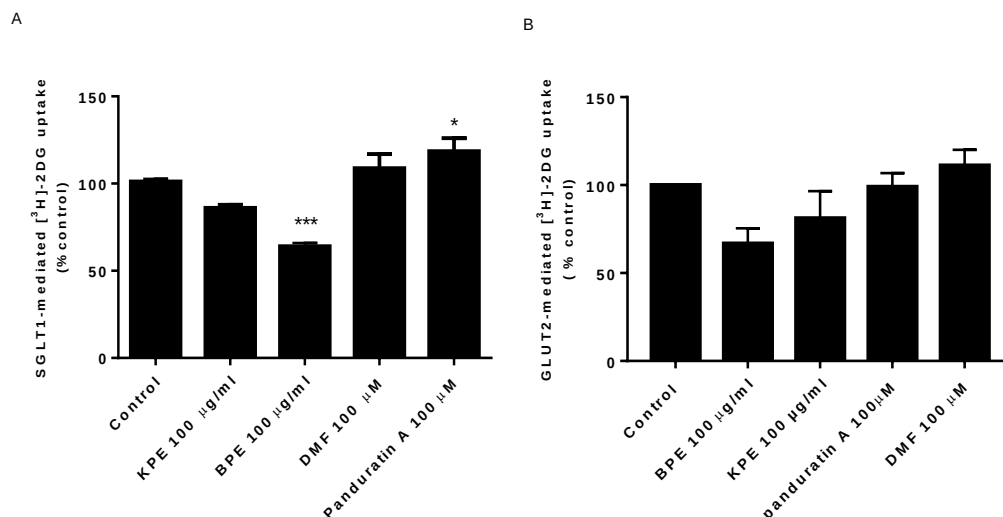
To test whether hit compounds direct inhibit transporter activity without causing toxicity, cell viability of 30 minutes' treatment of hit compounds were examined. Confluence HK-2 cells were treated with 0-200  $\mu$ M HD-ZWM-1081, HD-ZWM-1083, HD-ZWM-1084, DMF, panduratin A or 0-200  $\mu$ g/ml BPE or 0 – 500  $\mu$ g/ml KPE for 30 minutes. As show in figures 4.7 - 4.8, HD-ZWM-1081, HD-ZWM-1083, and HD-ZWM-1084 caused cytotoxicity to cells after exposing for 30 minutes, fortunately, KPE, BPE, DMF, and panduratin A had no effect on cell viability. This indicated that reduction of SGLT2- or GLUT2-mediated [ $^3$ H]-2DG uptake by HD-ZWM-1081, HD-ZWM-1083, and HD-ZWM-1084 was mediated by cytotoxicity and plant natural compounds (KPE, BPE, DMF, and panduratin A) direct inhibited SGLT2- or GLUT2-mediated [ $^3$ H]-2DG uptake.



**Figure 3.2** Effect of hit compounds on cell viability of HK-2 cells. Data are shown mean  $\pm$  SEM (\*\*\*\* $p$ <0.0001 compared with control).

### Effects of hit compounds on SGLT1 and GLUT2 inhibition in enterocyte (Caco-2 cell)

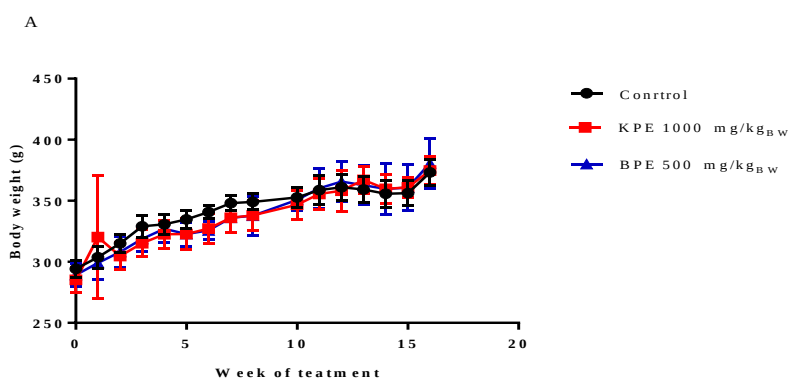
To examine the selectivity of hit compounds in SGLT2 inhibition, effect of hit compounds on SGLT1 inhibition was examined in intestinal cell line (Caco-2 cell). Glucose uptake assay was performed, cytochalasin B was used to limited GLUT2 activity. KPE and BPE insignificant inhibited SGLT1 activity but not GLUT2 in Caco-2 cells.

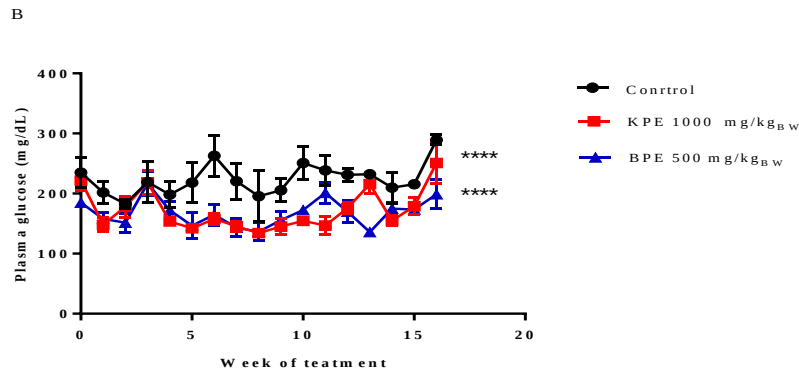


**Figure 3.3** Effect of KPE, BPE, DMF, and panduratin A on SGLT1- and GLUTs-mediated [ $^3\text{H}$ ]-2DG uptake in enterocytes (Caco-2 cell). Results were analyzed by one-way ANOVA following Dunnett's multiple comparisons test. Data are shown as mean  $\pm$  SEM (compared to control; \* $p < 0.05$ , \*\*\* $p < 0.001$ ).

### Effect of hit compounds on bodyweight and hyperglycemia in type 2 diabetic rat.

The aim of this experiment is to investigate the anti-hyperglycemic effect of hit compounds in type 2 diabetic rat. Goto Kakiziki (GK) rat was chosen as diabetic model in this study. GK rat is characterized as lean type 2 diabetic rat model with progressive insulin resistance and deficiency. GK rats with non-fasting plasma glucose equal or more than 200 mg/dl were included in experiment. The GK rats were treated with vehicle, 100 mg/kg<sub>BW</sub> KPE, and 100 mg/kg<sub>BW</sub> BPE for 3 months. At the end of 3 months, rats were treated with vehicle, 1000 mg/kg<sub>BW</sub> KPE, and 500 mg/kg<sub>BW</sub> BPE for 1 month. Plasma glucose and body weight were measured weekly to observe the effect of hit compounds on antihyperglycemia or body weight. As show in figure 4.13, KPE and BPE had no effect on body weight. KPE and BPE significant reduced plasma glucose of GK rats compared to control ( $p < 0.0001$ ).

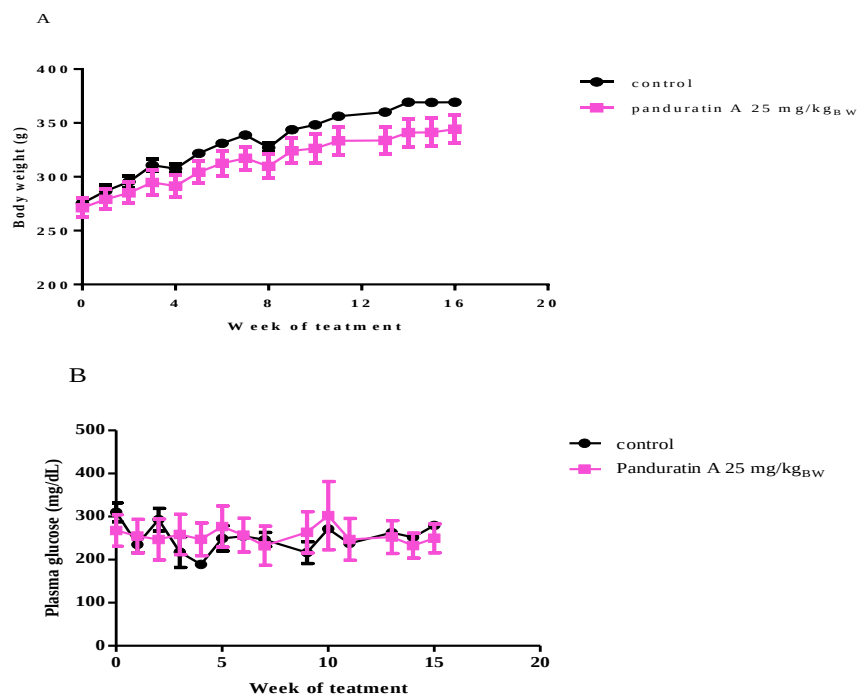




**Figure 3.4** Effects of KPE and BPE on plasma glucose and affect body weight of GK rat. Data are shown in Mean  $\pm$  SEM. Results were analyzed by one-way ANOVA following Dunnett's multiple comparisons test. Data are shown as mean  $\pm$  SEM of 6-8 animals (\*\*\*\* $p$ <0.0001 compared with control).

### Effect of panduratin A on bodyweight and hyperglycemia.

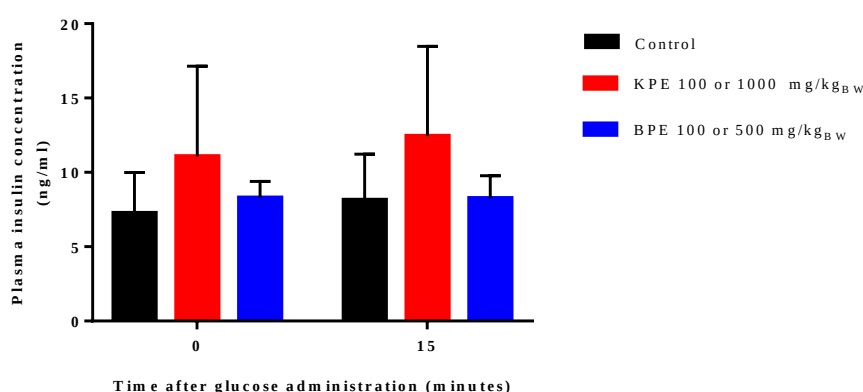
To determine whether panduratin A, a BPE component is the active compound reducing plasma glucose of GK rats, the effect of panduratin A on plasma glucose of GK rats was determined. Panduratin A did not reduce plasma glucose of GK rats (figure 4.15B). In addition, effect of panduratin A on body weight was examined, panduratin A reduced body weight but did not reduce plasma glucose (figure 3.5).



**Figure 3.5** Effect of panduratin A on plasma glucose and body weight of GK rat. Data are shown in Mean  $\pm$  SEM. Data were analyzed by Unpaired t test.

### Effect of hit compounds on insulin secretion

Insulin action is the major mechanism to defense rising in plasma glucose. Effect of KPE and BPE on insulin secretion was evaluated. OGTT was performed at the end of treatment (4 months). Rat sera were collected after glucose load 0 and 15 minutes. Plasma insulin level was assessed by insulin ELISA Kit. The result is shown in figure 4.20, the level of insulin in rats treated with KPE or BPE in both 0 and 15 minutes. This result indicated that treatment of KPE and BPE, did not affect plasma insulin level.



**Figure 3.6** Effect of KPE and BPE on affect insulin secretion. Data are shown in mean  $\pm$  SEM n=6-8. Result was analyzed by Two-way ANOVA followed by Tukey's multiple comparisons test.

### Discussion and conclusion

From 134 natural compounds screening, KPE and BPE showed the inhibitory effect on SGLT2 in human renal proximal tubular cell. Interestingly, DMF and panduratin A had low effective effect. These results indicated that the extracts might contain other active compounds on SGLT2 inhibition. In addition, KPE and BPE also inhibited GLUT2 that expresses at basolateral membrane. KPE and BPE may reduce glucose reabsorption by blocking both SGLT2 and GLUT2 activity at apical and basolateral membrane of renal proximal tubule, respectively. The inhibitory effect of KPE and BPE was predominantly on SGLT2 and GLUT2 in renal cells compared with intestinal cells indicating these compounds might not interfere glucose absorption in small intestine and they might not produce osmotic diarrhea from non-absorbable glucose as found in non-specific SGLT2 inhibitor, phlorizin. Although, marine compounds showed the higher inhibitory potencies than that of plant natural compounds, they also produced cytotoxicity which might limit further study for drug development. In vivo, KPE and BPE at low dose and high dose showed the lowering effect on plasma glucose without changing plasma insulin. Interestingly, the bioactive compound of BPE, panduratin A, had no effect on plasma glucose. These results were

similar as found in *in vitro* data. Although, KPE and BPE showed the plasma glucose in diabetic rats, the mechanisms responsible are unknown. The further studies concerning the mechanisms are required.

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#### Sub-project 4. Lipid lowering and anti-adiposity effects of naturally occurring compound

#### ABSTRACT

The incidence of obesity has substantially increased worldwide and has been received considerable attention as a major health hazard. It is common in aging population. The present study aimed to investigate lipid lowering effect of *Curcuma comosa* Roxb. (*C. comosa*) which contains phytoestrogen on adiposity and lipid metabolism in estrogen-deprived rats. Adult female rats were ovariectomized (OVX) and received daily doses of either a phytoestrogen from *C. comosa* [(3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol; DPHD], *C. comosa* extract, or estrogen (17 $\beta$ -estradiol; E2) for 12 weeks. Adipose tissue mass, serum levels of lipids and adipokines were determined. In addition, genes and proteins involved in lipid synthesis and fatty acid oxidation in visceral adipose tissue were analyzed. The results showed that ovariectomy for 12 weeks elevated level of serum lipids and increased visceral fat mass and adipocyte size. These alterations were accompanied with the up-regulation of lipogenic mRNA and protein expressions including LXR- $\alpha$ , SREBP1c and their downstream targets. OVX rats showed decrease in proteins involved in fatty acid oxidation including AMPK- $\alpha$  and PPAR- $\alpha$  in adipose tissue, as well as alteration of adipokines; leptin and adiponectin. Treatments with E2, DPHD or *C. comosa* extract in OVX rats prevented an increase in adiposity, down-regulated lipogenic genes and proteins with marked increases in the protein levels of AMPK- $\alpha$  and PPAR- $\alpha$ . These findings indicated that their lipid lowering effects were mediated via the suppression of lipid synthesis in concert with an increase in fatty acid oxidation and AMPK- $\alpha$  activity in adipose tissues, supporting the use of this plant for health promotion in the post-menopausal women.

In addition, DPHD also inhibited adipocyte differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) by suppressing the expression of genes involved in adipogenesis through the activation of ER and Wnt/ $\beta$  catenin signaling pathways. This finding suggests the potential role of DPHD in preventing bone marrow adiposity which is one of major factor that exacerbates osteoporosis in post-menopause.

**Keywords:** anti-adiposity, diarylheptanoid, phytoestrogen, ovariectomy, *Curcuma comosa*, adipocyte differentiation

## บทคัดย่อ

อุบัติการณ์ของโรคอ้วนที่เพิ่มขึ้นอย่างชัดเจนทั่วโลก เป็นเรื่องที่มีความสนใจอย่างมากเนื่องจากมีผลกระทบต่อสุขภาพ และโรคอ้วนพบมากในผู้สูงอายุ การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารจากธรรมชาติในลดไขมัน ได้ศึกษาฤทธิ์ของสารที่แยกได้จากว่านชักมดลูกซึ่งมีสมบัติเป็นไฟโตรเอสโตรเจน โดยศึกษาผลต่อการสะสมไขมันในเซลล์ กระบวนการเมแทบอลิซึม และการทำหน้าที่ของเนื้อเยื่อไขมันในหนูที่ขาดฮอร์โมนเอสโตรเจน ผลการศึกษาใช้หนูแรทที่ถูกตัดรังไข่ และได้รับการรักษาด้วยสาร phytoestrogen จาก *C. comosa* คือ (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol; DPHD หรือสารสกัดของพืช หรือฮอร์โมนเอสโตรเจนเป็นเวลา 12 สัปดาห์ โดยศึกษาถึงปริมาณเนื้อเยื่อไขมัน ระดับไขมันในเลือด และฮอร์โมนจากเซลล์ไขมัน นอกจากนี้ยังวิเคราะห์ถึงการแสดงออกของยีนและโปรตีน ที่เกี่ยวข้องกับกระบวนการสังเคราะห์ไขมัน และกระบวนการสลายไขมันของไขมันในช่องท้อง ยังได้วิเคราะห์ถึงการเปลี่ยนแปลงของเมทาโบไลต์ของไขมันในเซลล์ ผลการวิจัยพบว่าการตัดรังไข่เป็นเวลานาน 12 สัปดาห์ มีผลเพิ่มระดับไขมันในซีรัมสูงขึ้น เพิ่มมวลและขนาดของเซลล์ไขมัน การเปลี่ยนแปลงดังกล่าวมีการเพิ่มของยีนและโปรตีนที่เกี่ยวข้องกับการสังเคราะห์ไขมัน เช่น LXR- $\alpha$  และ SREBP-1c ในเนื้อเยื่อไขมัน อีกทั้งยังลดโปรตีนที่เกี่ยวข้องกับการออกซิเดชันของกรดไขมันได้แก่ AMPK- $\alpha$  และ PPAR- $\alpha$  และมีการเปลี่ยนแปลงของอะดิพอกิน เช่น leptin และ adiponectin ที่หลังจากเนื้อเยื่อไขมันอีกด้วย การให้สารเอสโตรเจน (E2), DPHD หรือสารสกัด *C. comosa* แก่หนูที่ถูกตัดรังไข่สามารถป้องกันการเพิ่มขึ้นของมวลเนื้อเยื่อไขมัน รวมทั้งลดการแสดงออกของยีนและโปรตีนในกระบวนการสังเคราะห์ไขมัน เพิ่มระดับโปรตีน AMPK- $\alpha$  and PPAR- $\alpha$  ในกระบวนการออกซิเดชันของกรดไขมัน ผลวิจัยแสดงให้เห็นว่าสาร DPHD และ สารสกัด *C. comosa* มีประสิทธิภาพดีเด่นในการออกฤทธิ์ลดการสะสมของเนื้อเยื่อไขมันโดยยับยั้งกระบวนการสังเคราะห์ไขมันและเพิ่มการสลายของกรดไขมัน ซึ่งสนับสนุนการใช้สาร และ พืช *C. comosa* ในการดูแลรักษาโรคที่เกี่ยวข้องกับการสะสมไขมันส่วนเกินในร่างกายในหญิงวัยหมดประจำเดือน

นอกจากนี้สาร DPHD ยังมีฤทธิ์ด้านการการจำแนกเซลล์ต้นกำเนิดของมนุษย์ในโพรงกระดูกไปเป็นเซลล์ไขมัน โดยไปลดการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างเซลล์ไขมัน ทำานกระตุ้นวิถีสัญญาณในเซลล์ที่เกี่ยวข้องกับตัวรับฮอร์โมนเอสโตรเจน และ Wnt/ $\beta$  catenin ฤทธิ์ลดการสะสมไขมันในโพรงกระดูกนี้ สนับสนุนการใช้พืชและสารจากพืช *C. comosa* ในการดูแลรักษาโรคที่เกี่ยวข้องกับการสะสมไขมันส่วนเกินในร่างกาย และโรคกระดูกพรุนในหญิงวัยหมดประจำเดือน

**คำสำคัญ:** ลดไขมัน ไดเอริลเฮปตานอย ไฟโตรเอสโตรเจน ตัดรังไข่ ว่านชักมดลูก พัฒนาการของเซลล์ไขมัน

## 1. INTRODUCTION

### 1.1 Statement and significance of the research problem, and objectives

Obesity is a disorder of energy balance caused by the energy intake exceeds the expenditure resulting in an excessive accumulation and expansion of adipose tissue. The increase of visceral adipose tissue, specifically in adipocyte size, contributes to a chronic state of low-grade inflammation affecting adipose tissue functionality. Dysfunction of adipose tissue has been reported to be a major cause of metabolic disorders including insulin resistance, type 2 diabetes, and cardiovascular diseases (Romeo, et al., 2012). The

prevalence of obesity in postmenopausal women has been reported to be higher compared to men of the same age (BaHammam, et al., 2016). Although many factors have been proposed to involve in the development of obesity in women (Vicennati, et al., 2009), aging and decline in estrogen level are the important contributing factors to increase the risk of obesity and metabolic diseases (D'Eon, et al., 2005).

The relationship between a systemic decline in estrogen level and an increase of adiposity has been observed in post-menopausal women (Lundholm, et al., 2004). Estrogen suppresses mRNA expression of genes involved in lipid synthesis including liver x receptor- $\alpha$  (Lxr- $\alpha$ ) and its positive regulator, sterol regulatory element binding protein 1c (Srebp-1c) (Lundholm, et al., 2008). In addition, estrogen also regulates lipid metabolism by non-genomic activation of 5'AMP-activated protein kinase  $\alpha$  (AMPK- $\alpha$ ); a crucial metabolic sensor for energy regulation (D'Eon, et al., 2005).

*Curcuma comosa* Roxb. (*C. comosa*) a plant in Zingiberaceae family, is an indigenous medicinal herb that has traditionally been used for treatment of postpartum uterine bleeding and relieving unpleasant symptoms in menopausal women (Anonymous, 1967, Pongboonrod, 1976). *C. comosa* rhizome extract contains a number of diarylheptanoids (Suksamrarn, et al., 2008). A non-phenolic diarylheptanoid (3*R*)-1,7-diphenyl-(4*E*, 6*E*)-4,6-heptadien-3-ol (DPHD) is identified as the major active constituent of *C. comosa* and possesses estrogenic-like activity (Winuthayanon, et al., 2009). In addition to the estrogenic-like activity, both *C. comosa* rhizome extract and DPHD exhibit several other pharmacological effects including lipid lowering effect in hypercholesterolemic animals (Charoenwanthanang, et al., 2011, Piyachaturawat, et al., 1999, Ratanachamnong, et al., 2012) and ovariectomized rats (Prasannarong, et al., 2012), anti-oxidant effect, anti-inflammatory activity and protective effect on bone loss (Jantaratnotai, et al., 2006, Sodsai, et al., 2007, Weerachayaphorn, et al., 2011). *C. comosa* also improves lipid, glucose metabolisms, and insulin sensitivity in ovariectomy-induced dyslipidemia rats which show the impairment of insulin sensitivity (Prasannarong, et al., 2012). However, the mechanism underlying these systemic perturbations and the treatment effect of *C. comosa* are not clear. Adipose tissue which is the largest energy reservoir in the body, participating primarily in the energy homeostasis may be affected under this condition.

In the present study, we investigated the effect of DPHD and *C. comosa* extract on lipid metabolism in adipose tissue of ovariectomy-induced estrogen-deprived rats. The expression levels of key transcription factors involved in lipid synthesis, their downstream targets, and 5'AMP-activated protein kinase  $\alpha$  (AMPK- $\alpha$ ) phosphorylation which is an integrating signaling regulator of lipid metabolism, were determined. We also investigated therapeutic potential of *C. comosa* by determining changes of lipid profiles in the ovariectomized rat model of estrogen-deficiency-induced hyperlipidemia after treatment with different components of *C. comosa* using an untargeted metabolomics approach.

### Objectives:

- (1) To investigate the effect of DPHD and *Curcuma comosa* extract on adipose tissue mass and adipocyte size in OVX rats. The expression level of gene and protein involved in lipid synthesis and fatty acid oxidation in visceral adipose tissue was also determined.
- (2) To investigate the effect of DPHD from *Curcuma comosa* on the adipogenic differentiation of mesenchymal progenitors, human bone marrow-derived mesenchymal stem cells (hBMSCs)

## 2. Materials and methods

### 2.1. Plant materials and Preparation of *C. comosa* extract and DPHD

*Curcuma comosa* Roxb., a member of Zingiberaceae family, is commonly known as Wan chak motluk in Thai. They were collected from Kampangsaen District, Nakhon Pathom, Thailand and subjected to taxonomic identification by Soontornchainaksaeng and Jenjittikul (2010). The voucher herbarium specimen number SCMU-300 was deposited at the Department of Plant Science, Faculty of Science, Mahidol University, Thailand. *C. comosa* rhizomes extract and its phytoestrogen diarylheptanoid (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (DPHD, Fig 1) were prepared according to previously described (Suksamrarn, et al., 2008). The purity of DPHD was estimated to be >98% by HPLC.

### 2.2. Animals and treatments

Adult females Sprague-Dawley rats (8-weeks-old), weighing between 200-220 g, were used. The sample size in each experiment was determined using Minitab® statistical 18 software. Based on power analysis, six animals per group were required. The rats were randomly assigned to either sham-operated control (SHAM, n=6) or bilateral ovariectomy (OVX, n=24). Animals were allowed to recuperate for 7 days after surgery. Ovariectomized rats were divided into 4 groups of six animals each as follows: OVX rats receiving vehicle (OVX control); OVX rats receiving 17 $\beta$ -estradiol (E2) at a dose of 10  $\mu$ g/kg BW, s.c.(OVX+E2); OVX rats receiving DPHD 50 mg/kg BW, s.c. (OVX+DPHD); OVX rats receiving *C. comosa* extract 500 mg/kg BW, i.g. (OVX+extract). E2 and DPHD were initially dissolved in absolute ethanol and then diluted in olive oil to give a final injection volume of approximately 100-200  $\mu$ l. The extract was directly suspended in 1% carboxymethyl cellulose to the specified concentration for oral ingestion of not more than 1 ml. All treatments were given daily for 12 weeks as indicated. All animal procedures were approved by the Animal Care and Use Committee, Faculty of Science, Mahidol University (protocol no. MUSC 56-031-293).



### **2.3. Serum lipids and adipokine measurements**

At the end of the treatment period, animals were fasted overnight (12 h). They were euthanized. Following adipose tissue dissection, whole blood was collected from the posterior vena cava. Serum levels of triglyceride (TG), total cholesterol, and low density lipoprotein cholesterol (LDL-c) were measured using automated enzymatic methods (Siemens Dimension RxL Max; Siemens Medical Solution Diagnostics, NY, USA). For adipokine measurements, serum was diluted to 100-fold and subjected to leptin, and adiponectin quantifications using adipokine enzyme-linked immunosorbent assay (ELISA) kit (R&D system, Minn, USA).

### **2.4. Adipose tissue mass and adipocyte morphometry**

Visceral adipose tissue mass was determined by weighing fat pads dissected from specific depots which were retroperitoneal, periovarian and mesenteric fat pads. For adipocyte morphometry, fresh retroperitoneal adipose tissue was fixed with 10% neutral buffered formalin and process for routine staining of paraffin section with hematoxylin and eosin (H&E). Digital images were acquired randomly under Olympus BX51 microscope. Adiposoft, an automated software authorized by Center for Applied Medical Research (CIMA), University of Navarra, Spain, was used to measure adipocyte area at least 100-120 adipocytes/rat, which were represented as the percentage of total number adipocytes in different diameters (in  $\mu\text{m}$ ).

### **2.5. Cell culture and differentiation**

Cell lines used were human bone marrow derived-mesenchymal stem cells (MSCs) which were purchased from Lonza (Allendale, NJ, USA). They were isolated from normal person (non-diabetic), adult female human bone marrow, age 22, by withdrawing marrow from the posterior iliac crest. Cells were cultured in Dulbecco's modified Eagle media (DMEM) with low glucose content (1g/L) containing 10 % fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin). They were incubated at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>.

### **2.6. Induction of cell differentiation**

Adipogenic differentiation of human MSCs was induced by culturing in human adipogenic differentiation (HAD) medium. The medium consists of DMEM (high glucose) with 10% charcoal-stripped FBS, antibiotics, and inducing reagents; 10  $\mu\text{g}/\text{ml}$  insulin, 0.5 mM 3-isobutyl-1 methyl xanthine (IBMX), 10 nM dexamethasone and 0.2 mM indomethacin. Briefly, human MSCs were seeded into 6 well-plates at a density of 1x10<sup>6</sup> cells per well. Two days after reaching confluence, the adipogenic differentiation medium was applied for culture

and the medium was replaced every 3 days until 14 days. During adipogenic differentiation, MSCs were treated with DPHD or 17  $\beta$ -estradiol (E2). To investigate the involvement of estrogen receptor  $\alpha$  (ER $\alpha$ ), MSCs were pre-treated with 100 nM of ICI 182, 780, an ER $\alpha$  antagonist for 2 h before receiving DPHD or E2.

Osteogenic differentiation of human MSCs were induced by culturing in osteogenic differentiation (HOB) medium. The medium consists of DMEM (low glucose) without phenol red, 10% charcoal-stripped FBS, 5 mM  $\beta$ -glycerol phosphate, and 25  $\mu$ g/ml of ascorbic acid. Briefly, human MSCs were seeded into 6-well plates at a density of  $1 \times 10^6$  cells per well. Two days after achieving confluence (day 0), HOB medium was applied for differentiation and they were maintained in osteogenic media for 21 days. The culture media was replaced every 3 days.

## 2.7. Real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis

Quantitative real-time PCR of each samples was done on the equal amount of cDNA using KAPA SYBR<sup>®</sup>FAST qPCR kit according to the manufacturer's instructions (KapaBiosystems) with the ABI PRISM 7500 Sequence Detection System and analysis software (Applied Biosystems). The target mRNA level was normalized by  $\beta$ -actin or GAPDH. The relative levels of transcript expression were quantified using the mathematical model described by Pfaffl, in which  $\text{ratio} = 2^{-(\text{pCt sample} - \text{pCt control})}$  when  $\Delta\text{Ct} = \text{Ct (vehicle)} - \text{Ct (treated sample)}$ , where E = efficiency of the primer set, calculated from the slope of a standard curve of log (ng of cDNA) versus the value for a sample that contains the target according to the formula  $E = 10^{-(1/\text{slope})}$  (Hewitt et al., 2005).

Western blot analysis was performed according to the previously described protocol and manufacturer's instruction (Bio-Rad Laboratories).

## 2.8. Whole genome Affymetrix GeneChip cRNA screening

Human MSCs were selectively induced to differentiate either to osteoblasts, or adipocytes. During differentiation, they were received 0.1  $\mu$ M DPHD or 10 nM E2, as positive control. On day 7 of osteoblast and adipocyte differentiation, total RNA from MSCs was extracted and used as starting materials to obtain labeled cDNAs. Genome wide screening was performed using Affymetrix GeneChip<sup>®</sup> MG430A 2.0 Array (Affymetrix, USA) as previously described (Robinson, et al., 2010). Briefly, single stranded cDNA was synthesized by reverse transcription using the poly (A) RNA presented in the starting total RNA sample. Single stranded cDNA was then converted into double stranded cDNA and purified using the Affymetrix Cleanup Module. An in vitro transcription (IVT) reaction was then carried out overnight in the presence of biotinylated UTP and CTP to produce biotin-labeled cRNA from the double stranded cDNA. The resulting cRNA is fragmented in the presence of heat and

Mg+2, and hybridized to the DNA array on glass, the Hu 133.2 probe set of 54675 assays with 20 replicates per target. Targets include segments of most known genes, with two or more probes per target. Presence of transcripts and differences in gene expression between control and treatment were determined from the signal and variation of each assay replicate with statistical confidence as indicated. Analysis included determination of effect on common metabolic pathways by MetaCore (www.genego.com, Clarivate, Analytics, Philadelphia, PA, USA) comparing effect of DPHD, E2, and control against a library of 121 intracellular pathways.

## 2.9. Statistical analysis

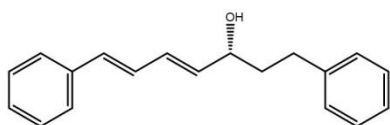
Data were expressed as means and standard error of means (mean  $\pm$  SEM). They were analyzed by using the statistical software package, GraphPad Prism version 5.0. Data among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. Statistical significance was considered when  $p < 0.05$ .

## 3. RESULTS

**Part I:** To investigate the effect of *C. comosa* treatment on adipose tissue mass and adipocyte size of OVX rats. The expression level of gene and protein involved in lipid synthesis and fatty acid oxidation in visceral adipose tissue was also determined.

### 3.1. Body weight, uterine weight, serum glucose and insulin, and lipids.

Table 4.1 shows general characters of all experimental groups. Ovariectomy for 12 weeks markedly increased the body weight with a decrease in uterine weight as compared to those in SHAM control. However, E2 treatment restored the body weight, and uterine weight of the OVX rats to the level of SHAM control whereas *C. comosa* (DPHD and *C. comosa* extract) treatment restored the body weight with a slight increase in the uterine weight, but still far below that of SHAM control. Levels of serum lipids including total cholesterol, triglyceride, and LDL cholesterol were significantly increased in the OVX rats ( $p < 0.05$ ). Treatments with E2, DPHD, or *C. comosa* extract for 12 weeks essentially prevented the increases in body weight, and serum level of total cholesterol, and LDL cholesterol in the OVX rats, indicating lipid-lowering activities of DPHD and *C. comosa* extract. However, only *C. comosa* extract significantly decreased serum triglyceride level ( $p < 0.05$ ).



**Fig. 4.1.** Structure of a non-phenolic diarylheptanoid (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol (DPHD) from *Curcuma comosa* Roxb.

**Table 4.1.** Effects of E2 and *C. comosa* on body weight, uterine weight and serum levels of lipids after 12-weeks of treatments.

| PARAMETERS               | SHAM        | OVX control               | OVX+E <sub>2</sub>        | OVX+DPHD                  | OVX+extract              |
|--------------------------|-------------|---------------------------|---------------------------|---------------------------|--------------------------|
| Body weight (g)          | 298.9±18.9  | 371.0±14.4 <sup>**</sup>  | 286.7±11.4 <sup>††</sup>  | 297.3±13.8 <sup>††</sup>  | 285.4±14.0 <sup>††</sup> |
| Uterine weight (mg/kgBW) | 2.42±0.04   | 0.31±0.07 <sup>**</sup>   | 3.00±0.02 <sup>††</sup>   | 0.87±0.05                 | 1.65±0.06 <sup>†</sup>   |
| Cholesterol (mg/dL)      | 107.0±11.10 | 145.33±19.5 <sup>**</sup> | 129.14±15.87 <sup>†</sup> | 115.83±13.76 <sup>†</sup> | 82±22.70 <sup>††</sup>   |
| Triglyceride (mg/dL)     | 32.67±10.33 | 47.167±13.26 <sup>*</sup> | 69.1.7±13.37              | 51.33±16.60               | 29±13.60 <sup>††</sup>   |
| LDL-cholesterol (mg/dL)  | 13.33±1.21  | 22.83±4.36 <sup>**</sup>  | 14.14±2.34 <sup>††</sup>  | 12.43±2.15 <sup>††</sup>  | 11.14±3.48 <sup>††</sup> |

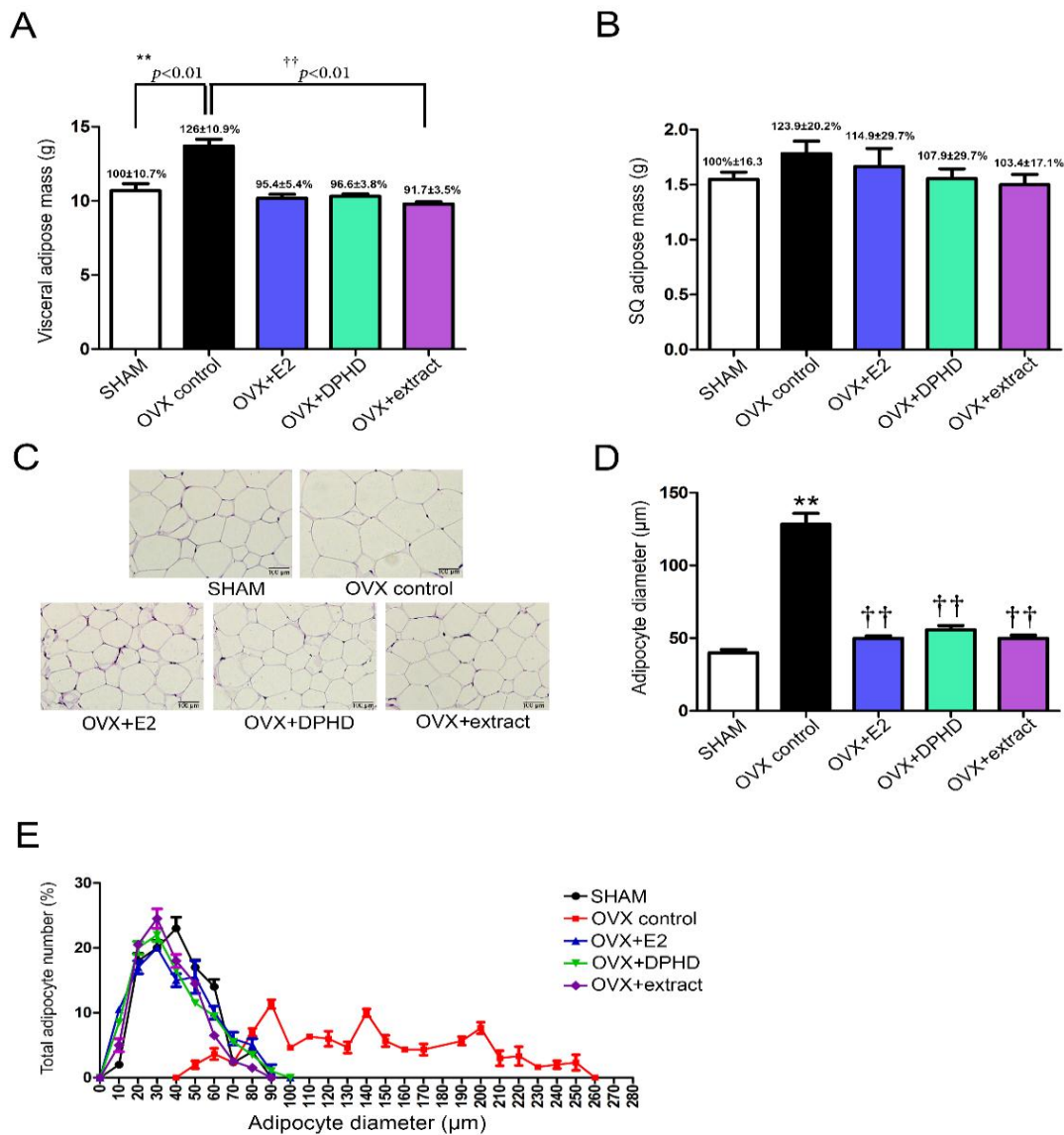
Each value is mean ± SEM obtained from 6 animals. Rats were assigned as sham operated control (SHAM), ovariectomized control (OVX control), OVX receiving E2 10 µg /kg BW (OVX+E2); OVX receiving DPHD 50 mg /kg BW (OVX+DPHD); and OVX receiving *C. comosa* extract 500 mg /kg BW (OVX+extract). \*p<0.05 and \*\*p<0.01 (vs. SHAM); <sup>†</sup>p<0.05 and <sup>††</sup>p<0.01 (vs. OVX).

### 3.2. Adipose tissue mass and adipocyte sizes

OVX rats had a greater visceral adipose tissue mass (13.7±1.2 g; 126.0±10.9%) than SHAM control (10.7±1.1g; 100±10.7%) (Fig 3.2A). E2 was used as a positive control, the mass was significantly reduced to control level after treatment with E2 (95.4±5.4%), DPHD (96.6±3.8%), and *C. comosa* extract (91.7±3.5%). There was a similar trend to modulate the subcutaneous adipose tissue mass by which ovariectomy caused an increase and the treatments decreased. However, there was no statistical significant compared to SHAM control ((P> 0.05) (Fig 3.2B). The increase of visceral adipose tissue mass in OVX rats was accompanied with an increase in the size of adipocytes (Fig3. 2C). As shown in Fig 3.2D, average adipocyte diameter significantly increased in OVX rats (128.4±53.12 µm) compared to that in SHAM (40±14.72 µm). Treatment with E2, DPHD or *C. comosa* extract reduced adipocyte diameter to the level comparable to that of SHAM. In Fig 3.2E, OVX rats had a greater number of adipocytes with larger cross-sectional area. The hypertrophy of adipocytes in OVX rats was a striking feature found in the visceral fat mass which was prevented by treatments with E2, DPHD or *C. comosa* extract.

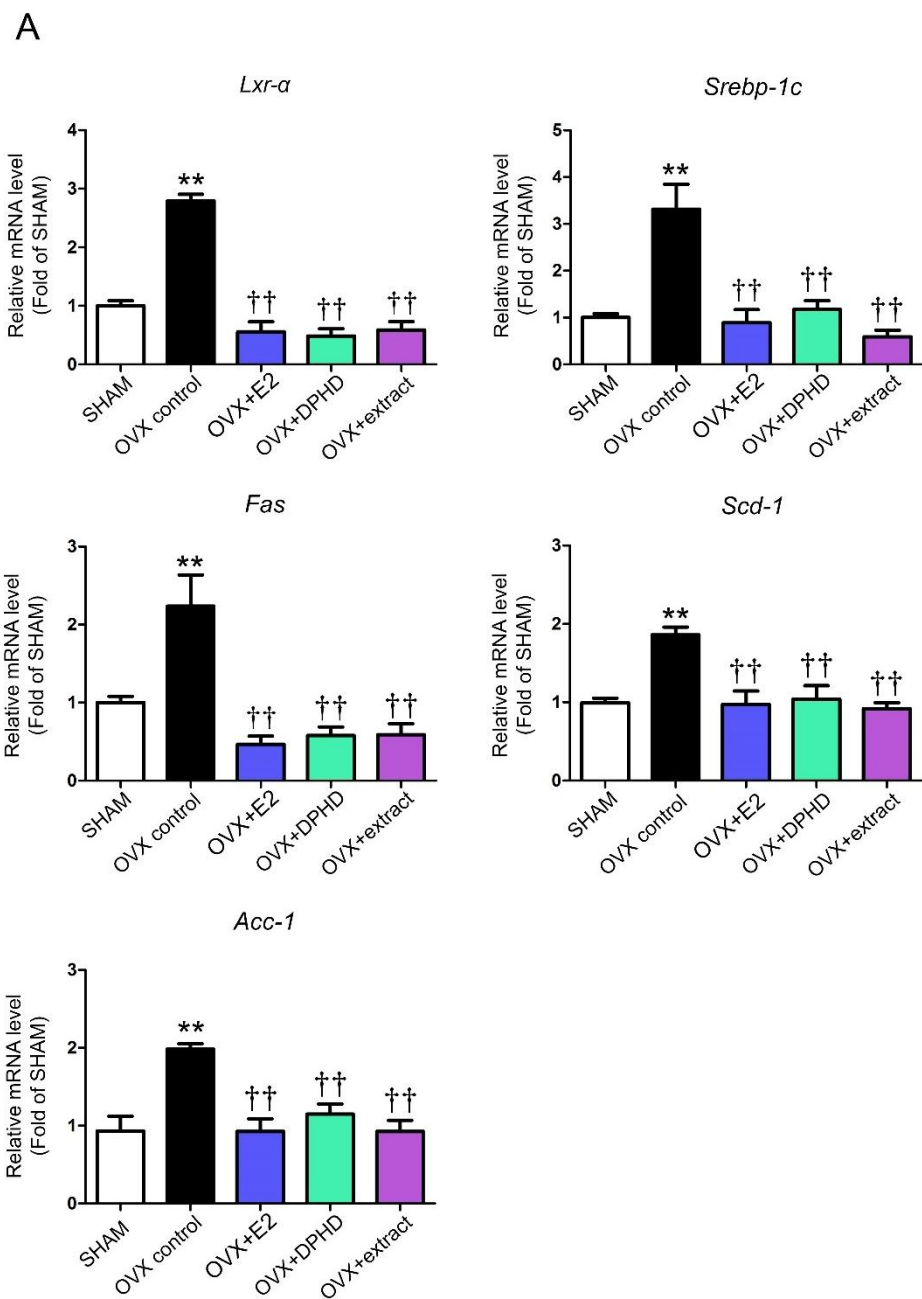
The effect of *C. comosa* in preventing the hypertrophy of adipocytes and the increase in adipose tissue mass of OVX rats were further investigated. The mRNA expression of genes involved in lipid synthesis including liver x receptor- $\alpha$  (*Lxr- $\alpha$* ), sterol regulatory element binding protein 1c (*Srebp-1c*), fatty acid synthase (*Fas*), stearyl-CoA desaturase 1

(*Scd-1*), and acetyl-CoA carboxylase 1 (*Acc-1*) were dramatically increased in the hypertrophic adipocytes of OVX rats compared to those in SHAM control. Treatment of E2, DPHD or *C. comosa* extract markedly suppressed the expressions of these elevated lipogenic genes to the levels of control (SHAM) (Fig 3.3A). In corresponding to the expressions of lipogenic mRNAs, levels of proteins involved in lipid synthesis including LXR- $\alpha$ , SREBP-1c, FAS, and ACC-1 in adipose tissue of OVX rats were also increased and they were decreased by treatment with E2, DPHD or *C. comosa* extract (Fig4. 3B).

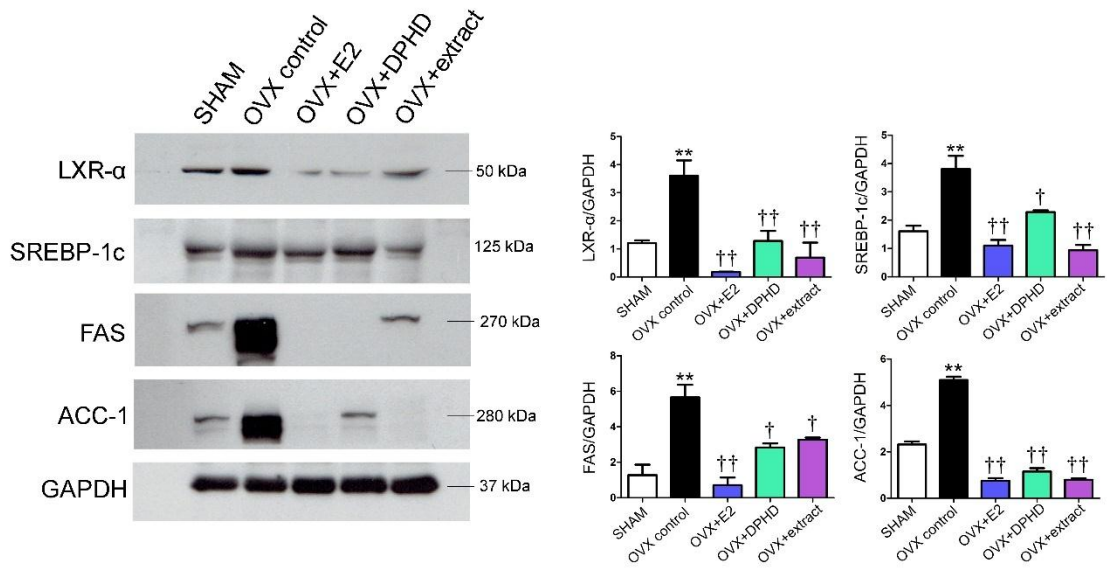


**Fig. 4.2.** Effects of treatments on adipose tissue masses from visceral (A); subcutaneous (B); and adipocyte size (C); average adipocyte diameter (D); and distribution of adipocyte size of visceral adipose tissue (E), in SHAM, OVX control, OVX+E2, OVX+DPHD and OVX+extract. Data are expressed as mean ± SEM obtained from 6 animals. \*  $p < 0.05$  and \*\*  $p < 0.01$  (vs. SHAM); †  $p < 0.05$  and ††  $p < 0.01$  (vs. OVX).

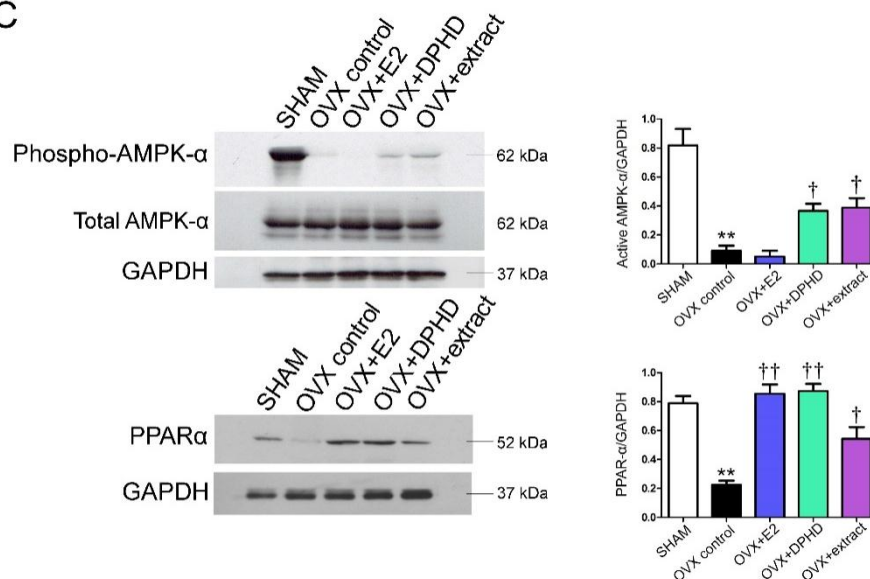
### 3.3. Expressions of genes and proteins involved in lipid synthesis and fatty acid oxidation in adipose tissue.



B



C



**Fig. 4.3.** Effects of treatments on the expressions of lipogenic mRNA: *Lxr- $\alpha$* , *Srebp-1c*, *Fas*, *Scd-1* and *Acc-1*.(A); level of lipogenic proteins: LXR $\alpha$ , SREBP1c, FAS, ACC-1, and SCD-1 along with densitometric values of protein bands normalized by GAPDH (B); and level of proteins involved in fatty acid oxidation: AMPK- $\alpha$  and PPAR- $\alpha$  with densitometric values of protein bands normalized by GAPDH (C) in SHAM, OVX control, OVX+E2, OVX+DPHD and OVX+extract. Data are expressed as mean  $\pm$  SEM from 6 animals. \* $p$ <0.05 and \*\* $p$ <0.01 (vs. SHAM); <sup>†</sup> $p$ <0.05 and <sup>††</sup> $p$ <0.01 (vs. OVX)

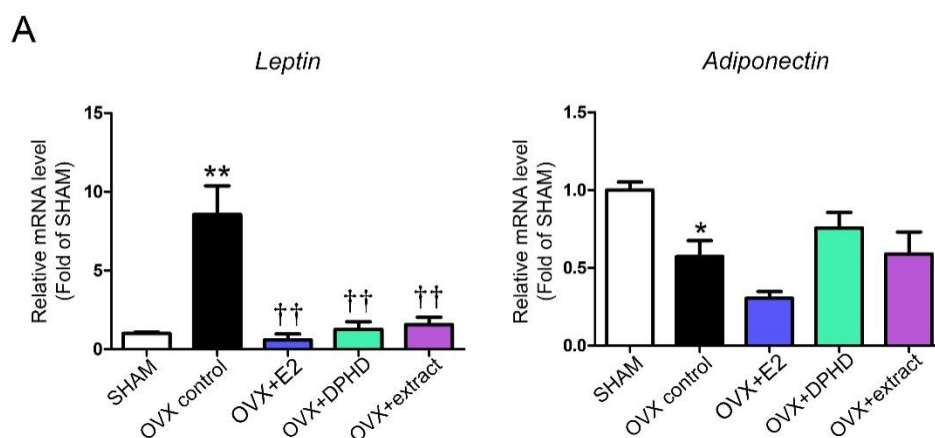
The potential role of proteins involved in fatty acid oxidation including AMPK- $\alpha$  and PPAR- $\alpha$  in mediating the anti-adiposity of *C. comosa* treatments was also determined. In

Fig. 4.3C, level of phosphorylated AMPK- $\alpha$  (Threonine 172) protein, a marker of AMPK- $\alpha$  activation was significantly decreased in the adipose tissue of OVX rats in respect to SHAM control, whereas *C. comosa* and DPHD demonstrated marginal increases in the active form of AMPK- $\alpha$ . However, E2 treatment had no effect on AMPK- $\alpha$  activity in adipose tissue. The level of PPAR- $\alpha$  protein was also significantly decreased in OVX rats and it was significantly increased to the level of SHAM control after treatment with E2, DPHD or *C. comosa* extract.

### 3.4. Expression and secretion of adipocytokines

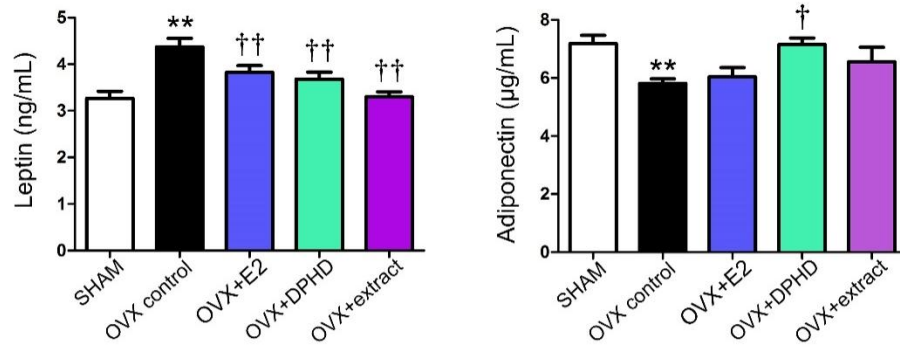
Hypertrophic adipocyte is often associated with adipokine dysregulation and insulin resistance (Jung and Choi, 2014). In OVX rats, the expression of *Leptin* mRNA in adipose tissue was highly upregulated whereas the expression of *Adiponectin* mRNA was downregulated. Treatment with E2 or *C. comosa* reduced the expression of *Leptin* mRNA to the level of SHAM control. On the other hand, DPHD or *C. comosa* extract failed to restore the level of *Adiponectin* mRNA whereas E2 treatment further decreased the expression of *Adiponectin* mRNA (Fig 4A). Concurrently, the circulating level of leptin was also increased in the OVX rats, but was decreased by treatment with E2 or *C. comosa*. On the contrary, the circulating level of adiponectin, which was decreased in the OVX rats, was restored only by DPHD (Fig 4.4B).

In addition to adipokines, which are involved in regulating food intake and insulin sensitivity, the expression of *Tnf- $\alpha$*  and *Resistin* mRNA that participate in inflammatory responses and insulin resistance were significantly increased in the visceral adipose tissue of OVX rats, indicating the presence of inflammation and insulin resistance in adipose tissue. Treatment with E2 or *C. comosa* significantly reduced the level of *Tnf- $\alpha$*  and *Resistin* mRNA (Fig 4.4C). Moreover, the increased level of TNF- $\alpha$  protein in OVX control was significantly decreased by treatment with E2, DPHD or *C. comosa* extract (Fig 4.4D).

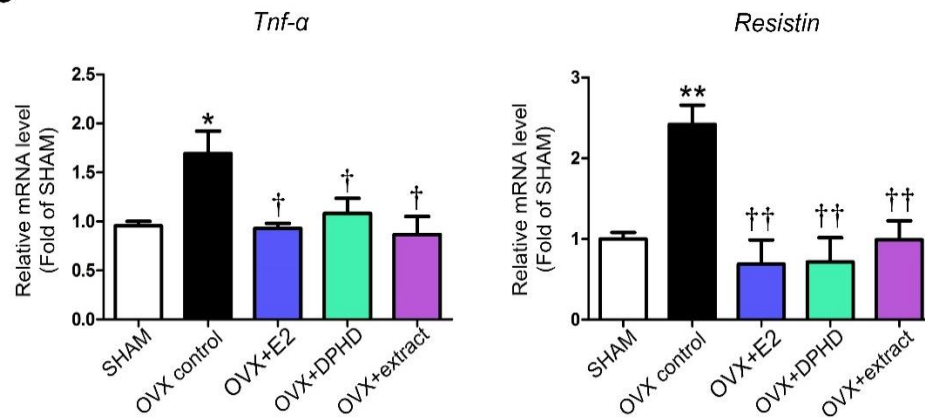




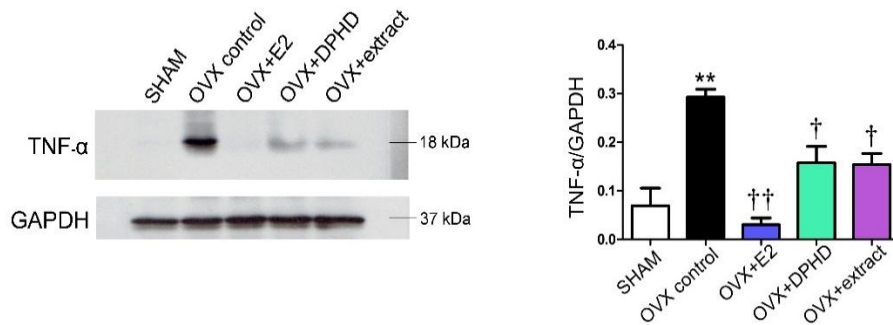
B



C



D



**Fig. 4.4.** Effects of treatments on the expressions of *Leptin* and *Adiponectin* mRNA determined by RT-qPCR (A); circulating leptin and adiponectin measured by ELISA (B); expression of *Tnf-α* and *Resistin* mRNA (C); and level of *TNF-α* protein along with densitometric values of protein bands normalized by GAPDH (D) in SHAM, OVX control, OVX+E2, OVX+DPHD and OVX+extract. Data are expressed as mean ± SEM of 6 animals. \* p<0.05 and \*\* p<0.01 (vs. SHAM); <sup>†</sup> p<0.05 and <sup>††</sup> p<0.01 (vs. OVX).

## Discussion and conclusion

The present study demonstrated the lipid lowering effect of *C. comosa* extract and its active phytoestrogen, DPHD, in estrogen-deficient rats. Both *C. comosa* extract and DPHD effectively prevented the gain of body weight, excessive visceral fat accumulation, enlargement of adipocyte, and metabolic disturbances in OVX rats similar to those by E2. In consistent with the reduced visceral fat mass, *C. comosa* down-regulated the expression of genes and proteins involved in lipid synthesis including LXR- $\alpha$ , SREBP-1c and their downstream targets. Moreover, they also increased proteins involved in fatty acid oxidation including AMPK- $\alpha$  and PPAR- $\alpha$  in adipose tissue of the OVX rats. Our data suggest that *C. comosa* reduced adiposity in OVX rats by inhibiting lipogenesis, in concert with promoting fatty acid oxidation and thereby improving adipokine secretion from adipose tissue. This is the first report showing the mechanism underlying lipid lowering effect of *C. comosa*.

Estrogen plays important roles in regulating energy and lipid homeostasis by both suppressing food intake and increasing energy expenditure (D'Eon, et al., 2005). Deficiency of estrogen is associated with increases in adiposity and risk of metabolic syndromes, particularly in postmenopausal women (Lundholm, et al., 2008). In the present study, ovariectomy markedly increased visceral adipose tissue mass. A similar trend was also seen in subcutaneous adipose tissue mass, although there was no statistically significant. Our finding is similar to the previous study reporting that ovariectomized (OVX) rats gain adipose tissue, specifically visceral adipose tissue with no significant change of subcutaneous adipose tissue (Clegg, et al., 2006). The marked increase in visceral adipose tissue mass was accompanied with up-regulation of key transcription factors responsible for lipid synthesis including LXR- $\alpha$  and SREBP-1c, which were suppressed by E2 or *C. comosa* treatments. The interplay between the LXR and ER ligand/receptor systems has been evidenced by the presence of ERE on LXR- $\alpha$  promoter (Han, et al., 2014). Decreased expressions of LXR- $\alpha$  and its positive regulator SREBP-1c found in E2 and *C. comosa* treatments might co-operatively suppress the *de novo* lipid synthesis which led to the reduction of adipose tissue mass. Our findings were consistent with the earlier report that E2 suppressed LXR- $\alpha$ -activated SREBP-1c expression (Han, et al., 2014). Although *C. comosa* contains a weak estrogenic-like activity as indicated by its weak uterotrophic effect (Table 4.1), it substantially suppresses the elevated expressions of LXR- $\alpha$  and lipogenic genes in OVX rats similar to those of estrogen (Fig. 4.3A).

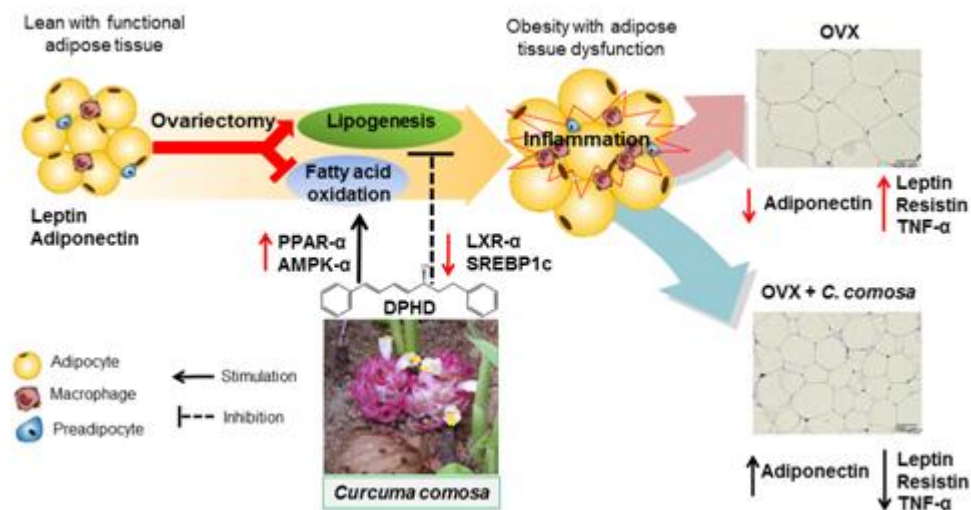
*C. comosa* treatment also increased the level of proteins involved in fatty acid oxidation including AMPK- $\alpha$ , an important energy sensor in metabolic tissue, and PPAR- $\alpha$ , which was decreased in the OVX rats. Activation of AMPK- $\alpha$  inhibits lipid synthesis by suppressing the expression of *Acc-1* mRNA and facilitating fatty acid oxidation in adipose tissue (Matejkova, et al., 2004, Sim and Hardie, 1988, Sullivan, et al., 1994). AMPK- $\alpha$  does not only influence lipid metabolism but also suppresses inflammation in adipose tissue (Viollet

and Andreelli, 2011). The activation of AMPK- $\alpha$  has been proposed to be a potential therapeutic target for the treatment of obesity and insulin resistance (Carling, et al., 2012). In this study, E2 and *C. comosa* treatments restored the level of PPAR $\alpha$  which acts synergistically with AMPK- $\alpha$  to increase fatty acid oxidation and suppress lipogenesis, contributing to the reduction of visceral adipose tissue mass.

*C. comosa* contains a weak estrogenic-like activity as indicated by its weak uterotrophic effect. The anti-lipogenic effect of *C. comosa* might, in part, be contributed by other pathways apart from estrogen signaling, particularly related to its anti-inflammatory activity. Recently, *C. comosa* has been shown to exhibit an anti-inflammatory activity in several experimental models. It suppresses the expression of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in aortic rings of OVX rats (Intapad, et al., 2012). Similarly, *C. comosa* extract is also found to exert the anti-inflammatory effect in hypercholesterolemic rabbits by decreasing the expressions of pro-inflammatory cytokines; IL-1, MCP-1, and TNF- $\alpha$  (Charoenwanthanang, et al., 2011). In the present study, *C. comosa* also reduced the expression of *Tnf- $\alpha$*  and *Resistin* genes which are associated with the inflammatory response. On the other hand, it also increased levels of adiponectin and AMPK- $\alpha$  which are anti-inflammatory factors. The improvement of lipid metabolism by *C. comosa* treatments was essentially related to the reduced adipose tissue mass which is likely contributed by its anti-inflammatory activity. In addition, the alterations in adipose tissues in the present study may account for the underlying systemic perturbations in the estrogen deficiency condition and after receiving the DPHD and *C. comosa* treatments in the previous metabolomic study (Vinayavekhin, et al., 2016). More importantly, DPHD gave rise to the same outcome as *C. comosa* extract, indicating that most of the modulating effects observed in *C. comosa* extract might be mainly responsible by DPHD.

In conclusion, we report herein for the first time the lipid lowering effect of *C. comosa* extract and its major active compound, DPHD, on adipose tissue in OVX rats. These compounds suppressed lipogenesis in concert with the improvement of fatty acid oxidation, which promoted metabolically healthy adipocytes with normal adipokine secretion. This study provides insight information for the development of *C. comosa* and DPHD as a dietary supplement for treatments of metabolic complications related with estrogen deficiency-induced adipose tissues accumulation.

### Graphical abstract



**Figure 4.5** Schematic diagram showing the anti-adiposity activities of *C. comosa* treatments in visceral adipose tissue of OVX rats. The *C. comosa* extract as well as DPHD, its major active compound, suppresses lipogenesis in concert with the improvement of fatty acid oxidation in adipose tissue in OVX rats. They promote metabolically healthy adipocytes with normal adipokine secretion.

## Part II: Inhibition of Adipogenic Differentiation of Human Bone Marrow Derived Mesenchymal Stem Cells by DPHD, a Phytoestrogen from *Curcuma comosa*

Adipocytes in bone marrow share a common precursor with osteoblast, so called mesenchymal stem cells (MSCs). In aging, a number of MSCs in the bone marrow, which are multipotent stem cells are decreased. In addition, they lose their ability to differentiate into osteoblasts but tend to differentiate into adipocytes, causing bone loss. Thus, any compound that has an ability to increase MSC pool and promote differentiation of osteoblasts with an inhibition on adipogenic differentiation may be the attractive therapeutic agent for the treatment of osteoporosis in aging and postmenopausal women.

DPHD, a phytoestrogen from *C. comosa* has previously been demonstrated to have a positive osteogenic effect in committed MC3T3-E1 pre-osteoblast (Bhukhai, et al., 2012) and in human osteoblasts (Tantikanlayaporn, et al., 2013). DPHD also provides adipogenic effect in 3T3-L1 pre-adipocytes (Unpublished data). However, its effect on the differentiation of uncommitted MSCs has not been investigated. The present study was designed to investigate the effect of DPHD on mesenchymal stem cell differentiation. As the differentiation of MSCs into mature adipocytes takes approximately 14 days whereas differentiation toward mature osteoblast requires additional 7 days, the experiments were conducted for 14 and 21 days.

## Results and Conclusion

DPHD treatment suppressed differentiation of MSCs into adipocytes while promoted differentiation into osteoblasts. In Fig. 4.6, after incubation in human adipogenic differentiation (HAD) medium for 7 days, MSCs differentiate into mature adipocytes, which was manifested by an increase in intracellular lipid droplet to  $3.01 \pm 0.4$  compared to non-induced MSCs (assigned as 1). E2, which was used as a positive control, significantly reduced lipid accumulation. Similar to E2, treatment with DPHD at concentrations of 0.1, 1, and 10  $\mu\text{M}$  during early period of differentiation (first 7 day of induction) also reduced the intracellular lipid accumulation to  $2.5 \pm 0.2$ ,  $2.4 \pm 0.2$ , and  $2.1 \pm 0.5$ , respectively. The intracellular lipid is stored in the form of triglycerides in adipocytes that are detected by the Oil Red O staining and triglyceride colorimetric assay. In addition to the morphological changes and lipid accumulations in the differentiated adipocytes, adipogenic genes and proteins were examined as markers of the differentiated cells. In Figure 4.7, levels of lipogenic proteins including PPAR $\gamma$  and FABP4 were markedly increased in HAD control compared to non-induced MSCs. DPHD inhibited adipocyte differentiation by suppressing the expression of adipogenic genes and protein including PPAR $\gamma$ , LPL and FABP4, thereby decreasing intracellular triglyceride accumulation. PPAR $\gamma$  is the key transcription factor that induces and enhances the expression of adipocyte-specific genes (Farmer 2005). The suppression of PPAR $\gamma$  expression by DPHD treatment led to inhibition of adipogenic gene expression including LPL and FABP4, and subsequently inhibited adipogenesis (Figure 4.10). As adipocytes and osteoblasts share a common progenitor cell, the differentiation of MSCs to adipocytes essentially suppresses their differentiation toward osteoblast and vice versa.

DPHD enhanced differentiation of MSCs into osteoblast by increasing the mRNA expression of osteogenic transcription factors including RUNX2 and OSX (encoded osterix), which are early and late osteoblast differentiation markers, respectively (Figure 4.8-4.9). Runx2 and osterix positively regulate osteogenic differentiation of MSCs. Accordingly, the expression of genes and proteins involved in anti-adipogenic and osteogenic effects of DPHD in MSCs were correlated with gene profiling obtained from wide genome expression screening (Fig.4.10). Surprisingly, DPHD partially restored the levels of cell proliferation factors during adipogenic differentiation. However, it suppressed the expression of genes encoding adipogenic transcription factor, PPAR $\gamma$  and its target genes, and many lipogenic proteins, thereby inhibiting adipocyte differentiation and maturation. The anti-adipogenic activity of DPHD in MSCs was similar to that of estrogen. Furthermore, the anti-adipogenic effect of DPHD was likely to be mediated through the cross-talk between estrogen receptor (ER) signaling and Wnt/ $\beta$ -catenin signaling pathway.

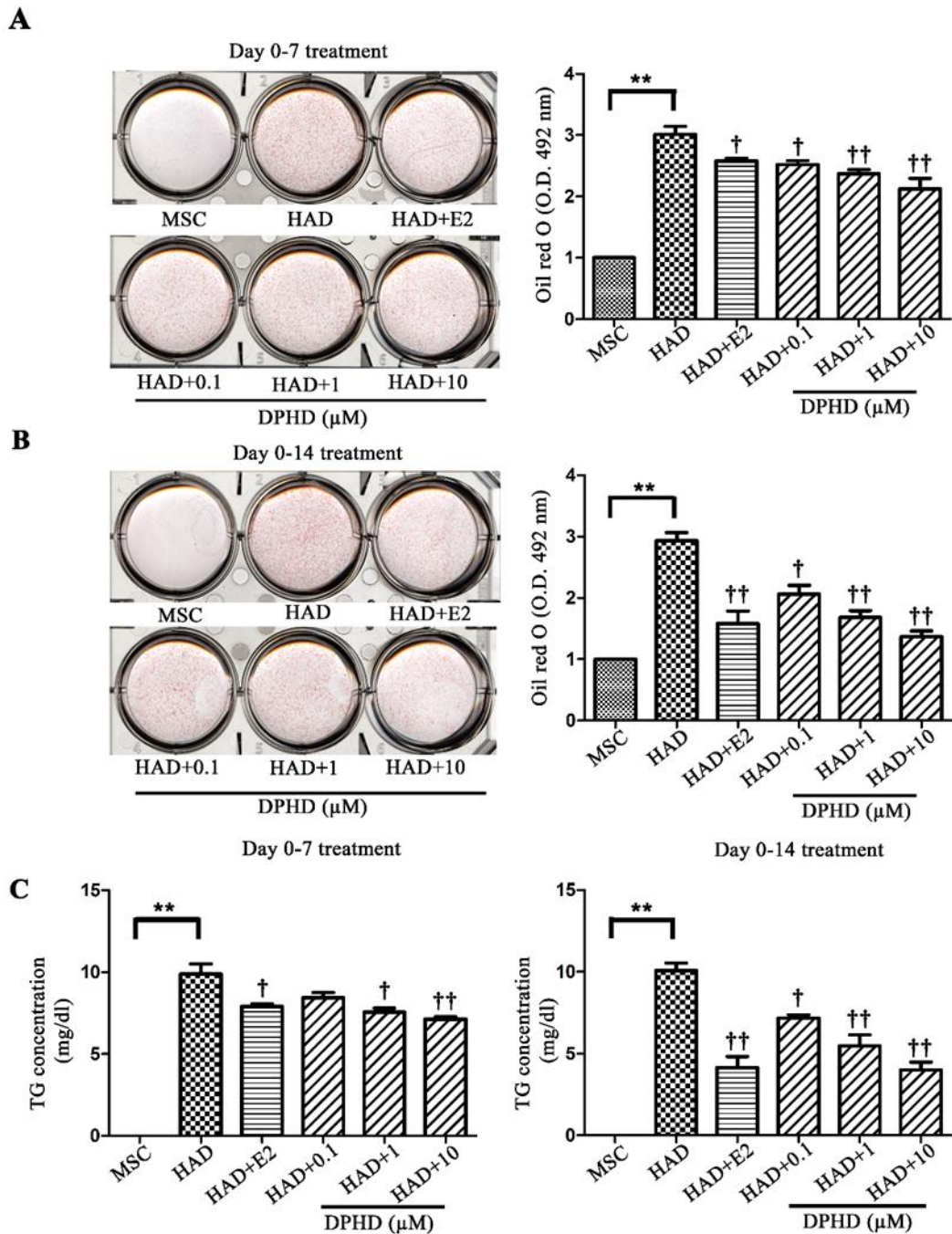


Figure 4.6 Effect of E2 and DPHD treatments from (A) 0-7 days and (B) 0-14 days on the accumulation of intracellular lipid by Oil red O staining, and (C) the intracellular triglyceride concentrations determined by triglyceride colorimetric assay on day 7 and 14 of adipogenic induction, respectively. Data are expressed as means  $\pm$  SEM obtained from 3 independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  (vs. MSC) and † $p < 0.05$  and †† $p < 0.01$  (vs. HAD)



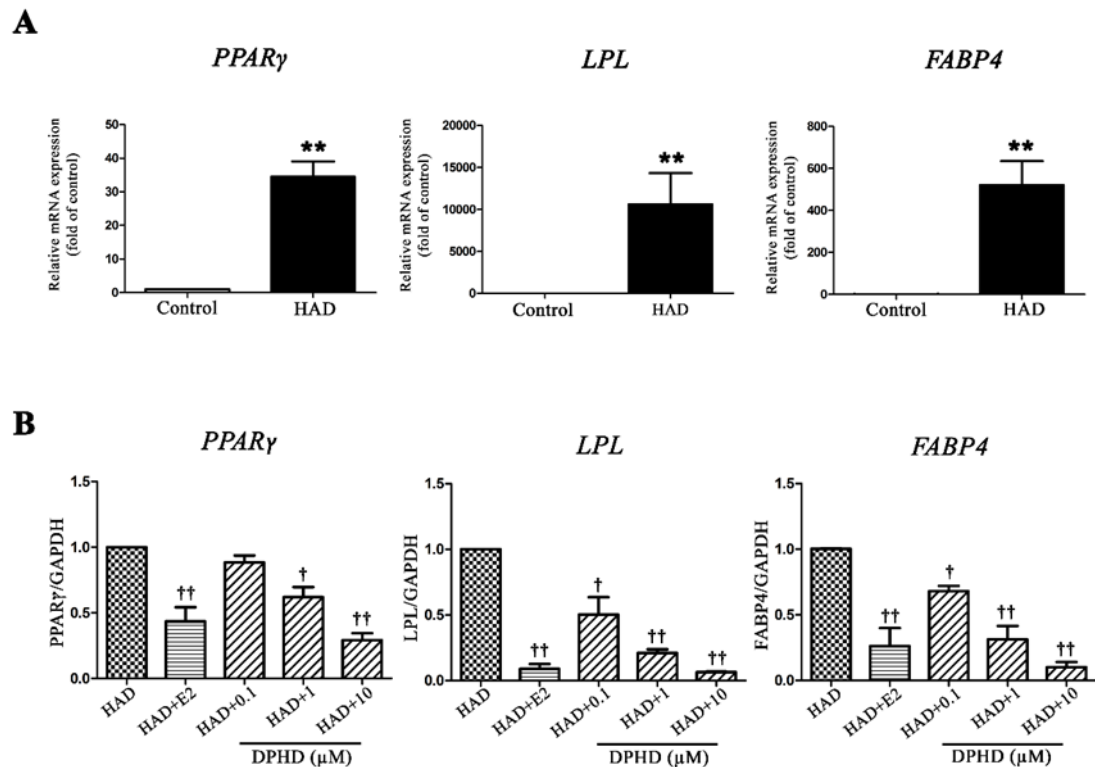


Figure 4.7 (A) The expression of adipogenic mRNAs: LPL, PPAR $\gamma$ , and FABP4 after incubating with human adipogenic differentiation (HAD) media for 7 days compared to control, non-induced MSCs and (B) the effect of treatments on the expression of adipogenic mRNA (LPL, PPAR $\gamma$ , and FABP4) compared to that of HAD control for 7 days. Data are expressed as mean  $\pm$  SEM obtained from 3 independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  (vs. MSC) and † $p < 0.05$  and †† $p < 0.01$  (vs. HAD)

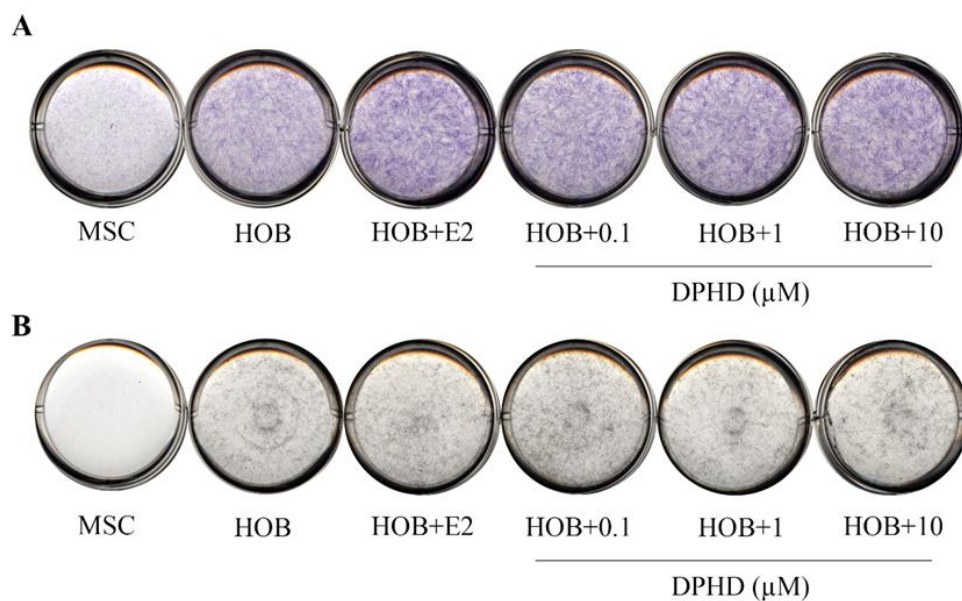


Figure. 4.8 Effect of treatments on (A) alkaline phosphatase activity determined by phosphatase staining and (B) calcium deposition determined by Von kosha staining in MSCs on day 21 of osteogenic induction.

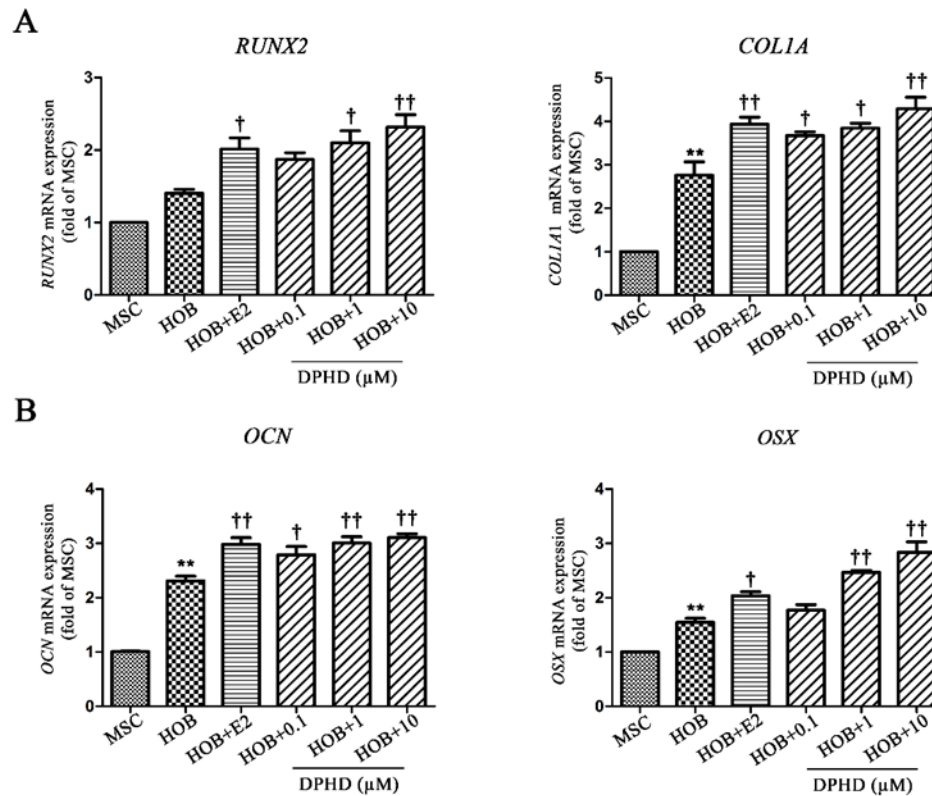


Fig. 4.9. Effect of E2 and DPHD treatments on the mRNA expression of osteogenic mRNA markers: (A) early markers at day-7: RUNX2 and COL1A1 and (B) late markers at day-21: OCN and OSX during osteogenic induction by real-time PCR. Each value is expressed as mean  $\pm$  SEM obtained from 3 independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  (vs. MSC) and † $p < 0.05$  and †† $p < 0.01$  (vs. HOB).

In the present study, DPHD enhanced differentiation of MSCs into osteoblast by increasing the mRNA expression of osteogenic transcription factors including RUNX2 and OSX (encoded osterix), which are early and late osteoblast differentiation markers, respectively. Runx2 and osterix positively regulate osteogenic differentiation of MSCs. This osteogenic effect of DPHD observed in MSCs was consistent the previous studies in which DPHD stimulates osteoblast differentiation of mouse osteoblast, and human preosteoblast (Bhukhai, Suksen et al. 2012), thereby promoting bone formation. Moreover, DPHD exerts a bone sparing effect in ovariectomy-induced trabecular bone loss and prevents deterioration



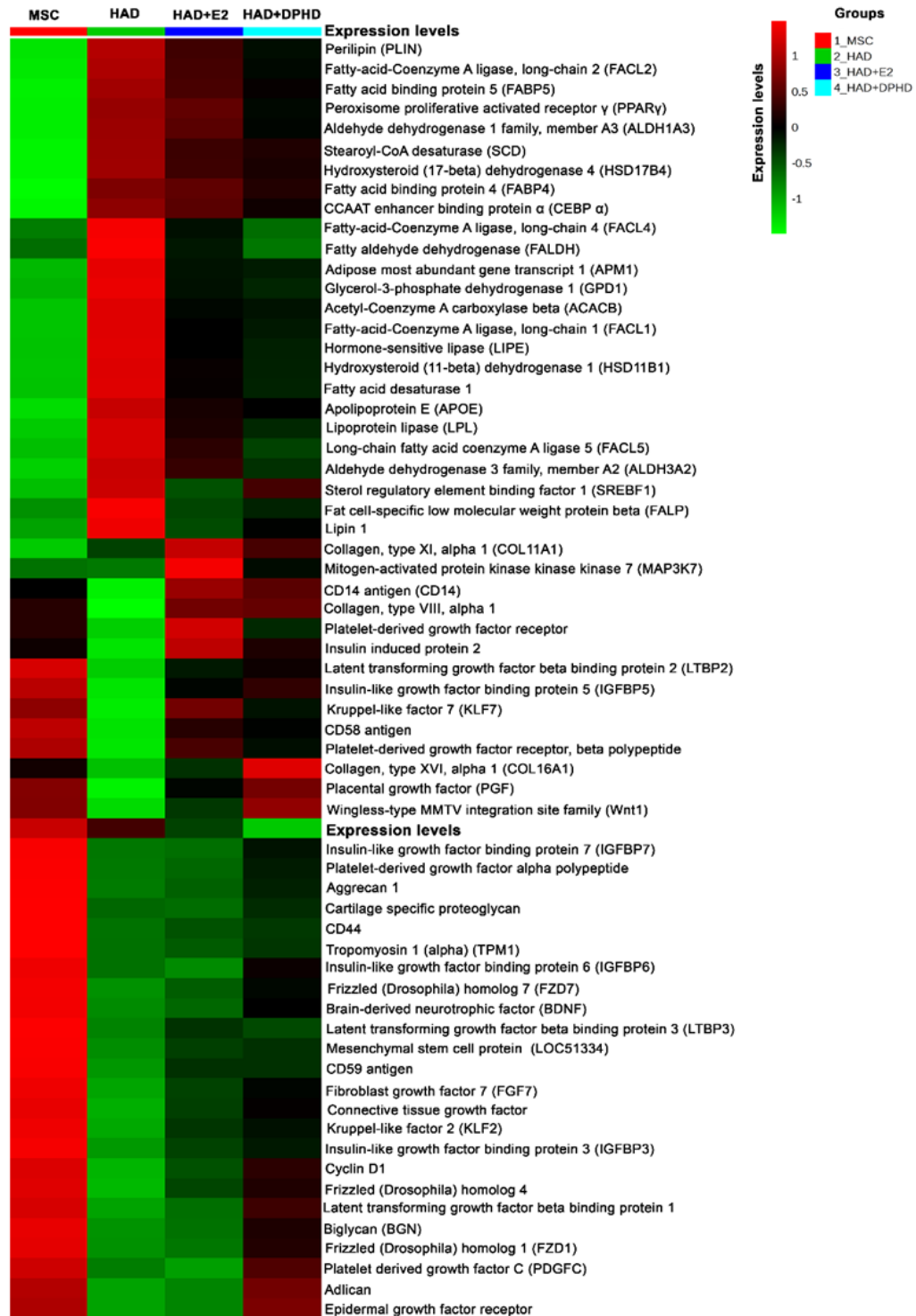


Figure 4. 10 Hierarchical clustering analysis of gene expression profile of the 63 candidate genes in non-induced MSCs, HAD induction (HAD control), HAD+E2, and HAD+DPHD (25 genes were decreased, and 38 genes were increased by treatments compared to HAD control) selected as relevant by Metabolyst

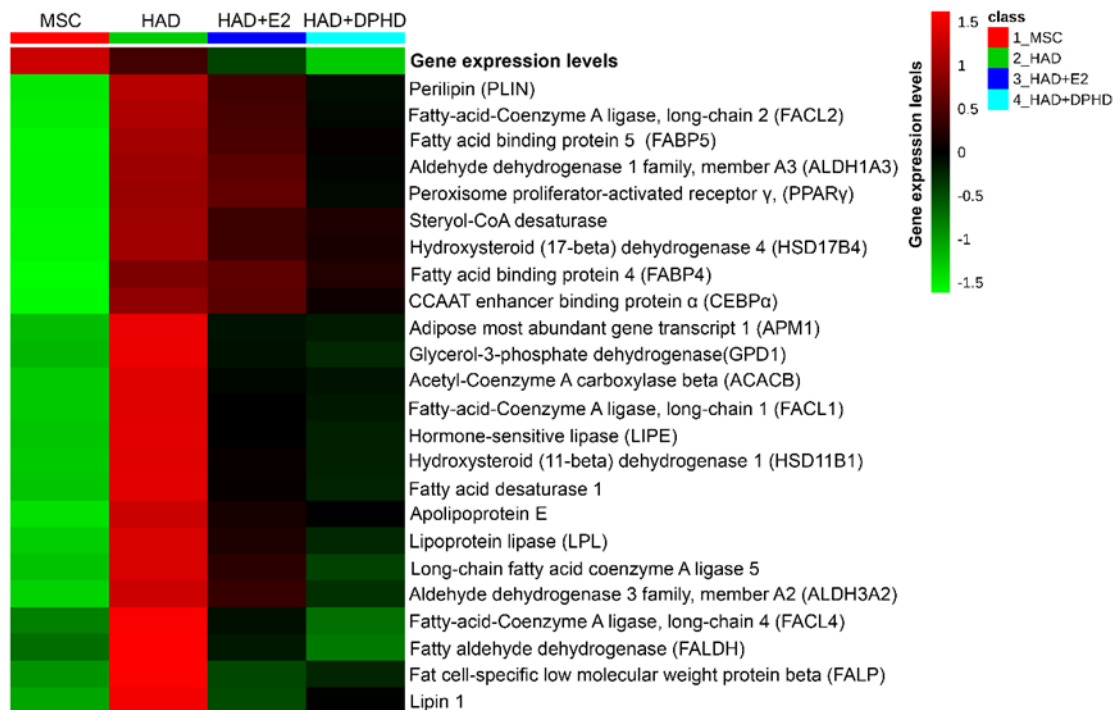


Figure 4.11. Hierarchical clustering analysis of 24 lipogenic genes in non-induced MSCs, HAD induction (HAD control), HAD+E2, and HAD+DPHD on day-7 of adipogenic differentiation selected as relevant by Metabolyst

of bone microarchitecture (Tantikanlayaporn, Wichit et al. 2013). Accordingly, the expression of genes and proteins involved in anti-adipogenic and osteogenic effects of DPHD in MSCs were correlated with gene profiling obtained from wide genome expression screening (Fig.4.10). Surprisingly, DPHD partially restored the levels of cell proliferation factors during adipogenic differentiation. However, it suppressed the expression of genes encoding adipogenic transcription factor, PPAR $\gamma$  and its target genes, and many lipogenic proteins, thereby inhibiting adipocyte differentiation and maturation. The anti-adipogenic activity of DPHD in MSCs was similar to that of estrogen. Furthermore, the anti-adipogenic effect of DPHD was likely to be mediated through the cross-talk between estrogen receptor (ER) signaling and Wnt/ $\beta$ -catenin signaling pathway. This is the first reported showing the anti-adiposity effect of *C. comosa* extract and its isolated compound DPHD in visceral adipose tissue of the OVX rats, which is the major cause of metabolic diseases. DPHD also increased proliferation of progenitor cells, human bone marrow-derived mesenchymal stem cells, and exerted the anti-adipogenic effect. Meanwhile, it suppressed bone marrow adiposity which is associated with osteoporosis in postmenopausal. This study provides insight health beneficial information for the development of *C. comosa* and DPHD as a dietary supplement to alleviate metabolic complications related to excessive adipose tissues accumulation in the body including insulin resistance and osteoporosis.

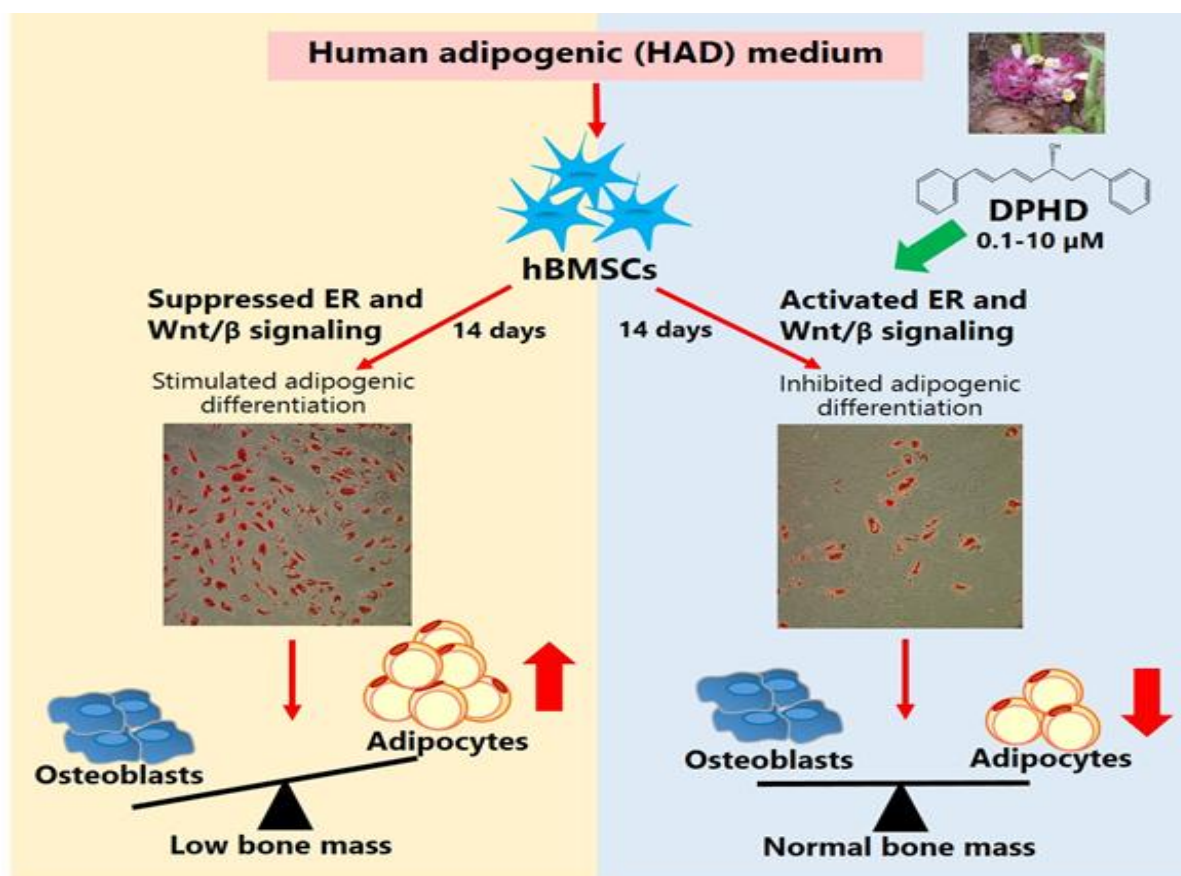


Figure 4.12. Schematic diagram showing the suppressing activities of DPHD from *C. comosa* on the differentiation of MSCs into adipocytes while promoted differentiation into osteoblasts. It indicates health beneficial effect of DPHD to reduce bone loss in aging population.

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## Sub-project 5: Neuroprotective effects of compounds

### ABSTRACT

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most two common neurodegenerative diseases related with aging. The cause of these two diseases are still unknown and treatment is unavailable. Oxidative stress occurred from free radical accumulation increased with age and consequences of metabolic disorders are believed to be a major cause of damage and death of neurons. Therefore, preventing oxidative stress could be a strategy preventing neurodegeneration. In this project, in collaboration with chemist from Thailand and China, we have tested several natural product derived compounds and modified compounds for their ability to prevent neurons from damages and death caused by both oxidative stress and factors known to induce neurodegeneration. We found that several plant-derived compounds showed high scavenging property and can protect neurons from oxidative stress induced cell damage and death. Interestingly, modification of a known anti-oxidant compound also yielded compounds with better activities. Among these, linking Alpha-lipoic acid (ALA) with an anti-ischemic drug 3-n-butylphthalide (NBP) produced compounds that contain significantly greater neuroprotective effect. Together the results from this study shade light to several possible compounds that can be further developed into novel therapeutic agents for treatment of neurodegenerative diseases.

**Keywords:** Neurodegenerative diseases, Alzheimer's disease, Parkinson disease, modified natural compounds, neuroprotection, antioxidant

### บทคัดย่อ

โรคอัลไซเมอร์และโรคพาร์กินสันเป็นโรคความเสื่อมของสมองที่พบบ่อยที่สุดในผู้สูงอายุ สาเหตุของโรคดังกล่าวยังไม่เป็นที่ทราบดีและปัจจุบันยังไม่มีวิธีรักษาให้หายขาด อย่างไรก็ตาม ปัจจุบันเป็นที่ทราบดีว่าภาวะเครียดที่เกิดจากออกซิเดชันเกิดจากการสะสมของอนุมูลอิสระที่เพิ่มมากขึ้นตามอายุ และจากโรคที่เกี่ยวข้องกับเมตาบอลิก เป็นสาเหตุสำคัญของการเสื่อมและตายของเซลล์ประสาท ดังนั้น การป้องกันไม่ให้เซลล์ประสาทถูกทำลายจากภาวะเครียดที่เกิดจากออกซิเดชันเป็นวิธีการป้องกันความเสื่อมของสมองที่สำคัญ งานวิจัยในโครงการนี้ ได้มีความร่วมมือกับนักวิจัยด้านเคมีจากประเทศจีน โดยได้พัฒนาโมเดลเพื่อทดสอบฤทธิ์ของสารที่ได้จากธรรมชาติ สารสังเคราะห์ชนิดใหม่ ตลอดจนสารที่ดัดแปลงโครงสร้างทางเคมี ในการป้องกันเซลล์ประสาทจากการถูกทำลายด้วยภาวะเครียดที่เกิดจากออกซิเดชัน ด้วยสภาวะที่เหนี่ยวนำให้เกิดโรคอัลไซเมอร์และโรคพาร์กินสัน ผลการทดลองพบว่าสารจากพืชสมุนไพรหลายชนิดมีฤทธิ์ต้านอนุมูลอิสระ และสามารถปกป้องเซลล์ประสาทจากสภาวะดังกล่าวได้อย่างมีประสิทธิภาพ ยกตัวอย่างเช่น การดัดแปลงโครงสร้างของกรดแอลฟาไลโปอิกที่นำมาเชื่อมต่อกับ NBP ซึ่งเป็นยาที่ปัจจุบันใช้เป็นยารักษาภาวะสมองขาดเลือด มีประสิทธิภาพในการป้องกันการทำลายของเซลล์ประสาทได้ดีมากเมื่อเทียบกับสารเดี่ยว ๆ นอกจากนี้ โครงการวิจัยยังได้ค้นพบโมเดลของเซลล์ประสาทแบบใหม่ เพื่อนำไปใช้ในการวิเคราะห์ผลของสารต่อการ

ปกป้องเซลล์ประสาทได้ในอนาคต ผลงานวิจัยจากโครงการนี้เป็นข้อมูลสำคัญที่จะนำไปสู่การพัฒนาใหม่สำหรับการป้องกันและรักษาโรคความเสื่อมของสมองได้ในอนาคต

**คำสำคัญ:** โรคความเสื่อมของสมอง โรคอัลไซเมอร์ โรคพาร์กินสัน สารธรรมชาติดัดแปลง การปกป้องเซลล์ประสาท ฤทธิ์ต้านอนุมูลอิสระ

## Introduction

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive and irreversible damage of the nervous system. The pathogenesis of these diseases is still unclear but several evidences suggest that oxidative stress, resulted from accumulation of free radicals and decreasing function of endogenous anti-oxidant system, is one of major causes of the degeneration [1-4]. The free radicals oxidize cellular components such as lipids, proteins, and DNA leading to neuronal damage and death. Mitochondrial electron transport chain generates reactive oxygen species (ROS) which toxic to neuron. The ROS that are generated by mitochondrial respiration, including hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^\cdot$ ), superoxide anion ( $O^{2-}$ ) [5, 6] are potent inducers of oxidative damage and mediators of ageing. Moreover, ROS particularly active in neurons as the excitatory amino acid and neurotransmitters serve as source of oxidative stress. In addition, neurons are post-mitotic cells and therefore, they are particularly sensitive to free radicals leading to neuronal dysfunction, neuronal cell death and eventually neurodegenerative diseases [7]. Currently there is no treatment for these diseases once they occur, therefore protecting neurons from such damages is the best way to prevent these neurodegenerative diseases.

Natural products from plants and marine microbes are sources of various biological active compounds. Several of these compounds demonstrated potent anti-oxidation activity. As oxidative stress is a major course of neurodegeneration, compounds derived from these natural products might be able to protect neurons from damages and death caused by oxidative stress or other related factor. Alpha-lipoic acid (ALA) is a naturally occurring compound that has been shown to exert antioxidant and anti-inflammatory properties. Several studies showed that ALA elicits neuroprotective effects both *in vitro* and *in vivo* models [8-16]. Synergistic protective effect has been observed if ALA is combined with other drugs in several different animal models of pathology [17-23].

In this study, a clinical anti-ischemic and neuroprotective drug 3-n-butylphthalide (NBP) [24] was conjugated with lipoic acid by an amide bond and determined its neuroprotective activities against oxidative stress-induced neuronal cell damage.

## Results

### Part I Determine neuroprotective effects of marine microbes derived compounds

#### 1.1 Screening for neuroprotective effects of marine microbes derived compounds against hyper-phosphorylated Tau-induced neurodegeneration

First, we tested for safety dose of the compounds derived from marine microbes to identify which concentrations are safe to use in neuronal cells (SH-SY-5Y). Most of the compounds are very safe in neuronal cells as shown in the table below. Then we test whether the compounds can prevent neuronal death induced by Okadaic acid, a toxin used to induce hyper-phosphorylated Tau, which mimics pathological cause of neuronal death found in Alzheimer's disease. However, none of these compounds could protect neurons from hyper-phosphorylated Tau-induced neurodegeneration.

Table 5.1. Cytotoxicity and neuroprotective test for marine microbes-derived compounds

| No. | Sample No.  | IC50 (uM)  | Neuroprotective effect |
|-----|-------------|------------|------------------------|
| 1   | HD-ZWM-1085 | 1.539e+010 | -                      |
| 2   | HD-ZWM-1087 | >50        | No                     |
| 3   | HD-ZWM-1089 | >50        | No                     |
| 4   | HD-ZWM-1090 | >50        | No                     |
| 5   | HD-ZWM-1091 | >50        | No                     |
| 6   | HD-ZWM-1092 | 6.179e+029 | -                      |
| 7   | HD-ZWM-1164 | >50        | No                     |
| 8   | HD-ZWM-1166 | 4.504      | -                      |
| 9   | HD-ZWM-1167 | 14.50      | -                      |
| 10  | HD-ZWM-1170 | 6.807      | -                      |
| 11  | HD-ZWM-1171 | >50        | No                     |
| 12  | HD-ZWM-1172 | >50        | No                     |
| 13  | HD-ZWM-1186 | 8.661      | -                      |
| 14  | HD-ZWM-1273 | 0.06643    | -                      |
| 15  | HD-ZWM-1274 | >50        | No                     |
| 16  | HD-ZWM-1281 | >50        | No                     |
| 17  | HD-ZWM-1289 | >50        | No                     |
| 18  | HD-ZWM-1290 | >50        | No                     |



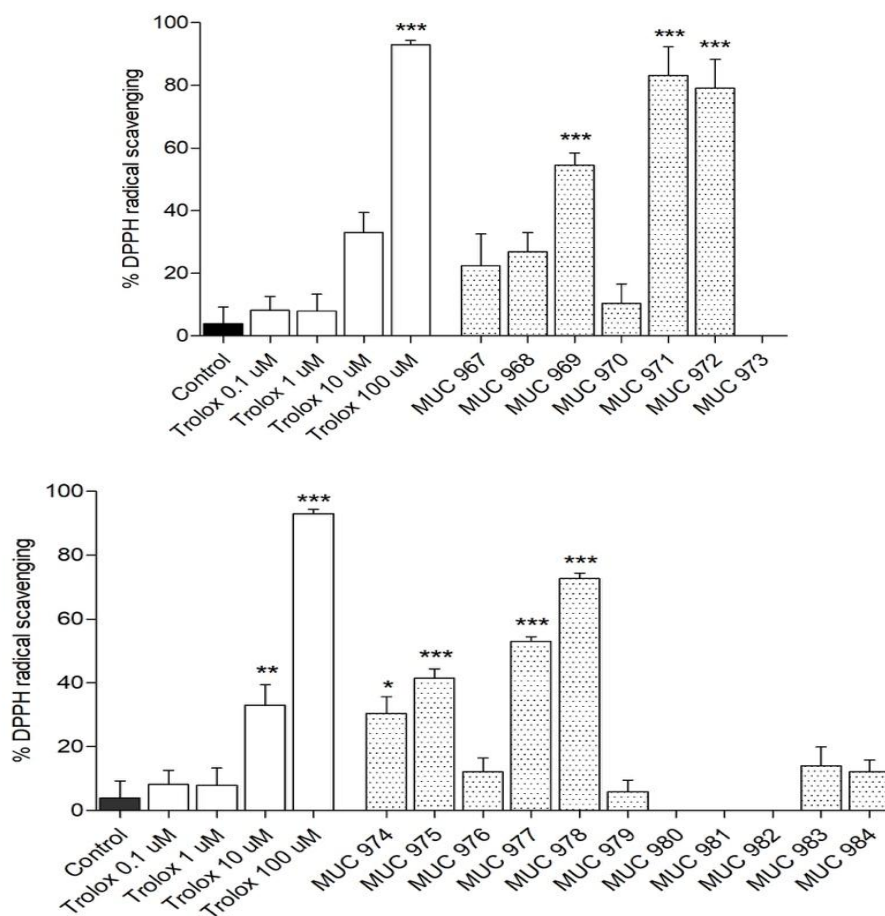
| No. | Sample No. | IC50 (uM) | Neuroprotective effect |
|-----|------------|-----------|------------------------|
| 19  | LWL-1      | >50       | No                     |
| 20  | LWL-2      | >50       | No                     |
| 21  | LWL-3      | >50       | No                     |
| 22  | LWL-4      | >50       | No                     |
| 23  | LWL-5      | >50       | No                     |
| 24  | LWL-6      | >50       | No                     |
| 25  | LWL-7      | >50       | No                     |
| 26  | LWL-8      | >50       | No                     |
| 27  | LWL-9      | >50       | No                     |
| 28  | LWL-10     | >50       | No                     |

## Part II Determine neuroprotective effects of plant-derived compounds

### 2.1 Screening for neuroprotective effects of plant-derived compounds against oxidative stress induced neurodegeneration

#### *2.1.1 Determine scavenging effect of plant-derived compounds*

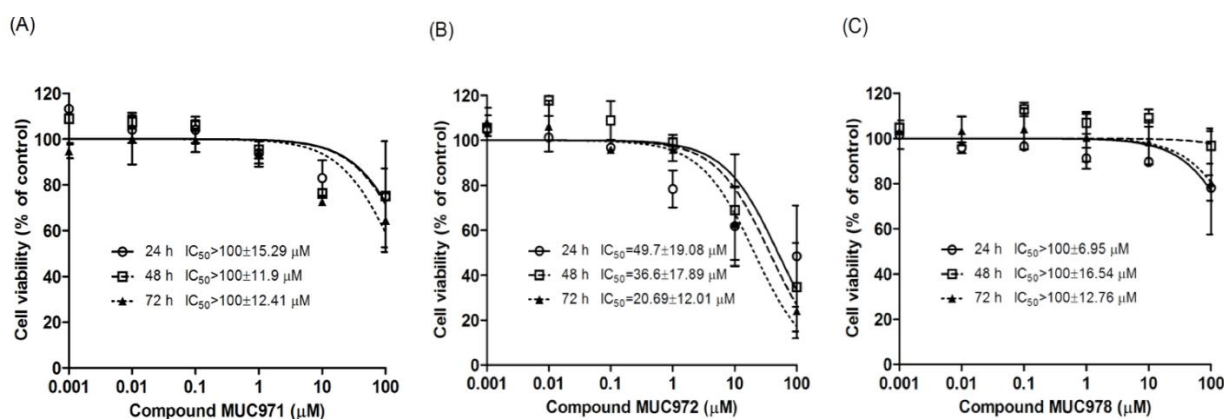
First, we screen for plant-derived compounds with ability to scavenge the free radicals using in vitro DPPH assay. Several of these compounds showed very high ability to scavenge the free radical. Among these compounds, the MUC 971, 972 and 978 elicit strongest effects. We therefore select these compounds for further investigation.



**Figure 5.1.** Graph showing scavenging effect of compounds from *Zingiber mekongense* (A) and *Gardenia sessiliflora* (B). Trolox was used as a positive control. Several compounds significantly decreased DPPH free radical. Data show as mean  $\pm$  S.E.M.,  $n=3$ . \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus control group (ANOVA).

### 2.1.2 Determine safety of the plant-derived compounds on neuronal cells

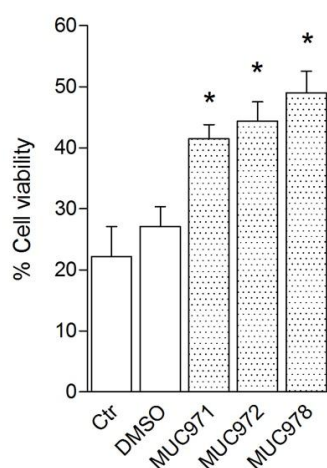
Next, we performed cytotoxicity test of the selected compounds derived from plant to identify their safe concentration that can be used in neuronal cells. Most of the compounds are quite safe to neuronal cells as shown in Figure 5.2.



**Figure 5.2.** Graph showing cytotoxicity test of MUC971, 972 and 978. All of these compounds are quite safe with  $IC_{50} > 50 \mu M$  for MUC972 and  $>100 \mu M$  for MUC 971 and 978.

### 2.1.3 Plant derived compounds prevent neuronal death against oxidative stress

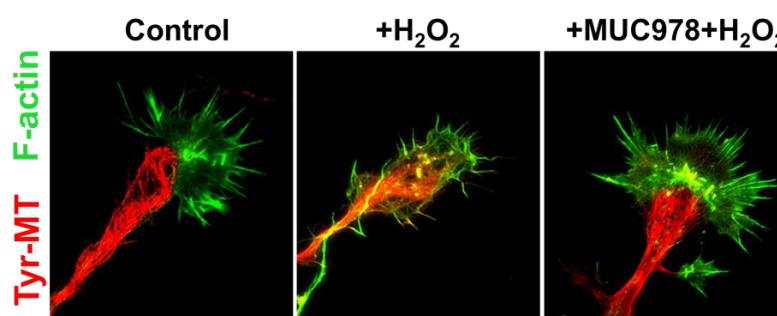
To determine whether the selected plant derived compounds can protect neurons against oxidative stress, CAD cells were pretreated with MUC 971, 972 and 978 (10  $\mu M$ ) for 24h before being exposed to  $H_2O_2$  for 12 h. Cell viability was assessed by MTT assay. We observed increased in cell viability in the presence of MUC 971, 972 and 978, with the strongest activity in MUC 978 (Figure 5.3).



**Figure 5.3.** Graph showing neuroprotective effect of MUC971, 972 and 978. The pretreated compounds increased cell viability of neuronal cells exposed to  $H_2O_2$ . Data are mean  $\pm$  SEM from three independent experiments. \* $p < 0.05$ , \* $p < 0.01$ , and \*\*\* $p < 0.001$  compared with  $H_2O_2$ -treated group.

#### 1.2.4 Plant derived compounds prevent neuronal growth cone damage against oxidative stress

Next, we selected MUC978 and determined whether it could protect primary neurons from damages induced by oxidative stress by using cortical neurons. One of the most sensitive structure in the neurons is the growth cone, a cytoskeletal enriched structure at the tip of the axons, which plays several crucial roles including neurite formation, axon guidance and neuronal branching [28]. Damages of growth cone therefore lead to neuronal dysfunction and eventually neuronal death. Growth cone is very dynamic and sensitive to the oxidative stress [29, 30]. In the presence of  $H_2O_2$  the growth cone of the cortical neurons shrank with disruption of both microtubules and actin filament (Figure 4). Interestingly, when the primary neurons were pretreated with MUC978 the growth cone structure was well preserved after  $H_2O_2$  exposure.



**Figure. 5.4** Representative images of the growth cone of cortical neurons before and after exposure to  $H_2O_2$  in the absence or presence of MUC978. The cells were pretreated with vehicle control (DMSO) or MUC978 (10  $\mu$ M) for 24 hours prior to exposure to  $H_2O_2$ . The cells were fixed and immunostained with tyrosinated tubulin antibody for dynamic microtubules (red) and Phalloidin for F-actin (green).

### Part III Determine neuroprotective effects of modified compounds

#### 3.1 Screening for neuroprotective effects of modified compounds against oxidative stress-induced neurodegeneration

##### 3.1.1 Determine safety of the modified compounds on neuronal cells

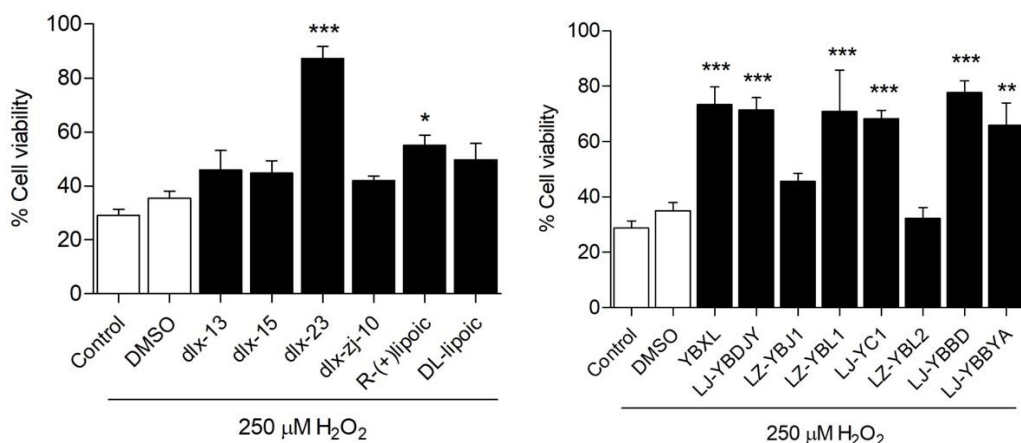
First, we test for safety of the modified known compounds including lipoic acid and organoseleniums derivatives. These compounds are synthesized and modified by our Chinese collaborators. All of these compounds are safe with neuronal cells.

Table 5.3. Cytotoxicity test for modified compounds in neuronal cells.

| IC <sub>50</sub> (μM) 72 hours exposure |     |
|-----------------------------------------|-----|
| Lipoic acid derivatives                 |     |
| dlx-13                                  | >50 |
| dlx-15                                  | >50 |
| dlx-18                                  | >50 |
| dlx-23                                  | >50 |
| dlx-zj-10                               | >50 |
| R-(+)-lipoic acid                       | >50 |
| DL-lipoic acid                          | >50 |
| Organoseleniums                         |     |
| YBXL                                    | >50 |
| LJ-YBDJY                                | >50 |
| LJ-YBJJY                                | >50 |
| LZ-YBJ1                                 | >50 |
| LZ-YBL1                                 | >50 |
| LJ-YC1                                  | >50 |
| LZ-YBL2                                 | >50 |
| LZ-YBG2(H)                              | >50 |
| LJ-YBBD                                 | >50 |
| LJ-YBBYA                                | >50 |

### 3.1.2 Determine the neuroprotective effect of modified compounds against neuronal cell death induced by oxidative stress

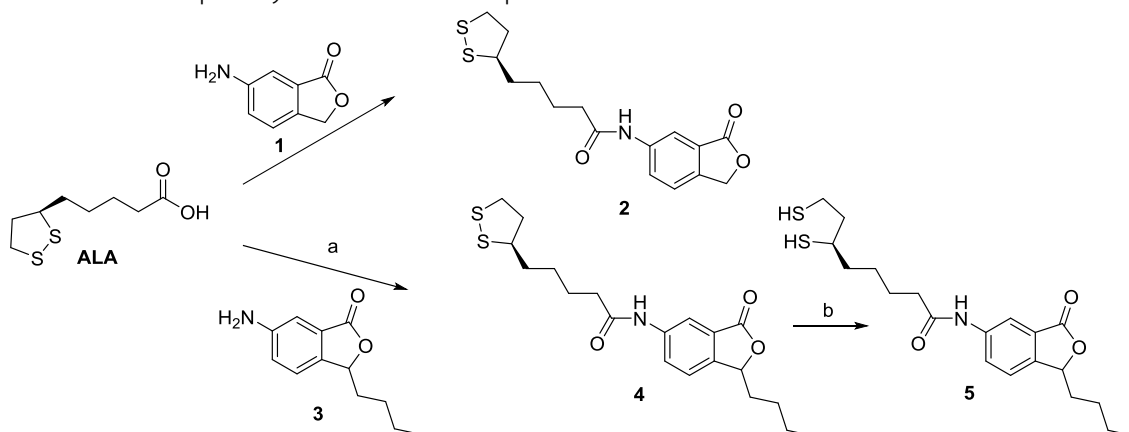
After we identify safe concentration range of the modified compounds for neuronal cells we tested these compounds for their ability to protect the neurons against oxidative stress induced cell death. We pretreated the cells with the compounds for 24 h before exposing with H<sub>2</sub>O<sub>2</sub>. Interestingly, several of these compounds could significantly protect the neuronal death compared to untreated cells.



**Figure 5.5.** Effects of ALA (A) and Organoselenium (B) derivatives on  $\text{H}_2\text{O}_2$ -induced CAD cells death. **(A)** The cells were treated with the maximum concentration that are safe to use in neuronal cells before being exposed to  $\text{H}_2\text{O}_2$  for 12 hours **(B)**. The values are presented as percentages of control. Data are means  $\pm$  SEM from three independent experiments. \* $p < 0.05$ , \* $p < 0.01$ , and \*\*\* $p < 0.001$  compared with  $\text{H}_2\text{O}_2$ -treated group.

### 3.1.3 Structural modification of Alpha-lipoic acid ALA

Among the modified compounds, the Dlx23 showed the best activity. Therefore, we decided to further investigate the Dlx23 for its effect and underlying mechanism. This compound is the ALA derivative, which was synthesized by the Chinese collaborator. Briefly, ALA was coupled with compound 1 [25] to provide the conjugated compound 2. Coupling of ALA and 6-amino-3-n-butylphthalide 3 [26] produced another conjugated compound 4 which was subsequently reduced to compound 5 (Dlx23).

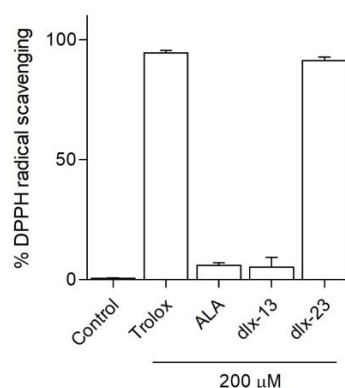


Reagents and condition:  
(a) EDC·HCl / DMAP / DCM / rt / overnight; (b)  $\text{NaBH}_4$ /MeOH/NaOH/ $\text{H}_2\text{O}$ .

**Figure 5.6** Structure and synthetic route of ALA derivatives

### 3.1.4 ALA derivative elicits stronger free radical scavenging property than the parent compound

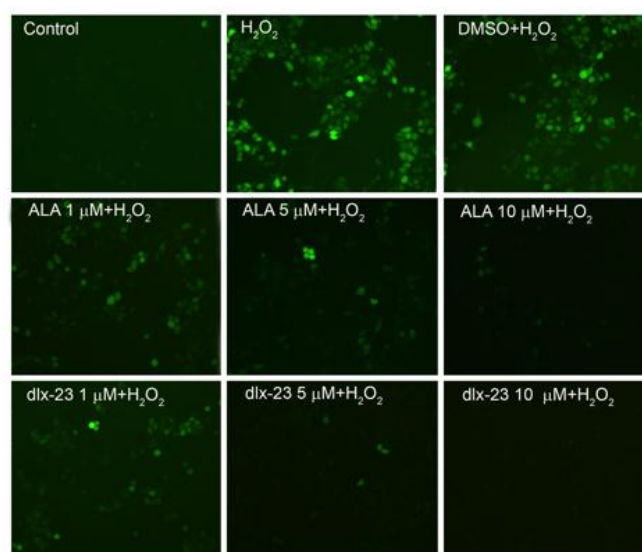
Next, we investigated whether neuroprotective effect of dlx-23 is elicited via its anti-oxidant activity. To examine anti-oxidant activity of ALA modified compounds the *in vitro* DPPH assay was used. Consistent with its neuroprotective effect, dlx-23 exerted antioxidant activity by scavenging DPPH radical *in vitro* (Figure 5.7).

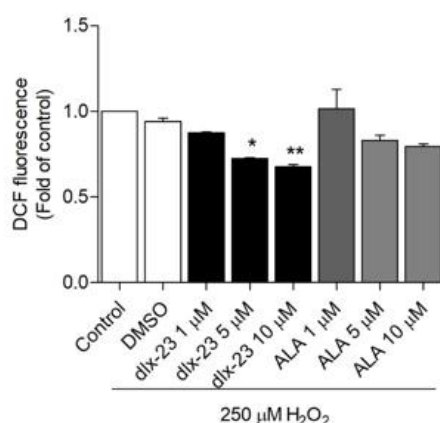


**Figure 5.7.** DPPH scavenging activity. Percentage of DPPH radical scavenging was calculated against control group. Data are means  $\pm$  SEM from three independent experiments.

### 3.1.5 ALA derivative elicits higher anti-oxidant property

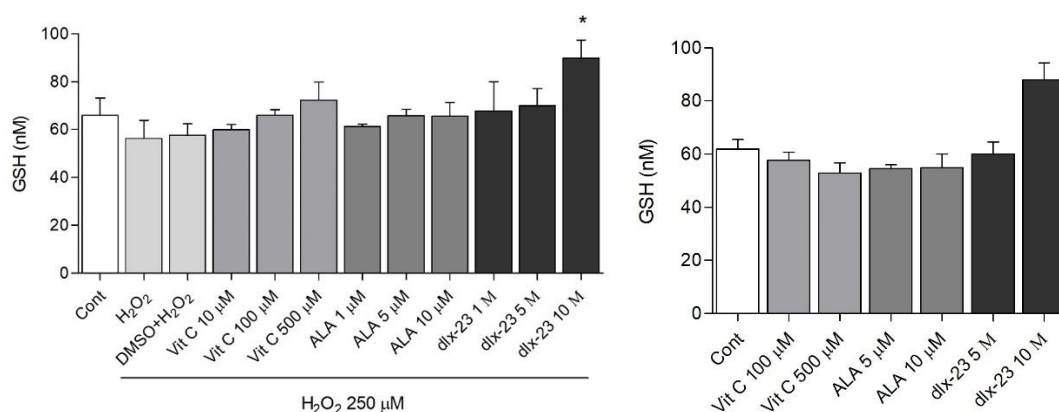
We further determined the ability of dlx-23 to prevent intracellular ROS accumulation. Intracellular ROS in CAD cells was significantly increased after treated with  $H_2O_2$  for 3 hours. Pretreatment with dlx-23 significantly reduced the ROS production induced by  $H_2O_2$  in CAD cells. This anti-oxidant effect was stronger compared to ALA at the same concentrations (Figure 5.8).





**Figure 5.8.** Effects of dlx-23 on  $\text{H}_2\text{O}_2$ -induced intracellular ROS accumulation. CAD cells were pretreated with dlx-23 or ALA at 1, 5, 10  $\mu\text{M}$  for 24 hours before exposed to  $\text{H}_2\text{O}_2$  for 3 hours. The DCF fluorescence signal was observed under fluorescence microscope.

Decreasing of GSH levels correlates with increasing oxidative stress. In this study, we further examined the effect of dlx-23 on intracellular GSH levels. As shown in Figure 9A, the cells exposed to 250 mM  $\text{H}_2\text{O}_2$  for 1 hour were slightly reduced intracellular GSH levels. Cells pretreated with vitamin C (a positive control) increased GSH levels in a concentration dependent manner. Although ALA maintains GSH levels, it was not significantly increased than those treated with  $\text{H}_2\text{O}_2$  alone. Pretreatment with dlx-23 efficiently maintains intracellular GSH at 5 mM and strongly increased the levels at 10 mM (lower concentration than that of vitamin C). Dlx-23 itself increases GSH expression in the cells at concentration of 10 mM (Fig. 5.9B). This result suggests greater effect of dlx-23 on intracellular GSH levels compared to its parent, ALA, and a well-known antioxidant, vitamin C.

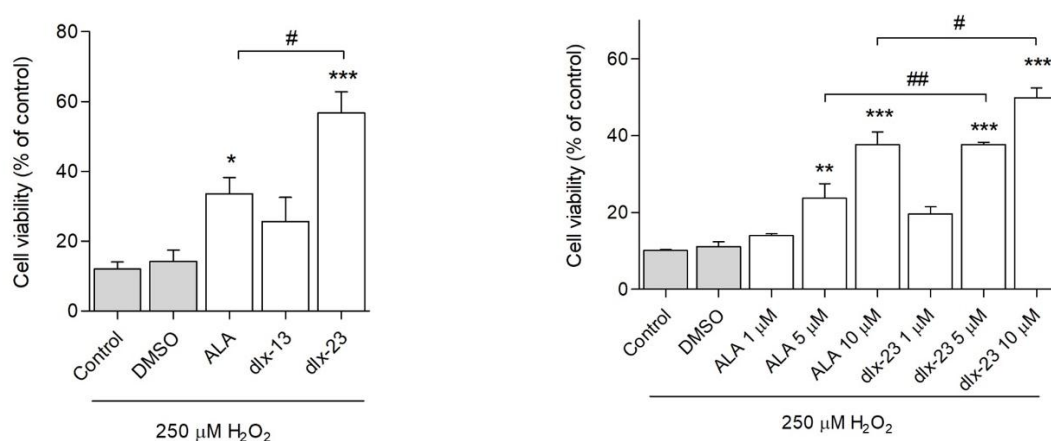


**Figure 5.9.** The effect of dlx-23 on intracellular GSH levels. (A) The cells were pretreated with indicated concentration of compounds for 24 hours before exposed to 250 mM  $\text{H}_2\text{O}_2$  for 1 hour. (B) The intracellular GSH levels after the cells exposed to the compounds alone for 24 hours.



### 3.1.6 ALA derivative prevents neuronal death against oxidative stress

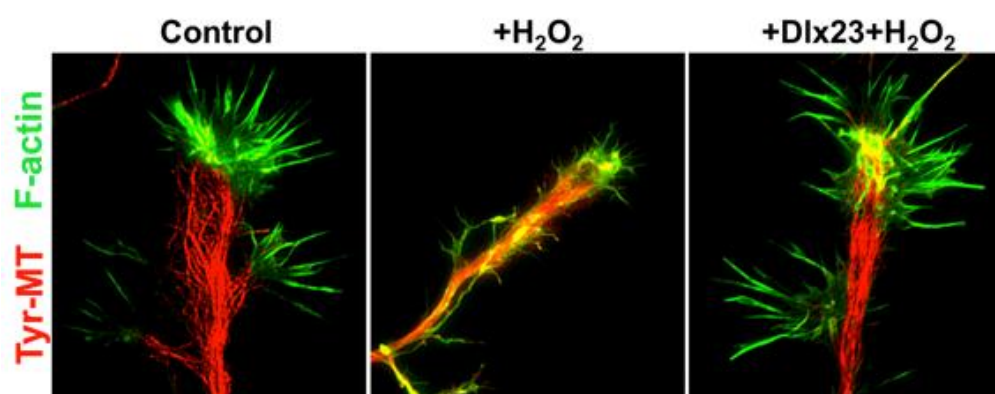
To determine the neuroprotective effect of ALA derivative compounds against oxidative stress-induced cell death,  $H_2O_2$  was used to generate free radical in the CAD cells, a CNS catecholaminergic cell line. The cells were pretreated with different concentration of the compounds or DMSO as a vehicle control before being exposed to  $H_2O_2$ . Cell viability was then determined by MTT assay. Exposure to 250  $\mu M$  of  $H_2O_2$  for 12 hours decreased the cell viability compared to control. Pretreatment with 10  $\mu M$  of dlx-23 prior to  $H_2O_2$  exposure significantly increased the cell viability (Fig. 5.10). We then further investigated different concentration of dlx-23 and found that its neuroprotective effect was a concentration-dependent manner (Figure 5.10B). These results suggest that compound **5** (dlx-23) exerts a stronger neuroprotective effect against  $H_2O_2$ -induced cell death compared to its parent compound (ALA).



**Figure 5.10.** Effects of ALA derivatives on  $H_2O_2$ -induced CAD cells death. **(A)** The cells were treated with maximum concentration with no toxic effect of compounds before exposed to  $H_2O_2$  for 12 hours **(A)**. Comparison of the effect of dlx-23 and ALA was showed a concentration dependent manner (1, 5, 10  $\mu M$ ) at 12 hours **(B)** The values are presented as percentages of control. Data are mean  $\pm$  SEM from three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, and \*\*\* $p$  < 0.001 compared with  $H_2O_2$ -treated group.

### 3.1.7 ALA derivative prevents neuronal growth cone damage against oxidative stress

As mentioned earlier, growth cone is one of the most sensitive structure in the neurons. We therefore ask whether the ALA derivative dlx23 could prevent growth cone damage induced by oxidative stress. Interestingly, when the primary neurons were pretreated with the dlx23 the growth cone structure was well preserved after  $H_2O_2$  exposure suggesting that the dlx23 can protect the primary neurons from  $H_2O_2$  induced growth cone damages.

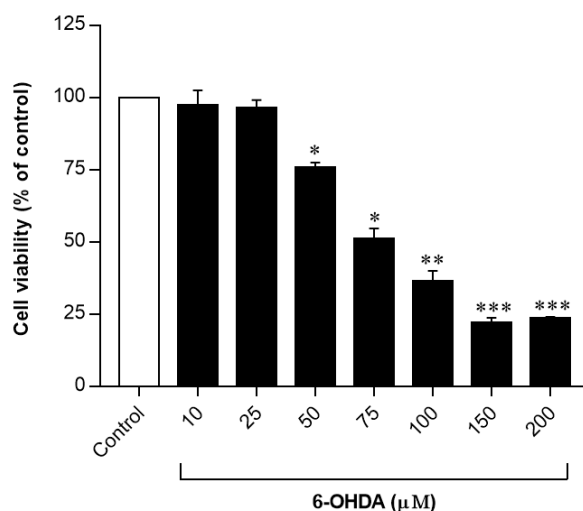


**Figure 5.11.** Representative images of the growth cone of cortical neurons before and after exposure to  $H_2O_2$  in the absence or presence of dlx-23. The cells were pretreated with vehicle control (DMSO) or dlx-23 (10  $\mu M$ ) for 24 hours prior to exposure to  $H_2O_2$ . The cells were fixed and immunostained with tyrosinated tubulin antibody for dynamic microtubules (red) and Phalloidin for F-actin (green).

### 3.2 The neuroprotective effects of modified compounds against toxin-induced Parkinson's disease

#### 3.2.1 6-OHDA induce cell death as a cell model of Parkinson's disease

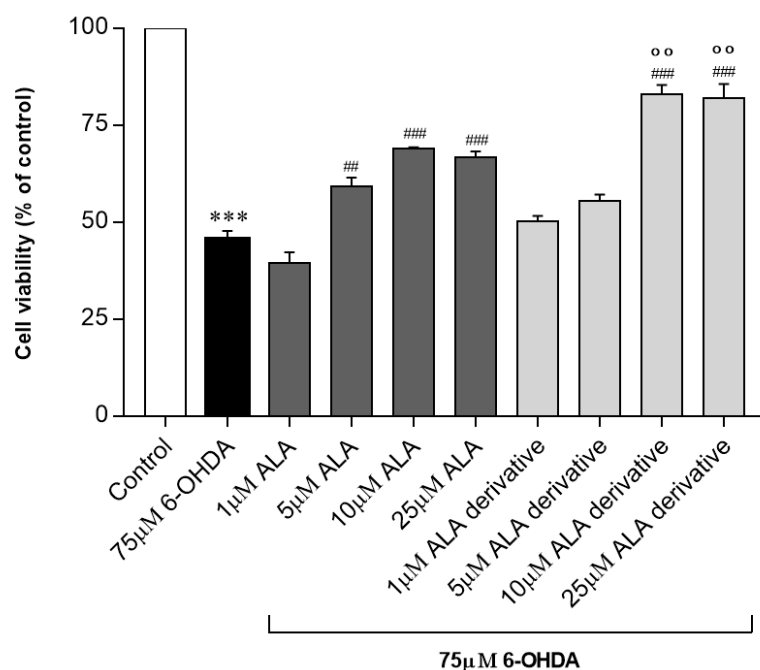
The toxicity of 6-OHDA was investigated by pre-treating cells with the fresh medium for 24 hours, similarly to pre-treatment condition, prior to 6-OHDA exposure for 24 hours. 6-OHDA was found to significantly decrease cell viability and this toxicity was dose dependent. A significant toxic effect was observed in groups that received 6-OHDA at 50, 75, 100, 150, and 200  $\mu M$  by  $75.89 \pm 1.59\%$ ,  $51.17 \pm 3.5\%$ ,  $36.39 \pm 3.52\%$ ,  $21.99 \pm 1.69\%$ , and  $23.55 \pm 0.46\%$  cell viability relative to control. Therefore, the cells exposed to 75  $\mu M$  6-OHDA was selected to induce toxicity and evaluate protection of ALA or its derivative against the toxicity of 6-OHDA



**Figure 5.12.** Effect of 6-OHDA on cell viability. The cells were treated with fresh media for 24 hours, followed by incubation with the different concentration of 6-OHDA (10, 25, 50, 75, 100, 150, and 200μM) for 24 hours. After treatment, MTT assay was performed to investigate viable cell. Data are percentage to control group and are mean  $\pm$  SEM of three independent experiment. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 versus control group (One-way ANOVA followed by Tukey's test).

### 3.2.2 Neuroprotective effect of ALA hybrid compound against 6-OHDA induce cell death in a cell model of Parkinson's disease

To investigate the neuroprotective effects of ALA and its derivative (dlx23) on 6-OHDA-induced cell death, SH-SY5Y cells were pretreated with either ALA or its derivative at 1, 5, 10, and 25μM for 24 hours prior to exposure to 75μM 6-OHDA for a further 24 hours. The viability of cells incubated with 75μM 6-OHDA for 24 h was  $45.43 \pm 2.3\%$  of the control value, and the viability significantly increased to  $59.1 \pm 2.36\%$ ,  $68.83 \pm 0.46\%$ , and  $66.79 \pm 1.38\%$  when cells were pretreated with ALA at 5, 10, and 25μM, respectively. Pretreatment with 10, and 25μM of ALA derivative for 24 hours showed protective effects against the damage caused by 6-OHDA to  $83.01 \pm 2.38\%$ , and  $82.06 \pm 3.59\%$ , respectively. The results showed that both ALA and its derivative dose-dependently protect cells from 6-OHDA-induced decreases in cell viability. However, the protective effect of the ALA derivative was significantly greater than ALA at 10, and 25μM.



**Figure 5.13.** Neuroprotective effect of ALA and its derivative. The cells were pretreated with either ALA or its derivative at the indicated concentration for 24 hours and then further incubated for 24 hours with 75µM 6-OHDA. Viability of cells was assessed by MTT assay. Data are expressed as percentages of control group and presented as mean  $\pm$  SEM of three independent experiment. \*\*\* $P$ <0.001 versus control group, # $P$ <0.01 and ### $P$ <0.001 versus 6-OHDA-treated group, °° $P$ <0.01 versus ALA-treated group (One-way ANOVA followed by Tukey's test).

## Conclusion

This study found neuroprotective effects in several compounds including compounds derived from plant as well as modified compounds. Some of these compounds demonstrate strong anti-oxidant activity and protect neuronal death and damage from oxidative stress. Several modified compounds also elicit the ability to protein neuronal death from both oxidative stress and factors known to induce neurodegeneration. Together the results from this study shade light to several possible compounds that can be further developed into novel therapeutic agents for treatment of neurodegenerative diseases.

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## Sub-project 6: Targeted drug delivery systems (TDDS) for anticancer activities

### ABSTRACT

Andrographolide analogue, namely 19-tert-butyl diphenylsilyl-8,17-epoxy andrographolide, or 3A.1, has been reported to be a potential anticancer agent for several cancer types. Due to its poor aqueous solubility, 3A.1 was incorporated within pH-sensitive amphiphilic chitosan derivatives (N-naphthyl-N,O-succinyl chitosan (NSCS), N-octyl-N,O-succinyl chitosan (OSCS) and N-benzyl-N,O-succinyl chitosan (BSCS). These 3A.1-loaded nanoparticles were nano-sized (<200 nm) and spherical in shape with a negative surface charge. 3A.1-loaded nanoparticles were produced by dropping method. 40% initial 3A.1-loaded NSCS exhibited the highest entrapment efficiency. The release of 3A.1 from these nanoparticles displayed a delayed release pattern. Under acidic conditions (pH 1.2) there was no free drug release. After the pH was adjusted to 6.8, a high cumulative 3A.1 release was obtained. In vitro anticancer activity against colorectal cancer cell HT-29 indicated that the 3A.1-loaded nanoparticles had significantly lower IC<sub>50</sub> than the free drug and promoted apoptosis. In addition, in vitro wound healing migration assay on head and neck cancer cell (HN-22) revealed that free 3A.1 and the 3A.1-loaded nanoparticles inhibited cell motility compared to untreated cells. Moreover, we synthesized folic acid conjugated NSCS (F-NSC) for active targeting. The result of anticancer activity against HT-29, which are overexpressed folate receptors on cell surface, clearly showed that 3A.1-loaded F-NSC had greater potency than unconjugated NSCS nanoparticles and also more accumulation into cancer cells through folate receptor-mediated endocytosis. These pH-sensitive amphiphilic chitosan nanoparticles decorated with folate may be promising nanocarriers for oral anti-cancer drug delivery to the targeted colon cancer sites.

**Keywords:** *Anticancer, Andrographolide analogue, nanocarrier decorated with folate*

### บทคัดย่อ

แอนโดรกราโฟไลด์สกัดได้จากฟ้าทะลายโจร มีการเพิ่มฤทธิ์ให้ดีขึ้นโดยการเตรียมให้อยู่ในรูปอนุพันธ์ต่างๆ หนึ่งในอนุพันธ์ของสารกึ่งสังเคราะห์ที่มีชื่อทางเคมี 19-tert-butyl diphenylsilyl-8,17-epoxy andrographolide หรือ 3A.1 มีฤทธิ์ต้านเซลล์มะเร็งที่ดีมากและใช้ได้ดีกับเซลล์มะเร็งหลายชนิด 3A.1 จึงเป็นสารที่มีศักยภาพต่อการนำไปใช้เป็นยาเคมีบำบัดตัวใหม่ได้ อย่างไรก็ตามปัญหาหลักของสารนี้ คือ การละลายน้ำที่ต่ำมาก ดังนั้นในการศึกษานี้จึงสังเคราะห์อนุพันธ์โคโตซานที่ไวต่อการเปลี่ยนแปลงพีเอช N-naphthyl-N,O-succinyl chitosan (เอ็นเอสซีเอส), N-octyl-N,O-succinyl chitosan (โอเอสซีเอส) และ N-benzyl-N,O-succinyl chitosan (บีเอสซีเอส) และเตรียมพอลิเมอร์ไกลโมเซลล์สำหรับนำส่งยา 3A.1 โดยบรรจุยาในพอลิเมอร์

ลิเมอร์กไมเซลล์ ด้วยวิธีทางกายภาพ ศึกษาผลของวิธีการเตรียม ชนิดของพอลิเมอร์และปริมาณตัวยาเริ่มต้น ต่อประสิทธิภาพในการบรรจุยาและความสามารถในการบรรจุยา นอกจากนี้ศึกษาขนาดอนุภาค รูปร่าง และการปลดปล่อยยา พบว่าวิธีการหดยสามารถบรรจุยาได้มากกว่าวิธีอื่น และพอลิเมอร์กไมเซลล์ที่เตรียมจากเอ็นเอสซีเอสและปริมาณตัวยาเริ่มต้นต่อพอลิเมอร์ร้อยละ 40 โดยน้ำหนัก มีความสามารถในการบรรจุยามากกว่าการใช้อนุพันธ์ชนิดอื่น ขนาดอนุภาคเล็กกว่า 200 นาโนเมตร และมีรูปร่างกลม การศึกษาการปลดปล่อยยา 3A.1 ในตัวกลางที่จำลองสภาวะทางเดินอาหารเป็นเวลา 8 ชั่วโมง พบว่าไม่มีการปลดปล่อย 3A.1 ในสภาวะจำลองกระเพาะอาหารพีเอช 1.2 และเมื่อปรับพีเอชของตัวกลางให้จำลองสภาวะลำไส้เล็กพีเอช 6.8 พบว่าการปลดปล่อย 3A.1 เพิ่มขึ้นอย่างมาก ทั้งนี้เนื่องจากผลของการแตกตัวเป็นไอออนของส่วนหมู่กรดซัลฟอนิก การทดสอบความเป็นพิษต่อเซลล์เอชที-29 พบว่า 3A.1 ที่บรรจุในพอลิเมอร์กไมเซลล์มีค่าความเป็นพิษต่อเซลล์สูงกว่ายาอิสระโดยเพิ่มการตายแบบอะพอพโทซิส การศึกษาการเคลื่อนที่ของเซลล์มะเร็งชนิดเอ็นเอช-22 พบว่าทั้ง 3A.1 อิสระและที่บรรจุในพอลิเมอร์กไมเซลล์สามารถยับยั้งการเคลื่อนที่ของเซลล์ได้ นอกจากนี้การนำโพลีมาควคู่กับเอ็นเอสซีเอส (F-NSC) เพื่อเพิ่มความจำเพาะกับเซลล์มะเร็งลำไส้ใหญ่เอชที-29 ซึ่งที่ผิวเซลล์มีตัวรับโพเลตสูง พบว่าสามารถเพิ่มประสิทธิภาพของระบบนำส่งได้ดีกว่าชนิดไม่มีโพลี ผลที่ได้แสดงให้เห็นว่า พอลิเมอร์กไมเซลล์เอ็นเอสซีเอสควคู่กับโพลีมีศักยภาพในการเพิ่มการละลายยา ควบคุมการปลดปล่อยและนำส่งยาไปบริเวณเป้าหมายที่เป็นมะเร็งลำไส้ใหญ่โดยการรับประทาน

*Keywords:* ยาด้านมะเร็ง ระบบนำส่งควบคู่กับโพลี อนุพันธ์แอนโดรกราโฟไลด์

## I. INTRODUCTION

### 1.2 Statement and significance of the research problem

Drug delivery systems (DDS) are processes or methods of pharmaceutical compounds' administration for an improved therapeutic effect in humans or animals body. DDS improve therapeutic efficacy through control of rate, time and place of drug release. There are commonly used routes of drug delivery including parenteral delivery, oral delivery, transdermal delivery, mucosal delivery (e.g. pulmonary, ocular, sublingual). Oral administration is the most widely used route of drug delivery due to its convenience in terms of self-administration, pain free and high patient compliance, especially in the case of chronic therapies. However, some properties of drugs are not suitable for oral route due to side effects, rapid metabolism, and poor solubility. The low solubility of drug, that is classified in Biopharmaceutical Classification System (BCS) class II and class IV, is a crucial obstacle due to low absorption in the gastrointestinal (GI) tract. Accordingly, the low solubility leads to low bioavailability (Li et al., 2009; Lu and Park, 2013). More than 40% of new chemical entities (NCEs) developed in pharmaceutical industry are practically insoluble in water (Kalepu, 2013). The techniques used for solubility enhancement of drug include particle size reduction, cosolvents, solid dispersions, complexation (Kumar, 2011). Recently, scientists have challenged to generate novel carriers of oral drug delivery for obtaining higher levels in bioavailability such as polymeric micelles, microemulsions, nanoparticles.



Polymeric micelles as a nano-sized carrier which is prepared through self-assembly of amphiphilic copolymers in an aqueous solution when a concentration of polymer is above the critical micelle concentration (CMC) value. The inner core is hydrophobic segment which entraps hydrophobic drugs while the outer hydrophilic shell stabilizes interface between the hydrophobic core and aqueous solution, protects the hydrophobic drugs from the environmental stimuli (e.g. gastric pH, enzyme, temperature) and decreases adverse effect of drugs on healthy cells and tissues (Ghaemy, 2014). Polymeric micelles bring some advantages for oral drug delivery such as encapsulating drug into inner core to avoid destruction in the GI tract, increasing efficient solubilization, releasing them in spatially controlled manner, which could enhance drug absorption (Kedar, 2010). Some widely used techniques for drug-incorporated polymeric micelles preparation and the efficacy of drug loading depends on the applied tools. They include techniques of chemical conjugation, physical entrapment, electrostatic and other methods. Physical entrapment is the simplest and the most convenient method and can be implemented via dialysis, O/W emulsion, dropping and evaporation. The hydrophobic drugs can be incorporated into the micelles inner core by hydrophobic interaction between entrapped drug molecules and the hydrophobic inner-core-forming polymer (Murthy, 2015). The hydrophobic interactions also work as a driving force for micelle formation such as hydrophobic force, electrostatic force,  $\pi$ - $\pi$  interaction and hydrogen bonding. Physical entrapment was selected to use in some studied such as dexamethasone-entrapped into PEGylated poly-4-(vinylpyridine) micelles by dialysis, O/W emulsion or evaporation (Miller, 2013), doxorubicin-loaded polyphosphazenes with poly(N-isopropylacrylamide-co-N,N-dimethylacryl amide) polymeric micelles by dialysis or O/W emulsion method (Qui, 2009), and preparation the polymeric micelles of methoxy poly(ethyleneoxide)-block-poly ( $\epsilon$ -caprolactone) (MePEO-b-PCL) containing cyclosporine A by co-solvent evaporation (Aliabadi, 2007). However, the polymeric micelles preparation with high drug loads remains challenging because different techniques may affect the efficacy of drug loading (Kim, 2010).

As it is known, pH levels in the GI tract vary from 1-3 in the stomach to 6-7.5 in the small intestine and the variation of pH has been utilized to control drug release from carriers (Xu, 2013). In oral administration, pH-sensitive polymeric micelle-based carrier can be made to improve the stability of micelles in the stomach and attain a controlled release in the intestine which improves oral bioavailability. The poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMAA) are generally used as pH-responsive polymers in pH-sensitive polymeric micelles in some studies. These polymers have pendant carboxyl groups with  $pK_a$  values of about 4-6 in the chain. For example, pH-responsive amphiphilic poly (acrylic acid-b-DL-lactide) (PAAc-b-PDLLA) was developed to incorporate prednisone acetate (Xue, 2009). This formulation of polymeric micelles showed minimizing drug release at pH 1.4 and burst release at pH 7.4. The pendant carboxyl groups in PAA moieties preserve collapsed states

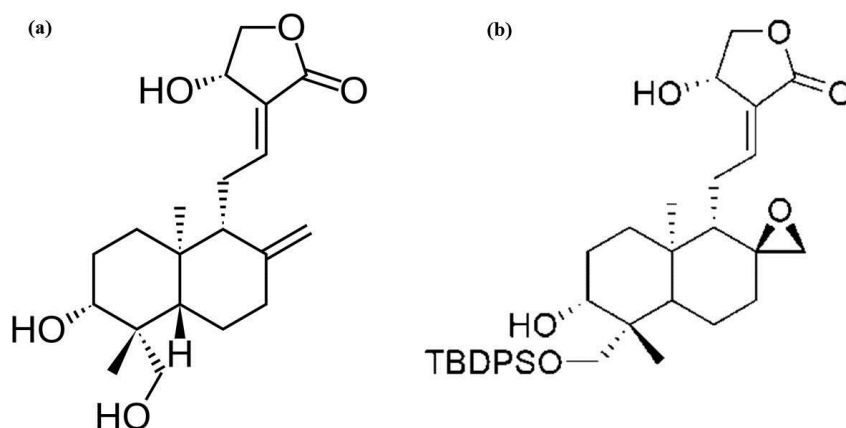
and are protonated in the low pH environment of the stomach; however, PAA swells in the intestines, ionizes, and releases protons (Sant, 2004). Yang et al. (2011, 2012) developed self-assembled, pH-sensitive micelles from amphiphilic copolymer brushes (e.g., poly(methyl methacrylate-co-methacrylic acid)-b-poly(poly(ethylene glycol) methyl ether monomethacrylate) [P(MMA-co-MAA)-b-PPEGMA] and poly(lactide)-b-poly (methacrylic acid)-b-poly(poly(ethylene glycol) methyl ether monomethacrylate) (PLA-b-MAA-b-PPEGMA) containing ibuprofen and nifedipine, respectively). They found that the drug release rates increased by modifying the MAA blocks.

Chitosan, is produced by Mucorales and other fungi, but on the whole it is manufactured by the deacetylation of chitin isolated from by-products of the marine fisheries. It is soluble in aqueous acidic solutions (pH < 6.0) (Muzzarelli et al., 2003; Muzzarelli et al., 2012), however, it cannot form polymeric micelles in water. Chitosan derivatives have been widely utilized in pharmaceutical applications since, they are biodegradable and less expensive (Bonferoni et al., 2014). Presently, chitosan derivatives in the form of polymeric micelles have been investigated for drug delivery. Several modified chitosans, for example *N*-lauryl-carboxymethyl-chitosan, *N*-phthaloylchitosan-g-mPEG, *N*-octyl-O-sulfate chitosan and *N,N*-dimethylhexadecylcarboxy methyl chitosan (DCMCs) have been utilized for the polymeric micelles preparation (Lia, 2014).

Many people are suffering from cancer which is an important lethal disease (Siegal, 2017). Colorectal cancer (CRC) is malignant tumors of colon and rectum with high mortality and morbidity in human beings. The statistics show that mortality rates from CRC have declined over the past decades as a result of better screening devices and more effective treatments. Unfortunately, in many cases, CRC patients are first diagnosed in the advanced stages making it hard to cure these patients. Therefore, early detection and optimal treatment such as surgery, radiotherapy, and chemotherapy, or combination therapy, should be able to improve patients' quality of life, and increase survival time with a higher cure rate. Chemotherapy is still the principal option for the treatment of advanced CRC or metastasis stage which cannot be treated by surgery alone (DiPiro, 2016). However, the clinical use of conventional chemotherapy is extremely restricted due to its low therapeutic efficacy, non-specific biodistribution, undesirable side effects, and high drug resistance, which may be the cause of treatment failure. It is therefore necessary to develop effective novel nanocarriers containing anticancer drugs with high potency against CRC, but low toxicity to normal tissues (Cisterna, 2016).

In recent years, plant-derived anticancer compounds have been developed to treat many types of cancer (Demain, 2011). Andrographolide (AG, Figure 1a) is a major active phytoconstituent derived from *Andrographis paniculata* Nees (Acanthaceae). It is widely used as a traditional herbal medicine in many countries such as India, China, and Southeast Asian countries, including Thailand (Hossain, 2014). This herb appears officially in the Thai Herbal

Pharmacopeia for the treatment of common colds and diarrhea. This natural compound has been revealed to demonstrate numerous pharmacological activities such as antibacterial and anti-inflammatory properties, and as an immune stimulant. Interestingly, many publications have confirmed that AG exhibits anticancer activity against various cancer cell lines but its anticancer potency is low (Shi, 2009; Varma, 2011; Aromdee, 2012). In order to improve its cytotoxic activity and obtain a potential anticancer compound, many researchers have attempted to modify the AG into various AG analogs (Nanduri, 2004; Sirion, 2012; Preet, 2014). One of them, a novel semi-synthetic AG analog, 19-*tert*-butyldiphenylsilyl-8,17-epoxy andrographolide (3A.1, Figure 1b) has recently been reported to demonstrate potent *in vitro* growth suppression of cancer cells including hepatocellular carcinoma, cervical carcinoma, cholangiocarcinoma, and colorectal carcinoma. However, the poor aqueous solubility ( $<1 \mu\text{g/mL}$ ) of 3A.1 remains one of the main problems that limits the effective use of the analog in cancer therapy (Nateewattana, 2014).



**Figure 6.1** (a) Chemical structure of andrographolide (b) andrographolide analog (3A.1)

In order to improve the aqueous solubility and therapeutic efficacy of the 3A.1, nanosized drug delivery system should be employed. Previous study revealed that curcumin can be loaded into amphiphilic N-benzyl-N,O-succinyl chitosan (BSCS) via dialysis method to increase solubility of curcumin. The BSCS consists of hydrophobic benzyl group and hydrophilic succinyl group that can form self-aggregation micelles in water during dialysis. Curcumin-loaded BSCS micelles show small particle size, highly negative charge, high water solubility, strong cytotoxicity to cervical cancer cells and high amount of drug release in physiological pH 5.5-7.4 (Sajomsang et al., 2014). To the best of our knowledge, there is no report in the literature about the drug delivery system used for 3A.1 analog. Therefore, this study aims to develop 3A.1-loaded pH-sensitive, amphiphilic self-assembled nanoparticles based on the amphiphilic CS graft copolymer for colorectal cancer treatment. Due to the properties of these self-assembled nanoparticles, they could improve the solubility of 3A.1 by

entrapping the drug into the hydrophobic inner core, while the hydrophilic shells are compatible with aqueous medium and help increase colloidal stability. The effects of preparation methods and hydrophobic cores on the drug loading were investigated. The surface morphology, size, charge, and drug release characteristics were also examined. Most importantly, the anticancer efficiency, apoptosis induction and antimigratory activity were also assessed.

Moreover, by coupling specific tumor-homing ligands such as folic acid to the surface of nanoparticles, an active targeting of anticancer drug to cancer cells overexpressed specific receptors for enhancing cellular uptake and minimizing side effects. Some tumor cells within solid tumors would overexpress folate receptor (e.g. breast, ovary, lung, brain, colorectal cancers), which can specifically recognize the folate molecule (oxidized form of folic acid). Thus, the targeting of folate-modified polymeric nanoparticles to these tumors is a promising strategy for active targeting treatment (Bahrami, 2015). Here, by coupling folate to the NSC nanoparticles (Fol-NSC) was developed and loaded optimal drug amount into polymeric micelles. The physicochemical characteristics were evaluated. Finally, *in vitro* antitumor activities of drug-loaded Fol-NSC nanoparticles against HT-29 colorectal cancer cells were also investigated, compared with drug-loaded NSC nanoparticles (unmodified nanoparticles).

### **1.3 Objectives of this research**

1. To synthesize pH responsive amphiphilic chitosan derivatives and formulate polymeric micelles for the 3A.1 drug delivery
2. To investigate the influence of the physical entrapment methods, type of amphiphilic chitosan derivatives, type and amount of drugs on entrapment efficiency, loading capacity and particle size of 3A.1-loaded polymeric micelles
3. To evaluate the drug release, anticancer efficiency, apoptosis induction and antimigratory activity of the 3A.1-loaded polymeric micelles
4. To compare anticancer activity of 3A.1-loaded polymeric micelles as passive targeting and 3A.1-loaded folate-modified surface of polymeric micelles as active targeting.

### **1.4 The research of hypothesis**

1. The amphiphilic chitosan derivatives can be synthesized and used to formulate 3A.1-loaded polymeric micelles.
2. The physical entrapment methods, type of amphiphilic chitosan derivatives, type and amount of drugs influence on the characteristics of polymeric micelles (i.e. particle size, size distribution, entrapment efficiency, loading capacity and stability).
3. Polymeric micelles can be used for the 3A.1 delivery to the targeted tumor site.

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5. *In vitro* releases study
6. *In vitro* cytotoxicity
7. Induction of cell apoptosis
8. *In vitro* cell migration
9. Short-term physical stability studies
10. Statistical analysis

### Materials

1. Acetonitrile HPLC grade (RCI Labscan Limited, Bangkok, Thailand)
2. Annexin-V FITC apoptosis kit (BD bioscience, USA)
3. Benzaldehyde (Sigma Aldrich<sup>®</sup>, St. Louis, MO, USA)
4. Chitosan (Degree of deacetylation; DDA 96%, MW 10-13 kDa) (OilZac Technologies Co., Ltd., Bangkok, Thailand)
5. Dimethyl sulfoxide (Fisher Scientific, UK Limited, UK)
6. Dulbecco's modified Eagle's medium (DMEM) (GIBCO<sup>®</sup>, Grand Island, NY, USA)

7. Fetal bovine serum (FBS) (GIBCO<sup>®</sup>, Grand Island, NY, USA)
8. Human colorectal adenocarcinoma cells (HT-29) cell line (Rockville, MD, USA)
9. N,N-dimethylformamide (DMF, 99.8%) (BrightchemSdnBhd, Malaysia)
10. 2-Naphthaldehyde (Sigma Aldrich<sup>®</sup>, St. Louis, MO, USA)
11. Octaldehyde (Sigma Aldrich<sup>®</sup>, St. Louis, MO, USA)
12. Oral squamous cell carcinoma (HN-22) was procured from the Faculty of Dentistry, Naresuan University.
13. Penicillin-streptomycin (GIBCO<sup>®</sup>, Grand Island, NY, USA)
14. Semi-synthetic andrographolide analog (3A.1) was kindly provided by the Faculty of Science, Burapha University.
15. Succinic anhydride (Sigma Aldrich<sup>®</sup>, St. Louis, MO, USA)
16. Sodium borohydride (Sigma Aldrich<sup>®</sup>, St. Louis, MO, USA)
17. Trypsin–EDTA (0.25 %) solution (GIBCO<sup>®</sup>, Grand Island, NY, USA)
18. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich<sup>®</sup>, St. Louis, MO, USA)
19. 19-*tert*-butyldiphenylsilyl-8,17-epoxy andrographolide (3A.1)

## Equipments

1. Analytical balance (Sartorius CP224S; Scientific Promotion Co., Ltd., Bangkok, Thailand)
2. Atomic-force microscope (AFM) (SPA400, Seiko, Japan)
3. Bruker AVANCE 500 spectrometer (Bruker, Switzerland)
4. CO<sub>2</sub> incubator (Heraeus HERA Cell 240, Heraeus Holding GmbH., Germany)
5. Dialysis bag (CelluSep<sup>®</sup> (6000–8000 MWCO) Membrane Filtration Products, USA)
6. Franz diffusion cells
7. Freeze-dryer (Model: Freezone 2.5, LABCONCO, USA)
8. Freezer/Refrigerator -20 °C, -80 °C, 5°C
9. Hot air oven (WTB Binder, Germany)
10. High performance liquid chromatography (HPLC) instrument (Agilent 1100 series, Agilent Technologies, USA)
11. HPLC column (Eclipse XDB-C18, 5 µm, 15 cm x 4.6 mm) (Santa Clara, CA, USA)
12. High voltage power supply (Model: Gamma High Voltage Research, USA)
13. Incubated shaker (Model: KBLee 1001, Daiki sciences, Bio-Active, Bangkok, Thailand)
14. Laminar air flow (BIO-II-A, Telstar Life Science Solutions, Spain)
15. Magnetic stirrer (Framo, Germany) and magnetic bar
16. Micropipette 0.1–2.5 µL, 2–20 µL, 20–200 µL, 100–1000 µL, 1–5 mL, and micropipette tip
17. Microcentrifuge (Microfuge 16<sup>®</sup>, Model: A46473, Beckman Coulter Inc., Germany)

19. Microcentrifuge tube (Eppendorf<sup>®</sup>, Corning Incorporated, NY, USA)
20. Microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA)
21. Nicolet 6700 spectrometer (Thermo Company, USA)
22. Nylon membrane filter (diameter 47 mm, pore size 0.45  $\mu\text{m}$ )
23. pH meter (Horiba compact pH meter B-212, Japan)
24. Probe-type sonicator (model CV 244, Sonics VibraCell<sup>TM</sup>, USA)
25. Sonicator bath
26. Sonicator probe-type (CV 244, Sonics VibraCell<sup>TM</sup>, Newtown, CT, USA)
27. Transmission Electron Microscopy (TEM) (Philips<sup>®</sup> Model TECNAI 20, Holland)
28. Vortex mixer (VX100, Model: Labnet, NJ, USA)
29. Water bath (HETOFRIG CB60; Heto High Technology of Scandinavia, Birkerød, Denmark)
30. Well-plate (96 Well plate) (Corning Incorporated, NY, USA)
31. ZetasizerNano ZS (Malvern Instruments, Malvern, UK)

## Methods

### 1. Synthesis of amphiphilic chitosan derivatives

The amphiphilic chitosan derivatives, i.e. *N*-naphthyl-*N,O*-succinyl chitosan (NSCS), *N*-octyl-*N,O*-succinyl chitosan (OSCS) and *N*-benzyl-*N,O*-succinyl chitosan (BSCS), were synthesized by reductive *N*-amination and *N,O*-succinylation. Two grams of chitosan was dissolved in 150 mL of 1% (v/v) aqueous acetic acid. Then, 100 mL of ethanol was added to the solution. 2-Naphthaldehyde, octanaldehyde or benzaldehyde (2.0 meq/GlcN) was added, and the reaction mixture was stirred at room temperature for 24 h. At this point, the pH of the solution was adjusted to 5 by adding 1 M NaOH, and 2.0 g of NaBH<sub>4</sub> (52.9 mmol) was added to the reaction mixture and stirred at room temperature for 24 h. The precipitate was collected by filtration, washed several times with ethanol, before being dried under a vacuum at room temperature to obtain the *N*-naphthyl chitosan (NCS), *N*-octyl chitosan (OCS) or *N*-benzyl chitosan (BCS). The *N,O*-succinylation was conducted using succinic anhydride. Briefly, 1.0 g of NCS, OCS or BCS was dispersed in 40 mL of *N,N*-dimethylformamide (DMF)/dimethyl sulfoxide (DMSO), (1:1 v/v), and 3.0 g of succinic anhydride (5.0 meq/GlcN) was added. The reaction was heated at 100°C under nitrogen atmosphere for 24 h. Next, the reaction mixture was cooled to room temperature and filtered to remove undissolved NCS, OCS or BCS. The clear solution was dialyzed with distilled water for 3 days to remove excess succinic anhydride and DMF/DMSO. The powdered NSCS, OSCS or BSCS were then obtained by lyophilization. The number average molecular weights ( $M_n$ ) of chitosan derivatives were evaluated based on the theoretical

molecular weight increase of the derivatives and the degree of substitution.

## **2. Characterization of pH sensitive amphiphilic chitosan derivatives**

### **2.1 Proton nuclear magnetic resonance ( $^1\text{H}$ NMR)**

The  $^1\text{H}$ -NMR spectra of chitosan and its derivatives were measured on a Bruker AVANCE 500 spectrometer (Bruker, Switzerland) using  $\text{D}_2\text{O}/\text{CD}_3\text{COOD}$  (99:1 v/v) solution and  $\text{DMSO-d}_6$ , respectively, at 10 mg/mL polymer concentrations. All measurements were performed at 300 K, using the pulsed accumulation of 64 scans and an LB parameter of 0.30 Hz. Tetramethylsilane was used as an internal standard.

### **2.2 Attenuated total reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)**

ATR-FTIR spectra were collected on a Nicolet 6700 spectrometer (Thermo Company, USA) using a single-bounce ATR-FTIR Smart Orbit accessory with a diamond internal reflection element (IRE) at ambient temperature ( $25^\circ\text{C}$ ). All spectra were taken from  $400\text{--}4000\text{ cm}^{-1}$ . Typically, 32 scans at a resolution of  $4\text{ cm}^{-1}$  were accumulated by using rapid-scan software in OMNIC 7.0 to obtain a single spectrum.

## **3. Characterization of polymeric micelles**

### **3.1 Critical micelle concentration (CMC)**

The CMC of graft copolymers in aqueous medium was determined using fluorescence spectroscopy with pyrene employed as a fluorescent probe. An aliquot (10  $\mu\text{L}$ ) of 1 mM pyrene solution in acetone was added to each vial of a series of aqueous polymer solutions (4 mL,  $0.5\text{--}3.9\times 10^{-3}\text{ mg/mL}$ ). The final concentration of pyrene in each sample solution was  $2.5\times 10^{-6}\text{ M}$ . The mixtures were sonicated for 15 min, heated at  $50^\circ\text{C}$  for 2 h, and then kept in the dark at room temperature overnight to equilibrate. Fluorescence spectra were recorded at an excitation wavelength of 335 nm, and the emission spectra were monitored over a range of 350–500 nm. The change in the intensity ratio of the first and third vibration bands ( $I_1/I_3$ ) at 373 nm ( $I_1$ ) and 382 nm ( $I_3$ ) in the emission spectra was used to investigate the shift in graft copolymer hydrophobic microdomains. The CMC was calculated after fitting the semi-log plot of the intensity ratio  $I_1/I_3$  vs. the concentration.

### **3.2 *In vitro* cytotoxicity**

The cytotoxicity of blank polymeric micelles was evaluated using an MTT cytotoxicity assay. The Caco-2 cells and HT-29 cells were cultured until 60–70% confluency in Dulbecco's modified Eagle's medium (DMEM) at pH 7.4 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acid solution and 0.1% penicillin-streptomycin solution in a humidified atmosphere (5%  $\text{CO}_2$ , 95% air,  $37^\circ\text{C}$ ). The cells were seeded into each well of 96-well plates and preincubated for 24 h at a seeding density of 10,000 cells/well. Then, the cells were treated with blank polymeric micelles at various



concentrations. After treatment, the solution was removed, fresh medium was added. Then, the mixture was incubated with MTT solution (final concentration 1 mg/mL) for 4 h at 37°C. The culture medium was removed, and the formazan crystals formed in the living cells were solubilized in 100  $\mu$ L DMSO. Relative cell viability was calculated based on the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, AOPUS01 and AI53601, Packard BioScience, CT, USA). The viability of non-treated control cells was arbitrarily defined as 100%.

#### **4. Drugs-loaded polymeric micelles**

##### **4.1 Physical entrapment methods**

###### **4.1.1 Dialysis method**

Overall, 5 mg of the amphiphilic copolymers (NSCS, OSCS and BSCS) and hydrophobic drugs (3A1) were dissolved in 2 mL of DMSO in a glass bottom container. Then, the mixture was stirred at room temperature until completely dissolved and transferred to dialysis bag (molecular weight cut-off, MWCO 6000-8000). The deionized water was replaced every 4 h for 24 h. The solution was centrifuged at 1000 rpm for 5 min. Then, the supernatant was filtered through a 0.45- $\mu$ m membrane filter and collected.

###### **4.1.2 O/W emulsion method**

The copolymers (NSCS, OSCS and BSCS) were prepared as for dialysis method. Then, drug was dissolved in DCM and was injected under constant stirring into 2 mL of blank polymeric micelles solution. Then, DCM was evaporated by overnight stirring at room temperature. The solution was centrifuged at 1000 rpm for 5 min. Then, the supernatant was filtered through a 0.45- $\mu$ m membrane filter and collected.

###### **4.1.3 Dropping method**

Five milligrams of copolymer and drugs were dissolved in 0.5 mL DMSO. The solution was slowly dropped into stirred water, and the mixed solution was stirred overnight. The final ratio of DMSO : water was 1:5. The mixture was then placed in a dialysis bag and dialyzed against deionized water overnight. The solution was centrifuged at 1000 rpm for 5 min. Then, the supernatant was filtered through a 0.45- $\mu$ m membrane filter and collected.

###### **4.1.4 Evaporation method**

Five milligrams of copolymer and drug were dissolved in DMF in a glass bottom container. The solution was mixed with acetone (1/3 of DMF) and stirred at room temperature under nitrogen gas flow until the solvent completely evaporated. Then, 3 mL of deionized water was added, and the solution was sonicated using a probe-type sonicator in a cycle with a sonication time of 5 min and a standby time of 5 min for 20 min. The solution was centrifuged at 1000 rpm for 5 min. Then, the supernatant was filtered through a 0.45- $\mu$ m membrane filter and collected.

## 4.2 Entrapment efficiency

The drug-loaded polymeric micelles of each method were dissolved in a mixture solution of DMSO : H<sub>2</sub>O (9:1). The amount of drug loaded into the polymeric micelles was determined using the high performance liquid chromatography (HPLC) (Agilent 1100 Series HPLC System, Agilent Technologies, USA) equipped with Eclipse XDB-C18 column (particle size 5  $\mu$ m, 15 cm  $\times$  4.6 mm) and DAD detector at 219 nm. The injection volume was 20  $\mu$ L. The isocratic elution was carried out with a mobile phase made of water and methanol (20:80, v/v), and a flow rate of 1.0 mL/min. Standard solutions with different drug concentrations were used to generate the calibrate curve. The entrapment efficiency and loading capacity were calculated with the following Eqs. (1) and (2), respectively.

$$\text{Entrapment efficiency (\%)} = (C_1/C_2) \times 100 \text{ (Eq.1)}$$

where  $C_1$  is the amount of drug in polymeric micelles and  $C_2$  is the initial of amount drug used for preparation

$$\text{Loading capacity (\mu g/mg)} = L_1/L_2 \text{ (Eq.2)}$$

where  $L_1$  is the amount of drug in micelles and  $L_2$  is the amount of graft copolymer used for preparation.

## 4.3 Characterizations of drugs-loaded polymeric micelles

### 4.3.1 Particle size

The micelle samples were diluted with distilled water prior to measure. The mean particle size and size distribution of the polymeric micelles with and without drugs were determined in triplicate at 25°C using the dynamic light scattering (DLS) (Malvern, Worcestershire, UK).

### 4.3.2 Morphology

Surface morphologies of the blank and 3A.1-loaded nanoparticles (unmodified and folate-modified NPs) were examined using a transmission electron microscopy (TEM) (Philips® Model TECNAI 20, Holland) at an accelerating voltage of 80 kV. The nanoparticle solutions were negatively stained with 1% uranyl acetate solution and dropped onto a formvar-coated copper grid before the observation.

### 4.3.3 The stability of drug-loaded micelles

The stability of drug-loaded micelles was determined by gel permeation chromatography (GPC) as described previously (Opanasopit et al., 2004). GPC was carried out using an Agilent HPLC system (Agilent 1100 series, USA) equipped with a Shodex<sup>®</sup> GFC SB804 HQ column at 40°C. Drug-loaded polymeric micelles freshly prepared solutions (50  $\mu$ L), were passed through a 0.45- $\mu$ m membrane filter and then injected into the column

and eluted with deionized water at a flow rate of 1 mL/min. Detection was performed by refractive index (RI) and UV detector.

## 5. *In vitro* releases study

In this study, 3A.1-loaded nanoparticles (unmodified and folate-modified NPs at 40 wt% of 3A.1 to polymer) prepared using the dropping method were selected for the release study. Briefly, 3A.1-loaded nanoparticles and drug suspension (the drug powder dispersed in deionized water) containing equal quantity of 3A.1 (40  $\mu\text{g}$ ) were added to microcentrifuge tubes containing release medium. A simulated gastric fluid (SGF  $\pm$  standard deviation) at pH 1.2 was used as the medium for the first 2 h, followed by a simulated intestinal fluid (SIF) at pH 6.8 for 6 h and a simulated colonic fluid (SCF) at pH 7.4 for the further 4 h. The release study was conducted at  $37 \pm 0.5$  °C in a shaking incubator at a speed of 150 rpm. The samples were collected at predetermined time intervals and filtered prior to the content determination. The release study was done in triplicate.

## 6. *In vitro* cytotoxicity

The anticancer activity of the 3A.1 free drug and 3A.1-loaded nanoparticles (unmodified and folate-modified NPs) on HT-29 cells (human colorectal cancer cells) was evaluated by MTT assay (Mosmann, 1983). Briefly, HT-29 cells ( $1 \times 10^4$  cells/well) were seeded in 96-well plates with DMEM supplemented with 10% FBS at pH 7.4 and cultured at 37 °C in a humidified 5% CO<sub>2</sub> incubator. After incubation for 24 h, the cells were treated with various concentrations (0.1 to 16.0  $\mu\text{g/mL}$ ) of the 3A.1-loaded nanoparticles (NSCS, OSCS, and BSCS; Fol-NSCS) and 3A.1 free drug, and further incubated for 36 h. After treatment, the medium was withdrawn and the cells were covered with fresh medium containing 0.5 mg/mL of MTT for 4 h. After that, the medium was taken out and the formazan crystals generated in the surviving cells were liquefied by adding 100  $\mu\text{L}$  of DMSO, after which the optical density (OD) of each well was analyzed using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA) at 550 nm. The percentage of cell viability was determined using the equation: Cell viability (%) =  $\text{OD}(\text{treated})/\text{OD}(\text{untreated}) \times 100$ . Moreover, half-maximal inhibitory concentration (IC<sub>50</sub>) values were assessed using GraphPad Prism version 5.01 (GraphPad Inc., USA). In a similar procedure, the cytotoxicity of the blank nanoparticles was also evaluated.

## 7. Induction of cell apoptosis

An apoptosis assay was carried out using a flow cytometer (BD, FACSC Canto, Becton Dickinson, CA, USA) with FITC-conjugated Annexin V/propidium iodide (PI) (BD Bioscience) co-staining (Reabroi, 2018). Briefly, HT-29 cells were sowed in 6-well plates and incubated in a

humidified 5% CO<sub>2</sub> incubator at 37 °C until 60–70% confluence. The cells were treated with 3A.1-loaded nanoparticles (NSCS, OSCS, BSCS, Fol-NSCS) and 3A.1 free drug in fresh medium (the final drug concentration was fixed at 3.5 µg/mL) at 37 °C for 24 h. After treatment, the cells were collected by adding EDTA solution as cell detachment agent and subsequently washed with medium. Then, the cells were gently pipetted to detach the cells. The treated cells were stained with Annexin V-FITC and PI in the dark for 15 min at ambient temperature, and immediately subjected to flow cytometry analysis. The data were evaluated using FACS diva software (BD Bioscience, USA). Both early apoptotic (Annexin V+/PI-) and late apoptotic (Annexin V+/PI+) cells were embraced in the cell apoptosis evaluation. The untreated HT-29 cells were used as negative control and the HT-29 cells treated with 2% Triton X-100 was used as positive control (Jain, 2017).

## **8. *In vitro* cell migration**

The effect of 3A.1-loaded nanoparticles and free 3A.1 on HN-22 (human oral squamous cancer) cell motility was performed using scratch wound healing assay as previously explained (Grada, 2017; Wongprayoon, 2018). Briefly, HN-22 cells were grown in 6-well culture plates with DMEM containing 10% FBS at pH 7.4 to achieve confluent monolayer. A monolayer of the cell in each well were gently scratched with a sterile 200-µL pipette tip to make three straight lines/well and rectangular cell-free spaces. Then, the culture medium was discarded and washed twice with PBS (pH 7.4) to remove debris and scratched cells. After that, the 3A.1-loaded nanoparticles (NSCS, OSCS, and BSCS) and free 3A.1 dispersed in fresh medium (3.0 µg/mL) were placed into each well and cultured at 37 °C under a humidified 5% CO<sub>2</sub> incubator for 24 h. Culture medium without drug and DMSO were used as control. After scraping at 0, 12, 18, and 24 h, the images (5 images/well) of the wound were captured at × 40 magnification using inverted microscope (Nikon<sup>®</sup> T-DH, Japan). The wound gap and wound area were quantified using JMicroVision version 1.2.7 and calculated to determine the cell migration rate and percentage of wound closure. The antimigratory activity was assessed in triplicate.

## **9. Short-term physical stability studies**

The stability of the 3A.1-loaded polymeric nanoparticles (NSCS, OSCS, and BSCS) was determined according to ICH guideline section Q1A R2. The nanoparticles samples were stored in glass bottles at 5 ± 3 °C, 60 ± 5 %RH (long-term condition) and at 25 ± 2 °C, 60 ± 5 %RH (accelerated condition) for 6 months. The particle size, zeta potential, and content of 3A.1 remaining in the drug-loaded nanoparticles were evaluated after storage for 0, 30, 60, 120, and 180 days. All data were measured in triplicate.

## **10. Statistical Analysis**

All experiments were accomplished in triplicate. Data were presented as mean ±

standard deviation (SD). The statistical significance of differences was assessed using one-way analysis of variance (ANOVA) followed by a LSD *post hoc* test (SPSS version 16.0 for Window, SPSS Inc., USA). The statistical significance of differences was noted when the *p* value <0.05 for all statistical tests.

## RESULTS AND DISCUSSION

### 1. Synthesis and characterizations of pH sensitive amphiphilic chitosan derivatives

The synthesis of the amphiphilic chitosan derivatives i.e. NSCS, OSCS and BSCS, was carried out by reductive N-amination and N,O-succinylation (Sajomsang et al., 2014) as shown in Figure 2. Overall, the N-alkyl or N-aryl chitosan were formed from the corresponding Schiff base intermediates before the reduction using sodium borohydride. The homogenous N,O-succinylation of NCS, OCS and BCS was carried out by using succinic anhydride in the mixture of solvents consisting of DMF and DMSO at 100°C. The successful synthesis of all amphiphilicchitosan derivatives was confirmed by <sup>1</sup>H NMR, ATR-FTIR and elemental analysis. The series of amphiphilicchitosan derivatives with different N-hydrophobic substituents were obtained as shown in Table 1. The degree of substitution (DS), defined as the number of N-hydrophobic groups, and the degree of N,O-succinylation (DSS), defined as the number of N,O-succinyl groups per hundred glucosamine units of chitosan, were determined by elemental analysis. The DS and DSS were calculated by comparing the C/N molar ratios obtained from the elemental analyses using Eqs. (3) and (4), respectively (Huo et al., 2012).

$$\text{DS of hydrophobic group (\%)} = \frac{(C/N)_{ACS} - (C/N)_{CS}}{X} \times 100 \quad (\text{Eq.3})$$

$$\text{DSS of succinylgroup(\%)} = \frac{(C/N)_{scs} - (C/N)_{ACS}}{4} \times 100 \quad (\text{Eq.4})$$

where (C/N)<sub>ACS</sub> represents the C/N ratio of amphiphilicchitosan derivatives, (C/N)<sub>CS</sub> represents the C/N ratio of chitosan, (C/N)<sub>scs</sub> represents the C/N ratio of succinylatedamphiphilicchitosanderivatives, and X represents the number of carbon atoms on the hydrophobic moieties of the chitosan backbone, which are 11, 8, and 7 for naphthyl, octyl, and benzyl substituents, respectively.

Both aliphatic and aromatic aldehydes at 2-fold molar ratios relative to the glucosamine (GlcN) residues of chitosan were used to study the DS. By varying the hydrophobic substituents on the chitosan backbone, the impact of the steric and electronic factors on this procedure could be elucidated. As shown in Table 1, the N-benzylation of

chitosan by benzaldehyde had a higher DS than the N-naphthylation and N-octylation by 2-naphthaldehyde and octanaldehyde, respectively, at similar molar ratios of aldehyde to GlcN. The DS order of N-hydrophobically modified chitosan was N-benzyl (0.69) > N-naphthyl (0.52) > N-octyl (0.47). This indicated that octanaldehyde was less reactive than benzaldehyde and 2-naphthaldehyde. This could be attributed to the relative stability of the Schiff base intermediate and steric hindrance effect. In the case of benzaldehyde and 2-naphthaldehyde, the Schiff bases were stabilized by resonance with the aromatic ring, while the Schiff base of octanaldehyde with chitosan could not be stabilized by the resonance effect. Compared with 2-naphthaldehyde, benzaldehyde showed lower steric hindrance, leading to a higher DS. Moreover, the hydrophobic moieties (i.e., the naphthyl, octyl, and benzyl groups) effectively substituted onto the primary amino groups, while the succinyl groups were added to both the primary amino and hydroxyl groups on the chitosan backbone. The  $M_n$  of all chitosan derivatives could be calculated based on the increase of theoretical molecular weight of the derivatives due to the DS of the hydrophobic groups and the DSS of the succinyl groups. The  $M_n$  of chitosan was determined to be 7,633 g/mol (47 repeating units) by GPC. The DS values of NCS, OCS, and BCS were calculated to be 0.54 (approximately 25 repeating units), 0.47 (approximately 22 repeating units), and 0.69 (approximately 32 repeating units), while the DSS values were 0.52 (approximately 24 repeating units), 1.13 (approximately 53 repeating units), and 1.07 (approximately 50 repeating units), respectively (Table 1). According to the DS and DSS, the hydrophobic/hydrophilic ratios on the chitosan backbone were 1.04, 0.42, and 0.65 for NCS, OCS, and BCS, respectively.

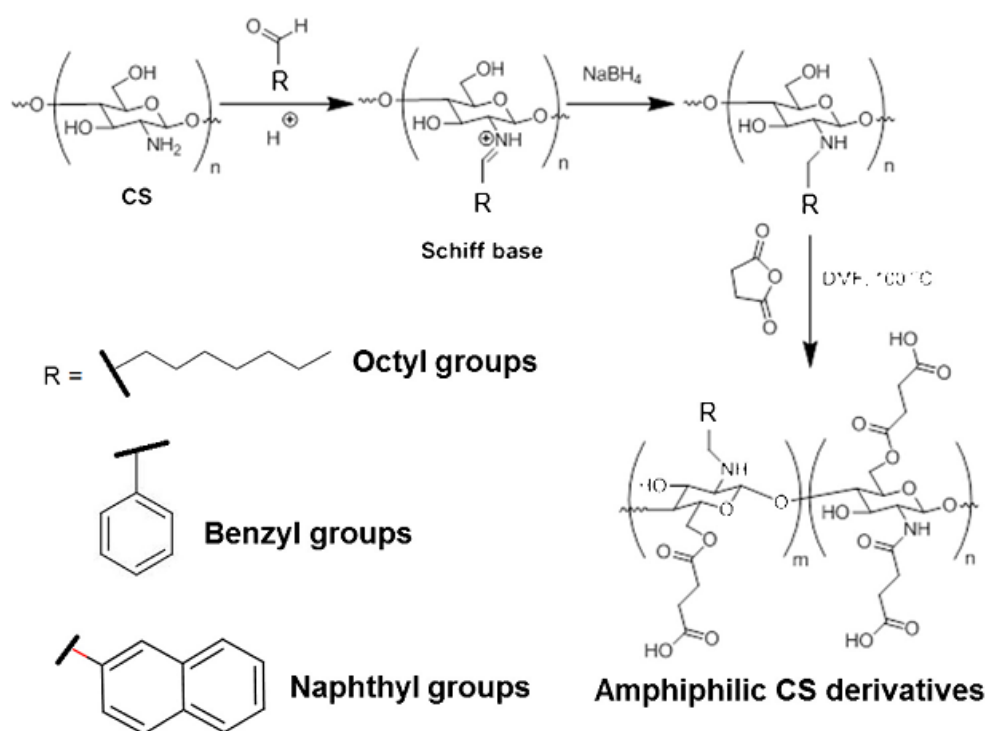


Figure 2 Synthesis scheme showing the formation of amphiphilic chitosan derivatives.

Table.6.1 Elemental analysis data for chitosan(CS) and its amphiphilic derivatives

| Sample | %C    | %H    | %N   | C/N   | DS <sup>a</sup> | DS <sup>b</sup> | DSS <sup>c</sup> | M <sub>n</sub> (g/mol) |
|--------|-------|-------|------|-------|-----------------|-----------------|------------------|------------------------|
| CS     | 44.3  | 7.87  | 8.38 | 5.28  | -               | -               | -                | 7633 <sup>d</sup>      |
| NCS    | 66.79 | 11.29 | 5.97 | 11.18 | 0.52            | 0.54            | -                | 10907 <sup>e</sup>     |
| OCS    | 49.49 | 12.68 | 5.47 | 9.04  | 0.37            | 0.47            | -                | 10046 <sup>e</sup>     |
| BCS    | 65.48 | 11.76 | 6.45 | 10.15 | 0.65            | 0.69            | -                | 10467 <sup>e</sup>     |
| NSCS   | 54.97 | 10.41 | 4.14 | 13.27 | -               | 0.54            | 0.52             | 13351 <sup>e</sup>     |
| OSCS   | 49.17 | 11.28 | 3.61 | 13.59 | -               | 0.47            | 1.13             | 15321 <sup>e</sup>     |
| BSCS   | 56.89 | 9.97  | 3.93 | 14.44 | -               | 0.69            | 1.07             | 15462 <sup>e</sup>     |

<sup>a</sup> The degree of *N*-substitution (DS) determined by <sup>1</sup>H-NMR

<sup>b</sup> The degree of *N*-substitution (DS) determined by elemental analysis

<sup>c</sup> The degree of *N,O*-succinylation (DSS) determined by elemental analysis

<sup>d</sup> Measured by GPC <sup>e</sup> Determined by elemental analysis

### 1.1 <sup>1</sup>H NMR characteristics

The <sup>1</sup>H-NMR spectrum of chitosan, NCS, OCS, BCS, NSCS, OSCS and BSCS are shown in Figure 3 and 4, respectively. In comparison with the <sup>1</sup>H NMR spectra of chitosan that contains no aromatic protons, the <sup>1</sup>H NMR NCS spectra and BC exhibited broad multiple protons at ranging from 7.42 to 7.89 ppm and at 7.32 ppm due to the presence of the naphthyl and benzyl groups, respectively. The OCS showed the proton signals at 0.82 ppm and 1.21 ppm due to the methyl and methylene protons of long chain hydrocarbon (Figure 4.2). Moreover, the proton signal at 2.45 ppm in NSCS, OSCS and BSCS represented the methylene protons of the succinyl moiety on the chitosan backbone (Figure 4) (Lim, 2013; Sajomsang, 2014). Based on the <sup>1</sup>H-NMR spectra, the DS values of NCS, OCS, and BCS were calculated using Eqs. (5)–(7), respectively. The DS values were 0.52, 0.37, and 0.65 for NCS, OCS, and BCS, respectively.

$$DS = \left( \frac{INap/7}{IH2-H6/6} \right) \quad (Eq.5)$$

where *INap* represents the total area (integration) of the *N*-naphthyl protons, and *IH2–H6* represents the peak area of protons C2–C6 on the chitosan backbone.

$$DS = \left( \frac{IMe/3}{IH2-H6/6} \right) \quad (Eq.6)$$

where  $I_{Me}$  represents the total area (integration) of N-methyl protons of the octyl group, and  $I_{H2-H6}$  represents the peak area of protons C2–C6 on the chitosan backbone.

$$DS = \left( \frac{I_{Ar}/5}{I_{H2-H6}/6} \right) \quad (\text{Eq.7})$$

where  $I_{Ar}$  represents the total area (integration) of N-benzyl protons, and  $I_{H2-H6}$  represents the peak area of protons C2–C6 on the chitosan backbone.

It is important to note that DS value determined by  $^1\text{H-NMR}$  method is less than elemental analysis method (Table 1), particularly in case of OCS. This difference was due to the limitation of solubility in  $^1\text{H-NMR}$  solvent. Moreover, the methylene protons of the succinyl groups overlapped with the solvent, DMSO- $d_6$ . Therefore, the DSS was determined using elemental analysis instead of  $^1\text{H-NMR}$  spectroscopy. These results from ATR-FTIR and  $^1\text{H-NMR}$  spectra were discussed to indicate the successful introduction of both functionalities onto the chitosan backbone.

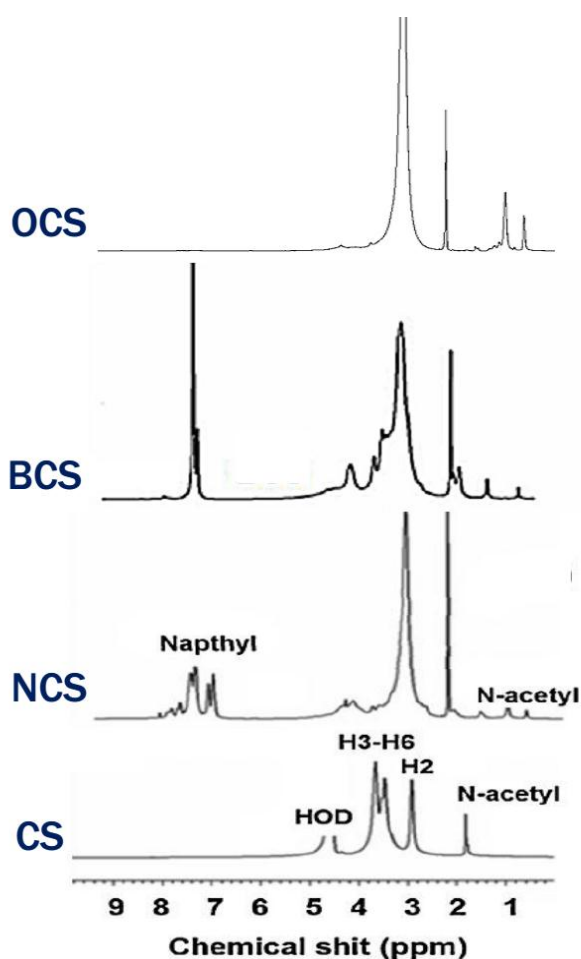




Figure 6.3  $^1\text{H}$  NMR spectra of NCS, BCS, OCS and CS

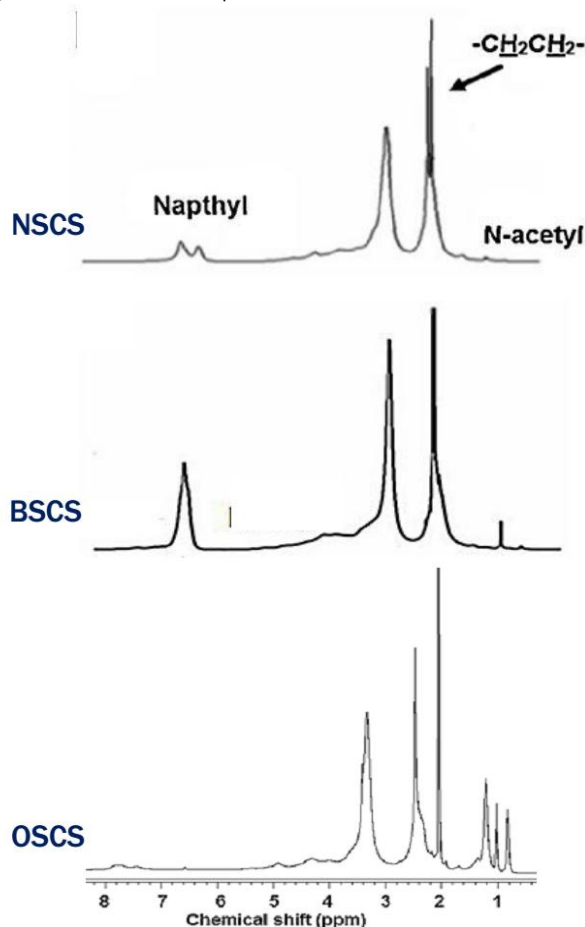


Figure 6.4  $^1\text{H}$  NMR spectra of NCS, BCS and OCS

## 1.2 ATR-FTIR characteristics

The ATR-FTIR spectra of CS, NCS, OCS, BCS, NCS, OCS and BCS are shown in Figure 6.5. The ATR-FTIR spectra of NCS, OCS and BCS were similar to that of CS and showed the additional absorption bands of functional groups (i.e., naphthyl, benzyl and octyl). The characteristic bands at 1632, 1490, 817 and 746  $\text{cm}^{-1}$  (NCS spectrum) and at 1600, 1494, 743 and 695  $\text{cm}^{-1}$  (BCS spectrum) were assigned to C=C stretching, C-H deformation (out of plane) for naphthyl and benzyl groups, respectively (Lim et al., 2013; Sajomsang et al., 2008) while the OCS spectrum had the strong absorption bands for C-H stretching of octyl groups at 2923 and 2856  $\text{cm}^{-1}$  (Figure 5a). After *N,O*-succinylation process, the NCS, OCS and BCS spectra exhibited the characteristic bands for C=O stretching of the succinic acid moiety at 1704  $\text{cm}^{-1}$  (NCS spectrum), 1714  $\text{cm}^{-1}$  (OCS spectrum) and 1715  $\text{cm}^{-1}$  (BCS spectrum) (Figure 6.5b).

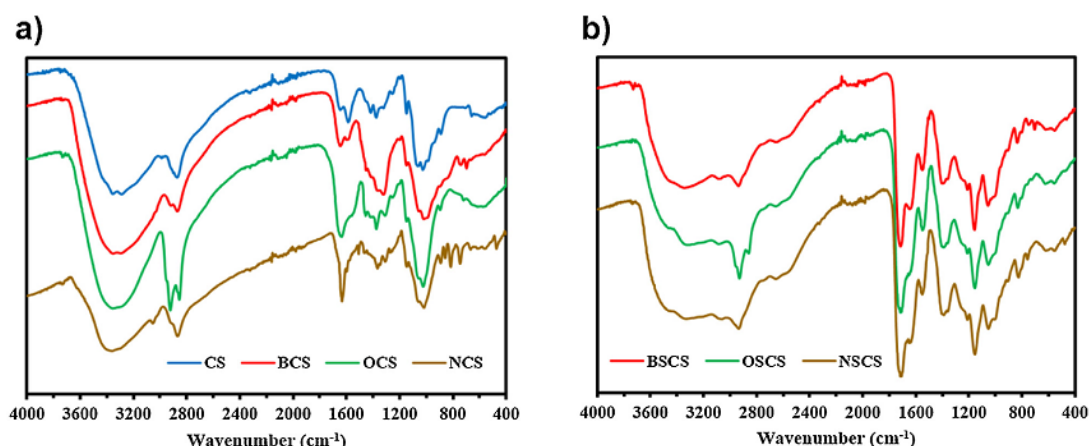


Figure 6.5(a) ATR-FTIR spectra of CS, BCS, OCS and NCS; (b) BSCS, OSCS and NSCS

## 2. Blank polymeric micelles

### 2.1 Critical micelle concentration (CMC)

The fluorescence assay was conducted to determine the concentration of NSCS, OSCS and BSCS polymer for micellization at the first taken place. Briefly, the hydrophobic pyrene probe was added to aqueous polymer solutions of increasing concentration and pyrene fluorescence spectra were recorded for all solutions. The change in the intensity ratio of the first and third vibration bands ( $I_1/I_3$ ) at 373 nm ( $I_1$ ) and 382 nm ( $I_3$ ) in the emission spectra was used to investigate the shift in graft copolymer hydrophobic microdomains. The plots of the intensity ratio  $I_1/I_3$  versus the concentration of polymer solutions of NSCS, OSCS and BSCS are shown in Figure 6 and the CMC value was determined for each copolymer solution from the intersection of two straight lines. When the concentration reached the CMC, the  $I_1/I_3$  ratio dramatically decreased. The CMC for NSCS, OSCS and BSCS was 0.0678, 0.0855 and 0.0575 mg/mL, respectively, and was lower than the CMC value of low molecular weight surfactants (Jiang et al., 2006). There is a covalent linkage in individual molecules within the hydrophobic core in polymeric micelles. This linkage prevents dynamic exchange of monomers between free solution and the micellar pseudo-phase which confers rigidity and stability to the polymeric micelles (Murthy, 2015).

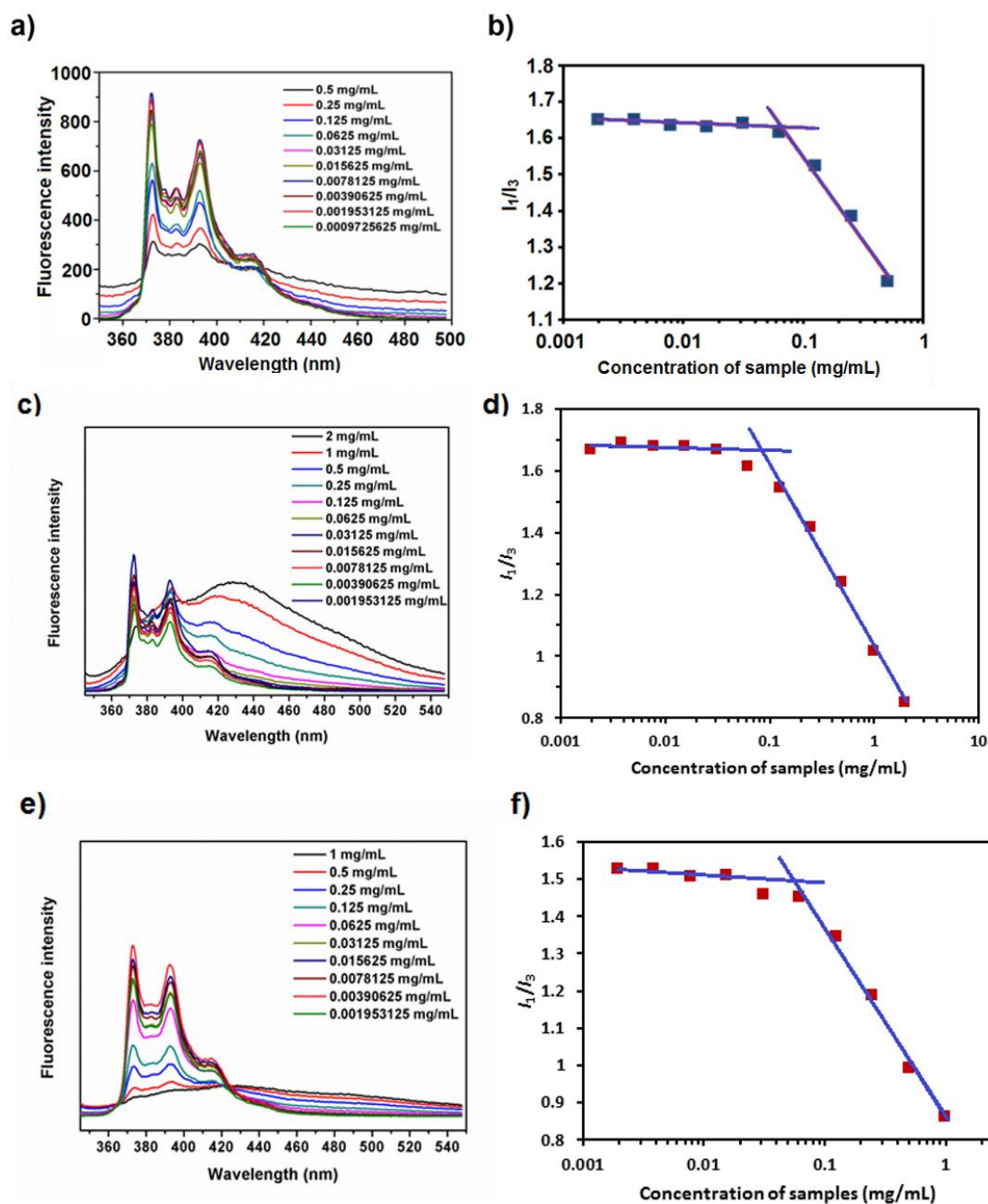


Figure 6.6 Fluorescence emission spectra of pyrene in water in the presence of NSCS (a), BSCS (c), and OSCS (e) polymeric micelles; a plot of the change in the intensity ratio ( $I_1/I_3$ ) from excitation spectra of pyrene in water at various concentrations of NSCS (b), BSCS (d), and OSCS (f).

## 2.2 *In vitro* cytotoxicity

As a major requirement, the polymer used to prepare the polymeric micelles should be non-toxic. Although chitosan is generally considered as a biodegradable and safe polymer, some case studies identified the toxic effects of chitosan derivatives (Ngawhirunpat et al., 2009). The Caco-2 cells, represented functional similarities to intestinal epithelium, are commonly used *in vitro* model for studies cytotoxicity or prediction of intestinal drug absorption. HT-29 cells, originally derived from a human colon cancer, were selected as model for the study of colon cancer. Thus, the cytotoxicity of blank NSCS, OSCS and BSCS micelles was determined using MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product (Calvello et al., 2016), performed with Caco-2 cells and HT-29 cells. The viability of Caco-2 cells treated with the various concentrations of all blank polymeric micelles is shown in Figure 6.7. The half maximal inhibitory concentration ( $IC_{50}$ ) value of the NSCS, OSCS and BSCS polymeric micelles for Caco-2 cells was  $3.08 \pm 0.15$ ,  $2.95 \pm 0.06$  and  $3.23 \pm 0.08$  mg/mL, respectively. No significant differences in the cytotoxicity were observed among the polymeric micelles in the Caco-2 cells. In a previous research, Chae et al. (2005) described low molecular weight chitosan (3.8–13 kDa; DDA 87–92%) at concentrations lower than 5 mg/mL revealed cell viability of Caco-2 cell > 80% after treating sample for 2 h. These results showed that all self-assembled polymeric micelles had low cytotoxicity in Caco-2 cells, indicating the excellent biocompatibility of the constituent polymers. As shown in Figure 6.8, blank NSCS, OSCS and BSCS micelles showed minimal cytotoxicity under concentration up to 0.5 mg/mL. The result indicated that blank micelles may be regarded as a safe drug carrier.

### 1. Preparation and Characterization of the 3A.1-Loaded Polymeric Nanoparticles

The blank nanoparticles and hydrophobic 3A.1-loaded nanoparticles based on the three types of amphiphilic chitosan graft copolymer (NSCS, OSCS, and BSCS) were prepared using different physical encapsulation techniques including dialysis, O/W emulsion, and dropping. These chitosan graft copolymers could generate core-shell structural nanoparticles which encapsulated the 3A.1 in the hydrophobic inner core by a self-assembly process.

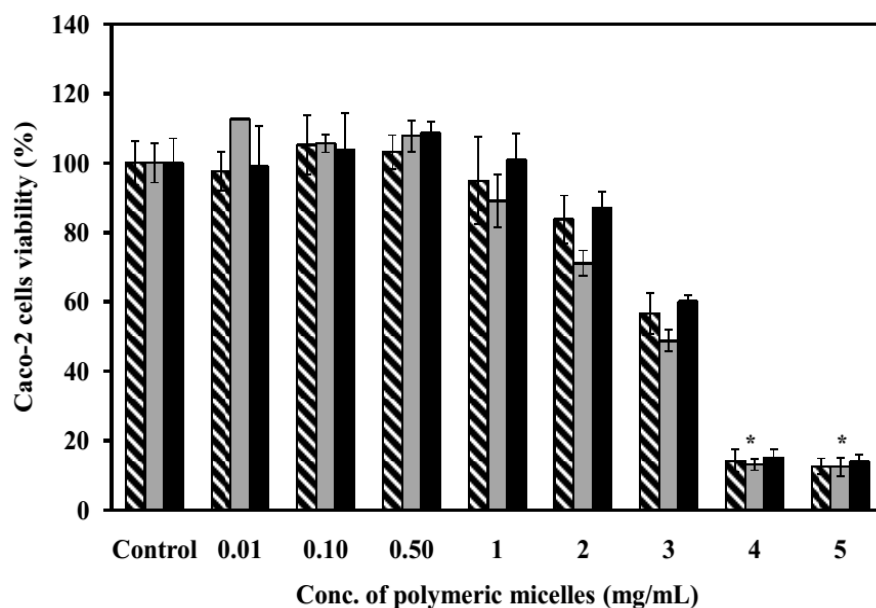


Figure 6.7 The percent cell viability in Caco-2 cells at varying concentrations of polymeric micelles; (▨) NSCS, (▤) OSCS, (■) BSCS. Each value represents the mean  $\pm$  SD of five wells. \* Statistically significant ( $p < 0.05$ ).

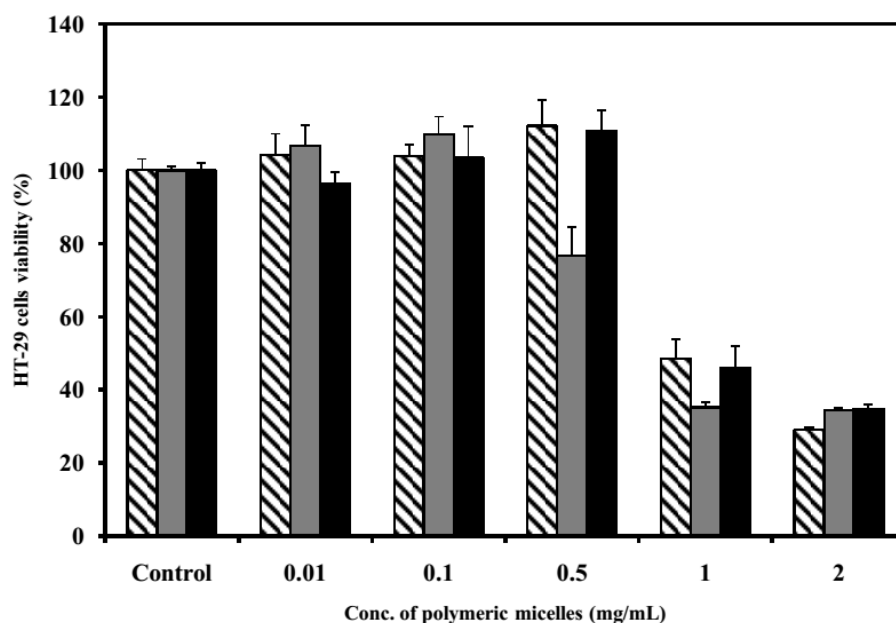


Figure 6.8 The present cell viability in HT-29 cells at varying concentrations of polymeric micelles; (▨) NSCS, (▤) OSCS, (■) BSCS. Each value represents the mean  $\pm$  SD of five wells.

### 3.1 Effects of physical entrapment methods

The effects of different physical entrapment methods on the physicochemical properties of the nanoparticles loaded with 3A.1 were investigated at a fixed initial ratio of 20 wt% drug to polymer. The parameters which were considered when selecting the optimal drug-loading method were particle size, zeta potential, entrapment efficiency (%EE), and drug-loading capacity (LC). All the data are listed in Table 6.2. Among the three drug-loading methods with different grafted hydrophobic moieties, the nanoparticles formed by the dropping method exhibited the smallest particle size with the diameter ranging from 90.33 to 165.63 nm, while the zeta potential values showed a highly negative surface charge (– 24.97 to – 28.40 mV). On the other hand, the 3A.1-loaded nanoparticles prepared by O/W emulsion method were found to be the biggest, with the lowest values of drug-loading content. The %EE and LC of 3A.1-loaded nanoparticles prepared by dropping method were  $72.59 \pm 8.61$  to  $80.13 \pm 5.01\%$  and  $145.17 \pm 17.21$  to  $160.27 \pm 10.03 \mu\text{g}/\text{mg}$ , respectively. These high values of drug content indicated that this method could efficiently entrap the hydrophobic 3A.1 anticancer drug within the cores of the nanoparticles. Therefore, the dropping method was selected to formulate 3A.1-loaded nanoparticles for further studies.

Table 6.2 The physicochemical properties of 3A.1-loaded nanoparticles with 20% initial drug added (NSCS, OSCS, and BSCS) prepared using three physical entrapment methods

| Chitosan derivatives | Method       | Particle size (nm) | Zeta potential (mV)     | Entrapment efficiency (%) | Loading capacity ( $\mu\text{g}/\text{mg}$ ) |
|----------------------|--------------|--------------------|-------------------------|---------------------------|----------------------------------------------|
| NSC                  | Dialysis     | $136.07 \pm 1.43$  | (–)<br>$25.10 \pm 0.22$ | $66.70 \pm 2.57$          | $133.39 \pm 5.13$                            |
|                      | O/W emulsion | $105.03 \pm 1.28$  | (–)<br>$24.90 \pm 1.34$ | $7.86 \pm 1.89$           | $15.72 \pm 3.78$                             |
|                      | Dropping     | $90.33 \pm 2.28$   | (–)<br>$24.97 \pm 1.11$ | $72.59 \pm 8.61$          | $145.17 \pm 17.21$                           |
| OSC                  | Dialysis     | $287.00 \pm 3.81$  | (–)<br>$29.60 \pm 0.33$ | $62.88 \pm 1.58$          | $125.77 \pm 3.17$                            |
|                      | O/W emulsion | $249.50 \pm 8.78$  | (–)<br>$25.80 \pm 0.29$ | $14.34 \pm 1.29$          | $28.67 \pm 2.59$                             |
|                      | Dropping     | $165.63 \pm 9.80$  | (–)<br>$26.67 \pm 0.82$ | $80.13 \pm 5.01$          | $160.27 \pm 10.03$                           |
| BSC                  | Dialysis     | $242.67 \pm 2.23$  | (–)<br>$26.77 \pm 0.46$ | $65.31 \pm 1.89$          | $130.62 \pm 3.77$                            |
|                      | O/W emulsion | $160.60 \pm 0.50$  | (–)<br>$32.37 \pm 1.54$ | $12.34 \pm 1.97$          | $24.68 \pm 3.95$                             |
|                      | Dropping     | $131.63 \pm 1.05$  | (–)<br>$28.40 \pm 0.68$ | $73.06 \pm 2.38$          | $146.13 \pm 4.76$                            |

### 3.2 Effects of initial 3A.1 and hydrophobic cores

The effects of the initial drug amount (5–40 wt% drug to polymer) and hydrophobic functionalities on the physicochemical properties of the amphiphilic nanoparticles were studied. The 3A.1-loaded nanoparticles were prepared by dropping method. The particle size, size distribution, and surface charge of the nanoparticles were determined by dynamic light scattering (DLS), as shown in Table 3. It was found that an

increase in the initial drug amounts resulted in an increase in the mean particle sizes (75.23 to 170.67 nm), which were bigger than those of the blank nanoparticles (66.26 to 132.33 nm). This might be due to the high amount of the drug molecule entrapped inside the nanoparticles. However, it should be noted that some aggregations occurred as the initial drug amount (wt% to polymer ratio) was increased to 80% (data not shown). At the initial drug-to-polymer ratio of 40%, the mean particle sizes of the 3A.1-loaded nanoparticles were ranked as follows: NSCS ( $102.53 \pm 0.60$  nm) < BSCS ( $153.97 \pm 1.20$  nm) < OSCS ( $170.67 \pm 1.72$  nm). The 3A.1-loaded NSCS nanoparticles exhibited the smallest particle size compared with other nanoparticles. In addition, the polydispersity indices (PDI) of all nanoparticles were lower than 0.3, indicating that the particle size distribution of these nanoparticles was narrow. The surface charges were also negative ( $-22.23 \pm 5.27$  to  $-32.07 \pm 3.07$  mV) which may be resulted from the appearance of negatively charged succinyl moieties on the nanoparticles that reduce nanoparticles aggregation, stabilize the nanoparticles in an aqueous medium, and display pH sensitivity feature. The morphology of the blank and drug-loaded nanoparticles was examined using a transmission electron microscope (TEM), and the resulting images are illustrated in Figure 9. In comparison with the blank nanoparticles, the TEM images of 3A.1-loaded nanoparticles (NSCS, OSCS, and BSCS) with 40 wt% initial drug added were spherical in shape, with a smooth surface and uniform size. Their mean sizes were in the nanometer range (approximately 40 to 90 nm in diameter). Notably, the mean particle sizes of the nanoparticles shown in the TEM images were smaller than those from the DLS technique. This is because the nanoparticles analyzed using TEM were performed in a dry state. On the other hand, those analyzed by DLS were carried out in an aqueous solution. These nanoparticles with a uniform size of not more than 200 nm could penetrate through the leaky vasculature of tumors and accumulate in tumor tissues *via* passive tumor targeting (You, 2016).

For characterization of Fol-NSCS nanoparticles, the results were expressed in Table 4. The surface modified polymeric nanoparticles were able to form nano-sized micelles in aqueous solution with folate molecule as targeting ligand, hydrophilic CS shells and hydrophobic naphthyl cores. The average diameters of 40% 3A.1 loaded Fol-NSCS nanoparticles ( $189.47 \pm 2.90$  nm) were bigger than unmodified NSC nanoparticles due to the steric structure of folic acid that was linked to succinic acid on CS structure. Both PDI value and zeta potential value of Fol-NSCS nanoparticles were similar with unmodified NSCS nanoparticles, which have narrow size distribution and optimal negative surface charge. In addition, TEM observation (Figure 10) also showed the Fol-NSCS nanomicelles dispersed in spherical shape with nano-size.



Table 6.3 The physicochemical properties of 20% initial 3A.1-loaded micelles (NSCS, OSCS and BSCS) prepared using three physical entrapment methods. Each value represents the mean  $\pm$  standard deviation from three independent experiments.

| 3A.1 to polymer (%) | NSCS            |       |                     | OSCS             |       |                     | BSCS             |       |                     |
|---------------------|-----------------|-------|---------------------|------------------|-------|---------------------|------------------|-------|---------------------|
|                     | Size (nm)       | PDI   | Zeta potential (mV) | Size (nm)        | PDI   | Zeta potential (mV) | Size (nm)        | PDI   | Zeta potential (mV) |
| 0                   | 66.2 $\pm$ 1.2  | 0.300 | (-) 30.5 $\pm$ 1.9  | 132.33 $\pm$ 6.6 | 0.131 | (-)17.6 $\pm$ 1.5   | 111.1 $\pm$ 1.05 | 0.160 | (-) 29.3 $\pm$ 0.3  |
| 5                   | 75.2 $\pm$ 1.3  | 0.164 | (-) 26.7 $\pm$ 0.9  | 150.43 $\pm$ 6.7 | 0.181 | (-) 29.2 $\pm$ 3.4  | 112.2 $\pm$ 0.67 | 0.138 | (-) 26.2 $\pm$ 0.6  |
| 10                  | 82.1 $\pm$ 3.5  | 0.185 | (-) 28.8 $\pm$ 2.3  | 161.81 $\pm$ 5.2 | 0.194 | (-) 27.0 $\pm$ 0.2  | 127.1 $\pm$ 2.32 | 0.185 | (-) 27.3 $\pm$ 1.5  |
| 20                  | 90.3 $\pm$ 2.2  | 0.255 | (-) 24.9 $\pm$ 1.1  | 165.63 $\pm$ 9.8 | 0.185 | (-) 26.6 $\pm$ 0.8  | 131.6 $\pm$ 1.05 | 0.208 | (-) 28.4 $\pm$ 0.6  |
| 40                  | 102.5 $\pm$ 0.6 | 0.162 | (-) 22.2 $\pm$ 5.2  | 170.67 $\pm$ 1.7 | 0.192 | (-) 32.0 $\pm$ 3.0  | 153.9 $\pm$ 1.20 | 0.195 | (-) 26.0 $\pm$ 0.4  |

Table 6.4 The physicochemical properties of blank and 40% initial 3A.1-loaded micelles and folate-conjugated micelles. Data represent the mean  $\pm$  standard deviation (n=3).

| Sample                        | Particle size (nm) | PDI   | Zeta potential (mV)  | Entrapment efficiency (%) | Loading capacity ( $\mu$ g/mg) |
|-------------------------------|--------------------|-------|----------------------|---------------------------|--------------------------------|
| Blank NSCS micelles           | 85.26 $\pm$ 1.21   | 0.383 | (-) 23.03 $\pm$ 1.11 | -                         | -                              |
| Blank Fol-NSCS micelles       | 343.80 $\pm$ 43.73 | 0.494 | (-) 28.50 $\pm$ 1.15 | -                         | -                              |
| 3A.1-loaded NSCS micelles     | 110.17 $\pm$ 1.13  | 0.170 | (-) 26.17 $\pm$ 1.44 | 90.84 $\pm$ 7.43          | 363.35 $\pm$ 29.74             |
| 3A.1-loaded Fol-NSCS micelles | 189.47 $\pm$ 2.90  | 0.174 | (-) 31.67 $\pm$ 0.39 | 68.44 $\pm$ 3.72          | 273.75 $\pm$ 14.91             |

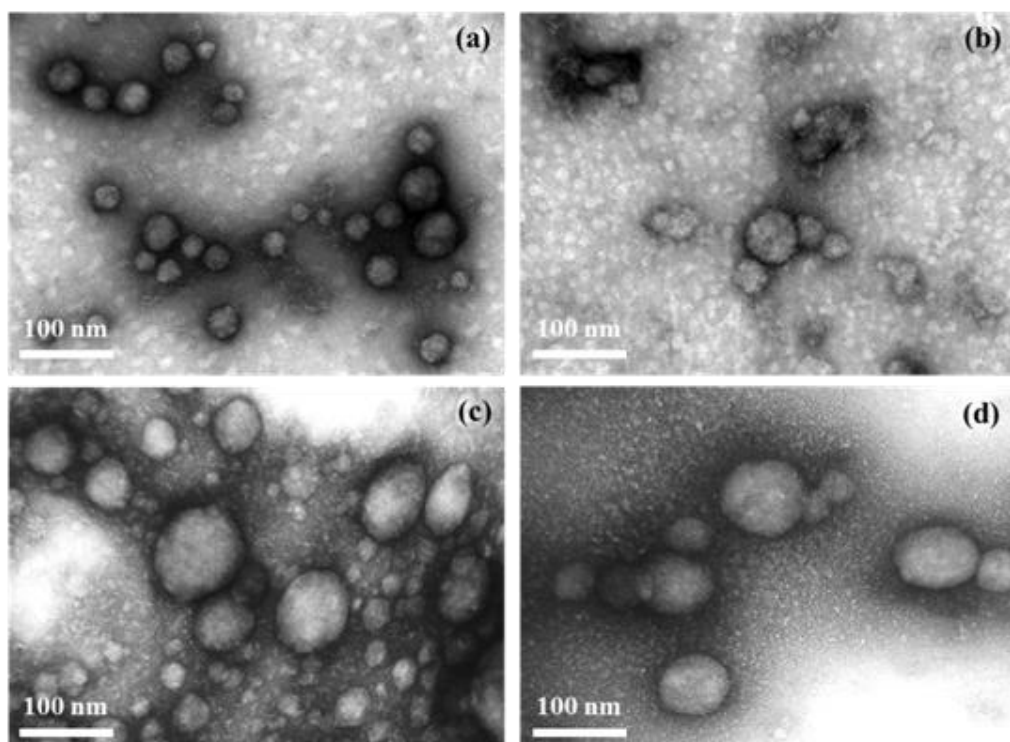


Figure 6.9 TEM images of nanoparticles prepared by dropping method: (a) blank NSCS nanoparticles, (b) 3A.1-loaded NSCS nanoparticles, (c) 3A.1-loaded OSCS nanoparticles and (d) 3A.1-loaded BSCS nanoparticles.

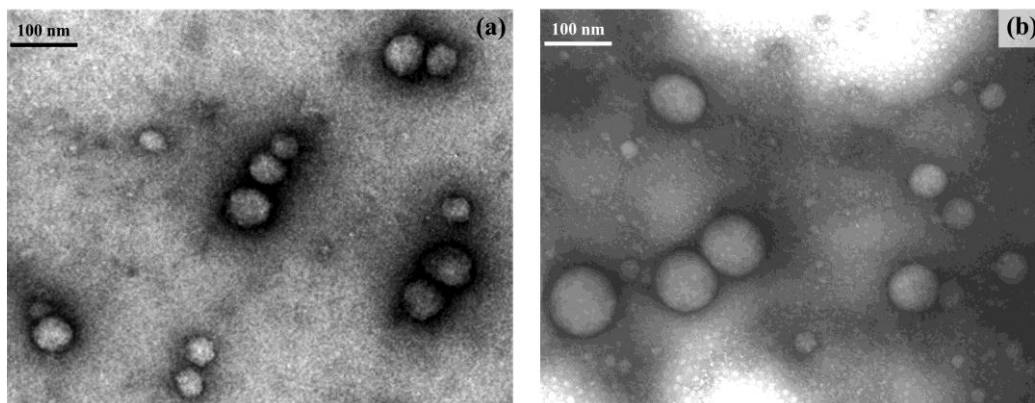


Figure 6.10 TEM images of nanoparticles prepared by dropping method: (a) 3A.1-loaded NSCS nanoparticles (b) 3A.1-loaded folate-conjugated NSCS nanoparticles.

A quantitative analysis of the drug content incorporated in the hydrophobic core of the nanoparticles was undertaken using HPLC. The effects of the initial drug amounts (5–40 wt% to polymer weight ratio) and the different grafted hydrophobic moieties (naphthyl,

octhyl, and benzyl groups) on the %EE and LC are presented in Figure 6.3. The x-axis represents the initial drug amount added to the nanoparticle formulations, and the y-axis represents the drug content in the nanoparticles in terms of %EE (Figure 11a) and LC (Figure 11b). The results revealed that the initial drug amount directly correlated with the drug loading content. An increase in the initial drug amount resulted in an increase in LC due to the hydrophobic interactions between the hydrophobic naphthyl, octhyl, and benzyl groups with the hydrophobic drug. Moreover, the NSCS nanoparticles with 40 wt% initial drug concentration demonstrated the highest value of LC compared with the other nanoparticles. These high levels of entrapped drug can be explained by the hydrophobic  $\pi$ - $\pi$  interactions between the hydrophobic moieties of the polymer and the aromatic rings of the drug. These were an important factor when entrapping the drug into the nanoparticles, therefore, the NSC nanoparticles which presented naphthyl groups (double aromatic rings) on the CS backbone created strong  $\pi$ - $\pi$  interactions which were greater than the BSC (benzyl group; single aromatic ring) and OSC (octhyl group; aliphatic hydrocarbon chain) alternatives. These results were in agreement with the previously investigated incorporation behavior of camptothecin by varying the degrees of the hydrophobicity of the nanoparticle inner cores (Opanasopit, 2004). Overall, these CS graft copolymers are beneficial when designing drug delivery systems due to their highly efficient drug encapsulation and capability of increasing drug solubility.

For drug loading content of Fol-NSCS nanoparticles, the 3A.1 hydrophobic anticancer agent was physically loaded into Fol-conjugated NSC nanoparticles by dropping method. Drug loading was performed at 40% feed weight to polymer ratio, while the results were shown in Table 6.4. Compared with unmodified NSCS nanoparticles, the conjugation of folate on NSCS nanoparticles could affect the %EE and LC. The %EE of Fol-NSCS nanoparticles were decrease to  $68.44 \pm 3.72$  % while LC were also decrease to  $273.75 \pm 14.91 \mu\text{g}/\text{mg}$ , indicating that Fol-NSC nanoparticles still had high drug loading content, probably due to good hydrophobic interaction.

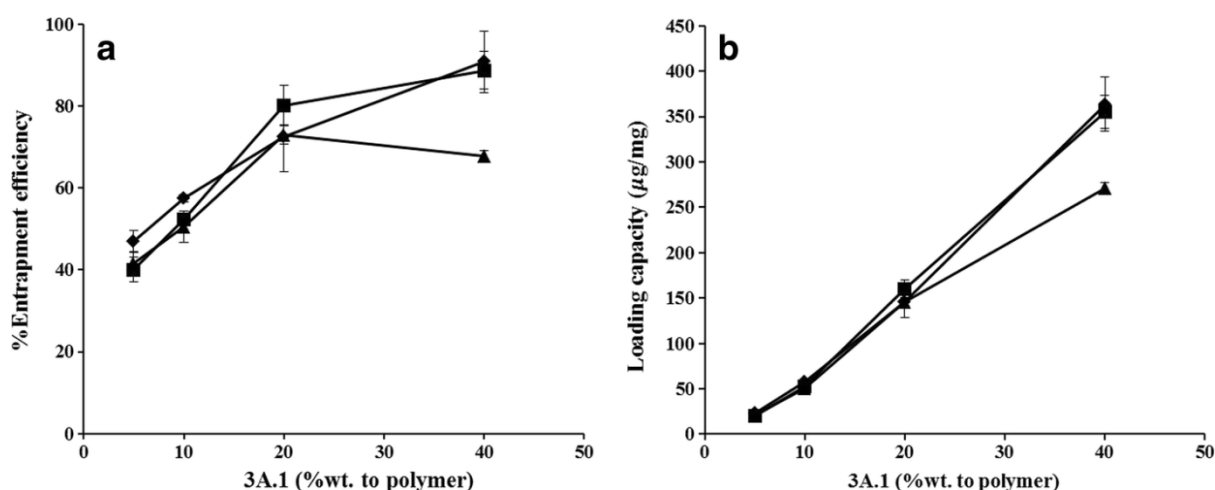


Figure 6.11 Effects of the initial drug loading ratio (5–40 wt% to polymer) on a the entrapment efficiency and b loading capacity of 3A.1-loaded nanoparticles prepared by dropping method; filled diamonds, NSCS; filled squares, OSCS; and filled triangles, BSCS. Data are expressed as the mean  $\pm$  standard deviation (n=3)

## 2. *In vitro* release

The *in vitro* release behaviors of the 3A.1 from the 3A.1-loaded nanoparticles (NSCS, OSCS, and BSCS) with 40 wt% initial drug added were examined at 37 °C in three stages with different pH imitated the condition in GI tract. A plot of the cumulative drug release from the 3A.1 free drug and 3A.1-loaded nanoparticles as a function of time is shown in Figure 12. In SGF (pH 1.2), there was no apparent amount of drug released from any 3A.1-loaded nanoparticles. When the release medium was changed to SIF (pH 6.8) for 6 h, the 3A.1 was considerably released from the 3A.1-loaded nanoparticles which significant difference in the release amounts was observed from the nanoparticles prepared from different CS derivatives, indicating that the release rate depended on the hydrophobic functionalities of the polymer. The percentages of drug release from these nanoparticles after 4 h were as follows: NSCS ( $84.10 \pm 2.43\%$ ) > BSCS ( $63.41 \pm 2.95\%$ ) > OSCS ( $52.03 \pm 3.33\%$ ). On the other hand, none of the 3A.1 free drug could be released through the simulated GI tract (SGF, SIF and SCF). This might be due to the poor aqueous solubility of 3A.1 in all types and pH values of release medium ( $< 1 \mu\text{g/mL}$ ).

Generally, the  $\text{pK}_a$  value of succinic acid is approximately 4.21 at 25 °C. In the gastric fluid (pH 1.2), the succinyl groups grafted onto the CS polymer were unionized, resulting in a tight arrangement of the polymer core-shell structure and protection of 3A.1 from the acidic environment. When the nanoparticles were exposed to the alkaline environment in SIF (pH 6.8) and SCF (pH 7.4), the succinyl groups grafted onto the CS polymer showed high ionization and created a negative charge, leading to lose compaction of the nanoparticle structures and promotion of the release of 3A.1 from the inner cores of the nanoparticles. In addition, the different hydrophobic moieties influenced the drug release in SIF and SCF. The extent of drug release reduced as follows: NSC nanoparticles > BSC nanoparticles > OSC nanoparticles ( $p < 0.05$ ). These results suggested that not only the  $\pi$ - $\pi$  interactions restrict water penetration but also other factors such as hydrophobicity, mobility/rigidity, hydrogen bonding, and steric factors, influencing the difference in drug release (Yokoyama, 2014). Hence, many drug release studies have inferred that these pH-sensitive nanoparticles could enhance the solubility of 3A.1 in SIF and SCF mediums, and different inner cores could influence the degrees of drug release. OSCS could retain 3A.1 within the inner core of the nanoparticles greater than the nanoparticles prepared from NSCS and BSCS. Therefore, it can

be assumed that the 3A.1-loaded nanoparticles (NSCS, OSCS, and BSCS) exhibited delayed release profiles useful for improving the bioavailability of 3A.1 for oral intestine/colon drug delivery.

The release behavior of 3A.1 from Fol-NSCS nanoparticles were investigated with the same condition compared with unmodified NSCS nanoparticles (Figure 13). This folate-conjugated NSCS still possessed pH-sensitive property with specific drug release at intestinal/colon sites. At time intervals (4–12 h), the Fol-NSCS nanoparticles showed an obviously lower release of 3A.1 than the unmodified NSCS nanoparticles. For example, about 52% and 80% of loaded 3A.1 could be release from Fol-NSCS nanoparticles and NSCS nanoparticles, respectively after 12 h. The lower content of drug release might be explained that conjugation of folic acid on NSCS polymer could enhance the strong hydrophobic interaction between the hydrophobic domain of polymer and drug. Therefore, Fol-NSCS nanoparticles for delayed release is helpful for site-specific release and decreasing drug leakage form nanoparticles before drug-loaded nanoparticles reach to target cells.

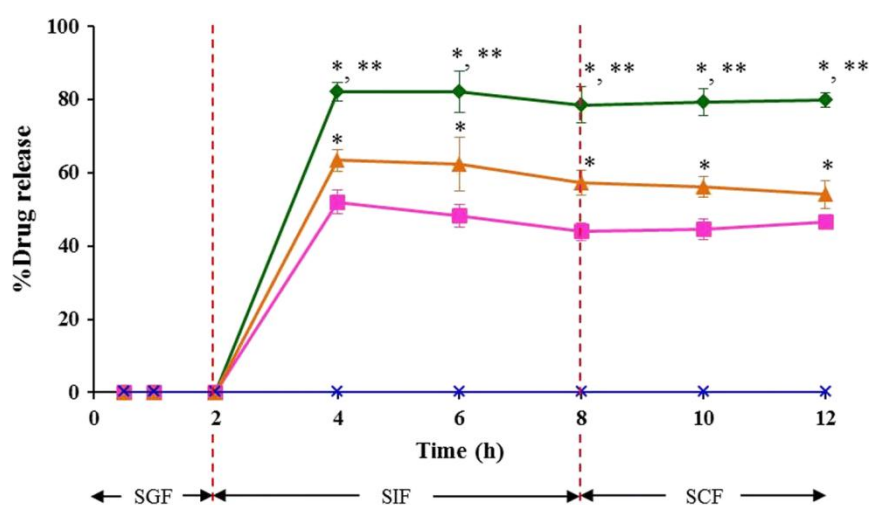


Figure 6.12 Release profiles of free 3A.1 and 3A.1-loaded nanoparticles (error marks) prepared by dropping method; filled diamonds, NSCS; filled squares, OSCS; and filled triangles, BSCS in simulated gastric fluid (SGF; pH 1.2, 0–2 h), simulated intestinal fluid (SIF; pH 6.8, 2–8 h) followed by simulated colonic fluid (SCF; pH 7.4, 8–12 h) ( $n = 3$ ).

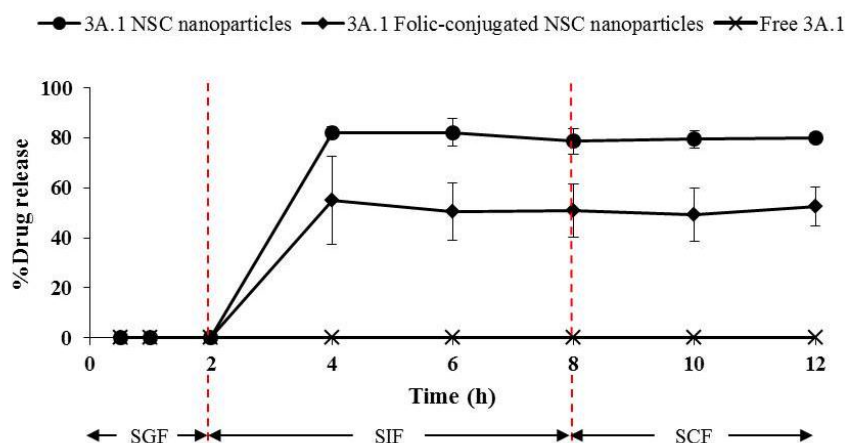


Figure 6.13 Release profiles of free 3A.1, 3A.1-loaded NSC nanoparticles and 3A.1-loaded Fol-NSC nanoparticles in SGF, SIF followed by SCF at 37 °C. Data represent the average values from triplicate experiments.

### 3. *In vitro* anticancer activity

The anticancer activity of 3A.1 free drug and 3A.1-loaded NSCS, OSCS and BSCS nanoparticles against human colorectal cancer (HT-29) cells was assessed using an MTT assay. The finding indicated that the %cell viability was dose-dependent. The half-maximal inhibitory concentration ( $IC_{50}$ ) was determined from the %cell viability-drug concentration relationship. The  $IC_{50}$  values of the 3A.1-loaded NSCS, OSCS and BSCS nanoparticles were  $1.024 \pm 0.071$ ,  $0.693 \pm 0.099$ , and  $0.538 \pm 0.025$   $\mu\text{g/mL}$ , respectively, which are significantly lower than that of the free drug ( $3.816 \pm 0.376$   $\mu\text{g/mL}$ ;  $p < 0.05$ ) (Figure 14). Cell viability of the blank nanoparticles remained at more than 80% even at high polymer concentrations up to 500  $\mu\text{g/mL}$ . No significant differences in the cytotoxicity were noticed between the blank nanoparticles prepared from different CS derivatives. These results clearly indicated that all the blank nanoparticles demonstrated minimal cytotoxicity in HT-29 cells, and the cytotoxicity was due to the presence of 3A.1.

To compare antitumor activities of non-conjugated and folated-conjugated NSC nanoparticles against HT-29 cell, which over-expressed folate receptors on cell surface (Bahrami, 2015), it is interesting that Fol-NSC nanoparticles exhibited obviously higher cytotoxicity than the non-conjugated nanoparticles. This lower  $IC_{50}$  value ( $0.412 \pm 0.048$   $\mu\text{g/mL}$ ) that was improved sharply by 2.5-fold compared to non-conjugated nanoparticles was attributed to the enhance cellular drug uptake via folate-receptor mediated endocytosis. The over-expressed folatereceptor on HT-29 cell facilitate the effective recognition of the folate ligand on surface of nanoparticles, leading to an increased cellular cytotoxicity. Therefore, conjugation of folic acid on nanoparticles has benefit to more deliver drug into cancer cell through active tumor targeting.

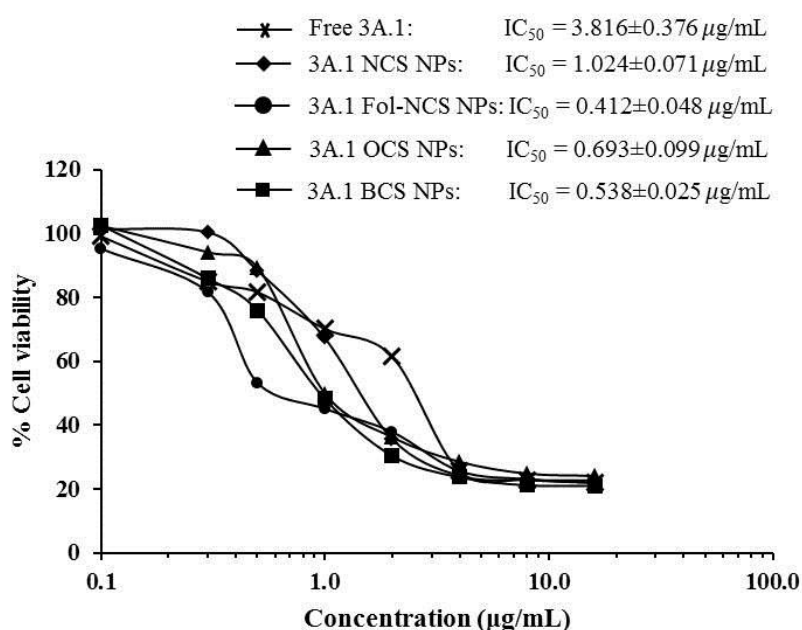


Figure 6.14 The percentage of cell viability (y-axis) of HT-29 cells after being exposed to various concentration drug-loaded nanoparticles or free 3A.1 (x-axis) and the IC<sub>50</sub> values

#### 4. Induction of cell apoptosis

To confirm that this drug can induce apoptosis in HT-29 colorectal cancer cells, a co-staining assay (annexin V-FITC and PI) was used to quantitatively analyze the degree of cell death, especially cell apoptosis, by flow cytometry analysis (Figure 15a). In this part, the cell apoptosis rate was calculated by the inclusion of the percentage of early apoptosis (Q4; annexin V-FITC+/PI-) and the percentage of late apoptosis (Q2; annexin V-FITC+/PI+) (Figure 15b). After incubation with the free drug and different formulations of 3A.1-loaded nanoparticles containing equivalent drug concentrations (at IC<sub>50</sub> of the free drug) for 24 h, the cell apoptosis rates of the drug-loaded NSCS, OSCS, and BSCS nanoparticles were  $45.20 \pm 3.27$ ,  $41.60 \pm 2.45$ , and  $40.50 \pm 2.04\%$ , respectively, which were significantly greater than those of the free drug ( $33.10 \pm 2.45\%$ ) and Triton X ( $9.50 \pm 1.63\%$ ). This degree of cell apoptosis is in concordance with the results of the *in vitro* anticancer activity. In addition, the percentage of cell necrosis of the cells treated with the drug-loaded nanoparticles was lower than that treated with free drug at this stage. This might be a beneficial property of the nanoparticles for growth suppression of cancer cells. Thus, 3A.1-loaded NSC, OSC, and BSC nanoparticles induced intense apoptosis in HT-29 colorectal cancer cells. Moreover, it should be noticed that Fol-NSCS nanoparticles had significantly higher cell apoptosis than unmodified NSCS nanoparticles due to the increase of drug into cell internalization via

receptor-mediated endocytosis. This result was in accordance with the *in vitro* anticancer activity.

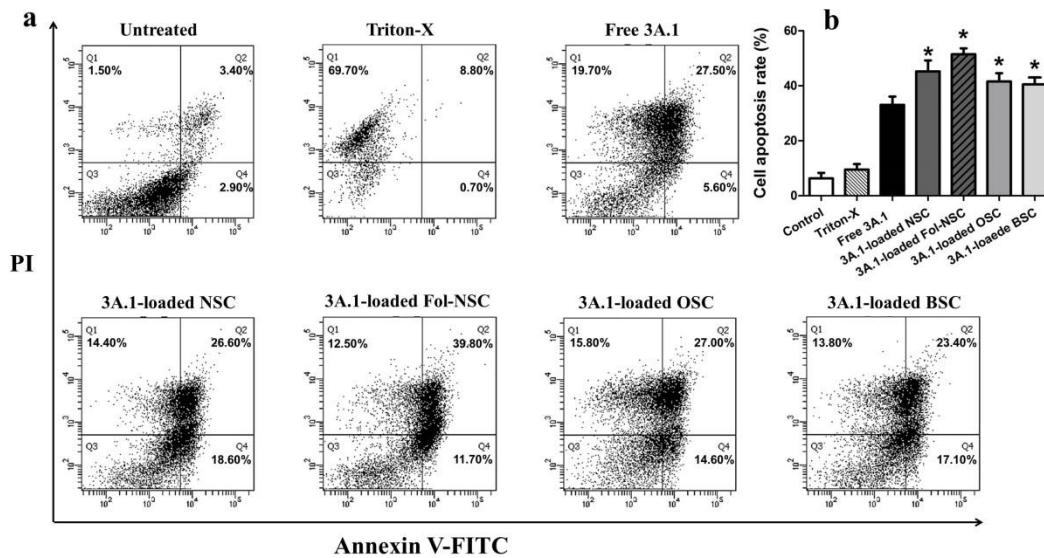


Figure 6.15 3A.1-loaded nanoparticles induced HT-29 cancer cell apoptosis examined by flow cytometry; **a** the cells treated with 3A.1-loaded nanoparticles, free 3A.1 (at the equivalent concentration), and Triton X for 24 h which are presented in four quadrants (Q) with dot plot. The number of the late apoptotic cells, early apoptotic cells, and living cells are displayed on Q2, Q4, and Q3, respectively, **b** percentage of cell apoptosis rate of the cells after treated with 3A.1-loaded nanoparticles, free 3A.1, and Triton X. Asterisk, statistically significant difference from free 3A.1 group.

## 5. *In vitro* antimigratory activity

The scratch wound migration assay was selected to assess the antimigratory effect of free 3A.1 and 3A.1-loaded nanoparticles on human oral cancer (HN-22) cells because of simple and cost-effective procedure. The wound gap images of cells after being treated with the 3A.1-loaded nanoparticles and free drug are shown in Figure 16a. After 24-h treatment, the scratch areas of untreated cell monolayer were almost fully closed, whereas both free 3A.1 and 3A.1-loaded nanoparticles tended to reduce the cell motility. In addition, the cell migration rate and %wound closure are illustrated in Figure 16b, c. respectively. The results found that free 3A.1 has significant slower cell migration rate than that of untreated cells and it also decrease %wound closure compared with untreated cell, indicating that 3A.1 could suppress cell motility. Moreover, both cell migration rate and %wound closure of 3A.1-loaded nanoparticles were not significant different to the free drug at the same concentration. From these results, it could be concluded that free 3A.1 might be one of the antimigration compounds and loading 3A.1 into the nanoparticles did



not affect its antimigratory activity. Therefore, 3A.1-loaded nanoparticles could be a potential carrier to reduce metastasis of oral cancer.

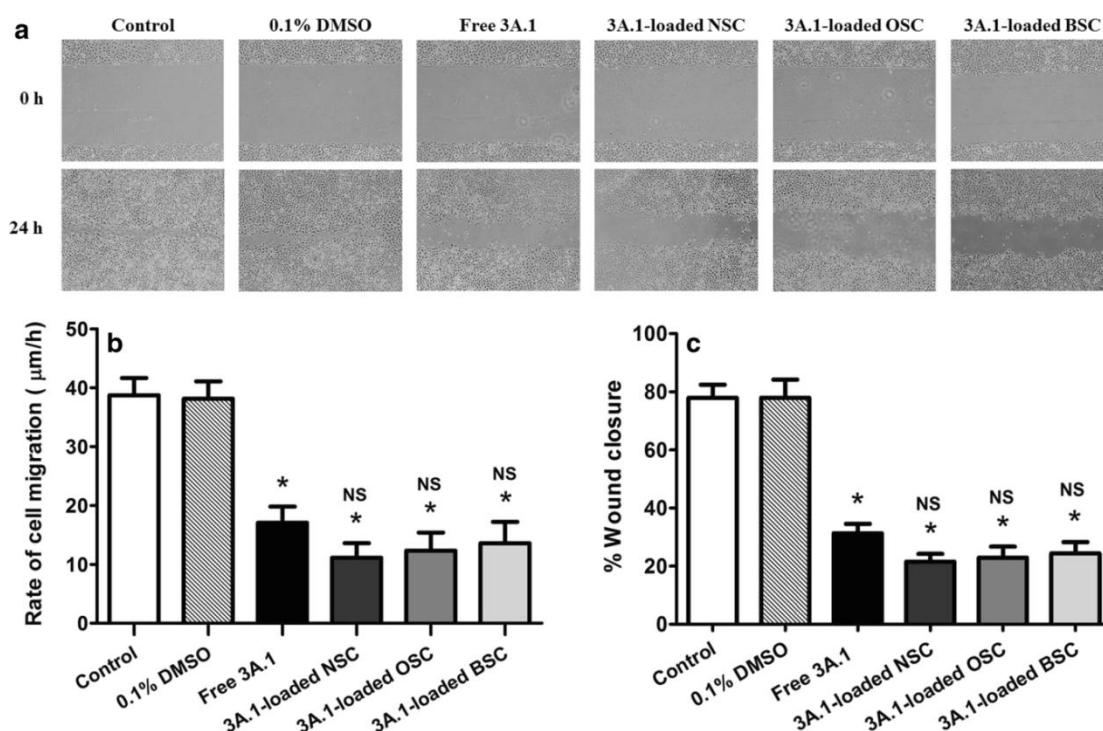


Figure 6.16 The antimigration of free 3A.1 and 3A.1-loaded nanoparticles prepared by dropping method on HN-22 cells presented as the value of **a** wound images taken by a microscope ( $\times 40$  magnification) at 0 and 24 h **b** cell migration rate and **c** %wound closure after 24-h treatment exposure. Asterisk, statistically significant difference from untreated control group; NS, not significantly different from free 3A.1 group.

## 6. Short-Term Stability

One of the main stability concerns regarding polymeric nanoparticles is particle aggregation. The physical stability of 3A.1-loaded nanoparticles (NSCS, OSCS, and BSCS) were examined under two conditions, an accelerated environment ( $25 \pm 2^\circ\text{C}$ ,  $60 \pm 5\% \text{RH}$ ) and a long-term environment ( $5 \pm 3^\circ\text{C}$ ,  $60 \pm 5\% \text{RH}$ ), for 180 days. The physical stability of the 3A.1-loaded nanoparticles was studied and the data, including particle size, PDI and percentage of drug remaining, is presented in Figure 17. The results indicated that the particle sizes of the nanoparticles stored at  $5 \pm 3^\circ\text{C}$ ,  $60 \pm 5\% \text{RH}$  were smaller than those stored at  $25 \pm 2^\circ\text{C}$ ,  $60 \pm 5\% \text{RH}$  over 180 days, the surface charges of the nanoparticles were not altered which a highly negative zeta potential value of approximately  $-30 \text{ mV}$

was remained. Interestingly, the mean particle size of these nanoparticles in both conditions still remained lower than 200 nm without nanoparticle aggregation. These results might suggest that the high negative charge of these nanoparticles helped to stabilize the nanoparticles in the aqueous solution through electrostatic repulsion. After storage at the long-term condition for 6 months, the total contents of 3A.1 in the NSCS, OSCS, and BSCS nanoparticles were 93.65, 87.22, and 85.67%, respectively. However, under the accelerated condition the drug content was found to be 81.64, 68.27, and 53.62%, respectively. The 3A.1-loaded nanoparticles kept under accelerated condition exhibited faster degradation than those stored under long-term condition. The 3A.1-loaded BSCS nanoparticles demonstrated poor stability, whereas the NSC nanoparticles showed better stability with the high values of %drug remaining after 6 months when stored under long-term condition. These results explained that naphthyl groups had stronger hydrophobic interaction between the hydrophobic groups of polymer and the hydrophobic drug than the benzyl groups do result in higher drug protection capability and decrease in drug leakage from the NSCS nanoparticles. Our data suggested that the nanoparticles should be stored in a refrigerator to maintain the particle size, zeta-potential, and the amount of drug remaining in the nanoparticles.

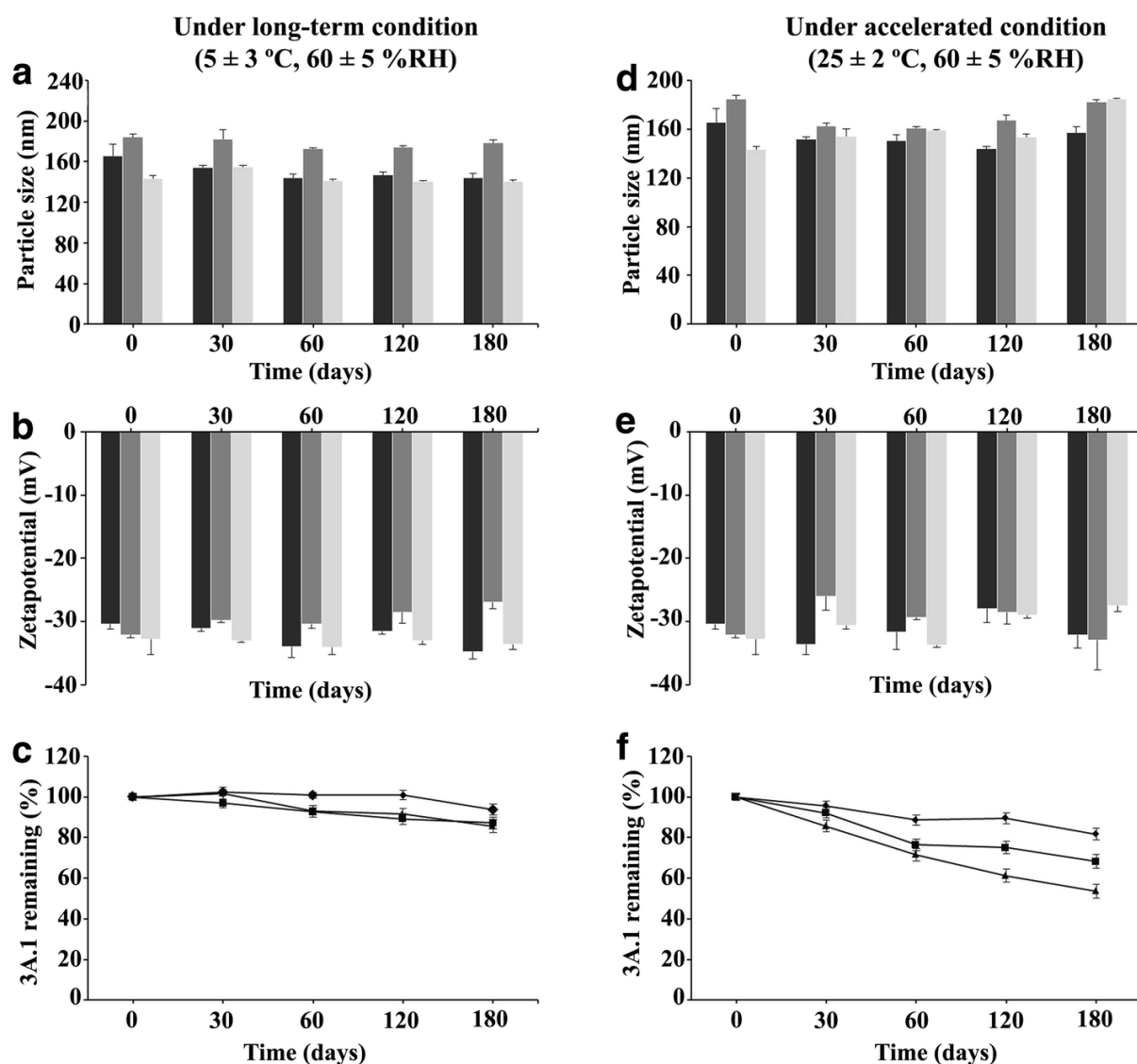


Figure 6.17 The short-term stability of 3A.1-loaded nanoparticles stored under long-term condition (left) compared with under accelerated condition (right) for 6 months; **a, d** particle size of NSCS nanoparticles (black-filled bars), OSCS nanoparticles (dark-gray-filled bars), and BSCS nanoparticles (light-gray-filled bars); **b, e** zeta-potential of NSCS nanoparticles (black-filled bars), OSCS nanoparticles (dark-gray-filled bars), and BSCS nanoparticles (light-gray-filled bars); **c, f** the amount of 3A.1 remaining in NSCS (filled diamonds), OSCS (filled squares), and BSCS (filled triangles) nanoparticles. All data are presented as the mean  $\pm$  standard deviation ( $n = 3$ )

#### IV. CONCLUSION

In the present study, pH sensitive amphiphilic chitosan derivatives, i.e. N-naphthyl-N,O-succinyl chitosan (NSCS), N-octyl-N,O-succinyl chitosan (OSCS) and N-benzyl-N,O-succinyl chitosan (BSCS) were synthesized and prepared as polymeric micelles to improve the solubility of poorly water soluble drug and to control the drug release. Semi-synthetic andrographolide (3A.1) was selected as a hydrophobic drug for colorectal cancer treatment. The influence of the physical entrapment methods, type of amphiphilic chitosan derivatives, amount of drugs on entrapment efficiency, loading capacity, particle size and stability of drugs-loaded polymeric micelles were evaluated. In addition, the *in vitro* cytotoxicity and their release behavior were investigated. The results showed that 3A.1 was successfully incorporated into self-assembled polymeric nanoparticles based on amphiphilic chitosan derivatives (NSCS, OSCS, and BSCS). These nanoparticles were formed by self-assembly in an aqueous solution and their hydrophobic part helped to solubilize the hydrophobic drug within the nanoparticles. The 3A.1-loaded NSCS nanoparticles with 40 wt% drug added prepared by a dropping method revealed the highest drug loading capacity, favorable properties (spherical shape, nanometer-size, negative surface charge) and good physical stability in a refrigerator. The release behavior of the 3A.1 depended on the pH of the medium, and high drug amounts were specifically released at intestinal/colon sites. Importantly, the 3A.1-loaded nanoparticles clearly showed great anticancer activity against colorectal cancer HT-29 cells and potent apoptotic induction, compared with free 3A.1. Moreover, the 3A.1-loaded nanoparticles also inhibited cell migration of oral cancer HN-22 cells. Therefore, these 3A.1-loaded pH-sensitive polymeric nanoparticles could be promising nanocarriers of the hydrophobic anticancer agent 3A.1 for oral intestine/colon drug delivery. Furthermore, compared with the non-conjugated polymers, the folate-conjugated NSCS polymers showed much more anticancer activity and highly potent apoptotic induction, suggesting that the folate-conjugated were active-targeting drug nanocarriers for colon tumor therapy via folate mediation.

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กิจกรรมที่ได้วางแผนและได้ดำเนินการตลอดโครงการ

| Objectives                                                                                                                                                                                                            | Output                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. สกัดแยกสารจากพืช และ marine microbes ดัดแปลงโครงสร้าง และสังเคราะห์สาร เพื่อเป็น compound library ใช้ในการนำไปทดสอบฤทธิ์                                                                                           | 1. ได้สกัดแยกสาร ดัดแปลงโครงสร้างและสังเคราะห์สารไว้ใช้เป็น compound library 725 สารจากพืชไทย และจาก marine microbes และพืชของจีนประมาณ 300 ตัว ให้โครงการย่อยทำการทดสอบฤทธิ์ชีวภาพต่างๆ                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| 2. ทดสอบฤทธิ์ และกลไกการออกฤทธิ์ต้านมะเร็ง (anti-cancer effects and mechanisms)                                                                                                                                       | 1. พบสารมีฤทธิ์ยับยั้งการแบ่งตัว เพิ่มจำนวนของเซลล์ชนิดต่างๆ<br>2. ได้ศึกษากลไกการออกฤทธิ์เชิงลึกของสารบางตัวในการต้านมะเร็ง กระเพาะ ลำไส้ใหญ่ และทวารหนัก<br>3. นำส่งสารที่ทราบกลไกการออกฤทธิ์เชิงลึกบางตัวให้กลุ่มวิจัยพัฒนาระบบส่งยาดำเนินการต่อไป สารที่ไม่เป็นพิษต่อเซลล์ ได้นำไปศึกษาฤทธิ์อื่นๆ                                                                                                                                                                                                                                                                                                                              |
| 3. ศึกษาค้นหาฤทธิ์ต้านเบาหวาน (Anti-diabetes type 2) และฤทธิ์ลดความเป็นพิษของยาต้านมะเร็ง cisplatin                                                                                                                   | 1. ค้นพบสารที่มีฤทธิ์ยับยั้งการขนส่งกลูโคสในเซลล์ไขมันมนุษย์<br>- สารบริสุทธิ์จากนักวิจัยไทย จำนวน 7 ตัว<br>- สารบริสุทธิ์จากนักวิจัยจีน จำนวน 4 ตัว<br>2. สารสกัดจากนักวิจัยไทยจำนวน 2 สารสกัด มีผลดีเด่นในการลดระดับน้ำตาลในเลือดของหนูเบาหวาน<br>3. ค้นพบสารบริสุทธิ์จากนักวิจัยไทย มีฤทธิ์ลดความเป็นพิษต่อเซลล์ไตของยาต้านมะเร็ง cisplatin จำนวน 1 ตัว                                                                                                                                                                                                                                                                         |
| 4. ศึกษาฤทธิ์ของสารต่อการสร้าง และการเมทาโบลิซึมของเนื้อเยื่อไขมันในสัตว์ทดลอง และศึกษาฤทธิ์ของยาต่อการจำแนกตนเองของเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกมนุษย์ (human bone marrow-derived mesenchymal stem cells, MSCs) | 1. พบฤทธิ์ดีเด่นของสาร DPHD ต่อการ metabolism ในเนื้อเยื่อไขมัน และการออกฤทธิ์ป้องกันพยาธิสภาพในสัตว์ทดลองที่ดัดรังไข่เป็นเวลานาน ซึ่งเป็นแบบจำลองของสตรีวัยหมดประจำเดือน มีภาวะผิดปกติของไขมันในเลือด<br>2. พบกลไกการทำงานของสาร และสารสกัดจากพืช มีศักยภาพดีเด่นในการลดการสร้างไขมันและเร่งการใช้ไขมัน ลดการสะสมไขมันของร่างกาย กระตุ้นการหลั่งอะดิโปไคน์ ช่วยเพิ่มความไวของเนื้อเยื่อในการตอบสนองต่อฮอร์โมน insulin เพื่อลดภาวะดื้อต่อฮอร์โมน insulin<br>3. พบว่าสาร DPHD มีฤทธิ์ยับยั้งการจำแนกตนเองของเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกมนุษย์ไปเป็นเซลล์ไขมัน โดยยับยั้งการแสดงออกของยีนที่จำเป็นต่อกระบวนการสร้างเซลล์ไขมัน |
| 5 ตรวจสอบฤทธิ์ของสารบริสุทธิ์ที่แยกได้จากพืชต่อการป้องกัน การเสื่อมของเซลล์ประสาท                                                                                                                                     | พบฤทธิ์ของสารอนุพันธ์ของ lipoic acid ต่อการป้องกันการเสื่อมและการตายของเซลล์ประสาทจากอนุมูลอิสระและปกป้อง microtubules และ actin filament ใน growth cone จากเซลล์ประสาทไม่ให้ถูกทำลายด้วย oxidative stress                                                                                                                                                                                                                                                                                                                                                                                                                         |



| Objectives                         | Output                                                                                                                                                                                                                                                                                                                                                                                            |
|------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 6. พัฒนาระบบนำส่งยาในรูปแบบลิโพโซม | <ul style="list-style-type: none"> <li>- การเตรียมตำรับลิโพโซม</li> <li>- การตรวจสอบสมบัติทางเคมีฟิสิกส์ของตำรับลิโพโซม</li> <li>- การวิเคราะห์หาปริมาณยาที่กักเก็บในตำรับลิโพโซม</li> <li>- การศึกษาการปลดปล่อยยาในหลอดทดลอง (<i>In vitro</i> drug release)</li> <li>- ได้พัฒนาระบบส่งยาของสารต้านมะเร็งลำไส้ที่ศึกษากลไกการทำงานแล้ว ให้ไปยังเป้าหมาย ลดความเป็นพิษต่อเซลล์ข้างเคียง</li> </ul> |

### ผลผลิตที่ได้รับจากงานวิจัย (Output-Outcome) (IRN580W0004)

#### ก. ผลงานวิจัยได้รับการตีพิมพ์ในวารสารระดับนานาชาติ จำนวน 37 ชิ้น (โครงการเครือข่ายวิจัยการค้นคว้าและพัฒนาจากสารธรรมชาติ)

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- (2) Teeratas Kansom, Rungnapha Saeeng, Pawinee Piyachaturawat, Theerasak Rojanarata, Tanasait Ngawhirunpat and Praneet Opanasopit. "Preparation and Characterization of Semi-synthetic andrographolide analogue-loaded Liposomes for Cancer Therapy" Seoul International Conference on Engineering and Applied Science (SICEAS), 7-9 Feb 2017, Marriott Seoul Times Square, Seoul, South Korea.
- (3) Pawinee Piyachaturawat, Antiproliferative and Anticancer Mechanisms of Styryl Lactones through Inhibition of Wnt/ $\beta$ -catenin Signaling. The 6th China-Thailand Joint Workshop on Natural Products and Drug Discovery 16 – 20 October, 2016 Aonang Cliff Beach Resort, Krabi, Thailand (Oral presentation)
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- (5) Praneet Opanasopit, Water Soluble Chitosan Derivatives for Applications as Drug/Gene Carriers by The 6th China-Thailand Joint Workshop on Natural Products and Drug Discovery 16 – 20 October, 2016 Aonang Cliff Beach Resort, Krabi, Thailand (Oral presentation)
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- (7) Arthit Chairoungdua, Induction of Cholangiocarcinoma Cell Death through Topoisomerase II $\alpha$  Inhibition by Altholactone and Halogenated Benzoate Derivatives, The 6th China-Thailand Joint Workshop on Natural Products and Drug Discovery 16 – 20 October, 2016 Aonang Cliff Beach Resort, Krabi, Thailand (Poster presentation)

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- (9) Witchuda Saengsawang, *Phyllanthus taxodiifolius* Beille extract disrupted cytoskeletal dynamics of cancer cells. The 6th China-Thailand Joint Workshop on Natural Products and Drug Discovery 16 – 20 October, 2016 Aonang Cliff Beach Resort, Krabi, Thailand (Poster presentation)
  
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- (11) Teeratas Kansom, Warayuth Sajomsang, Rungnapha Saeeng, Pawinee Piyachaturawat, and Praneet Opanasopit. Preparation and characterization of semi-synthetic andrographolide analogue-loaded amphiphilic chitosans micelles for cancer therapy. FAPA Congress 2016. November 9-13, 2016. BITEC Bangna, Bangkok, Thailand.
  
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- (16) Teeratas Kansom, Rungnapha Saeeng, Tanasait ngawhirunpat, Theerasak Rojanarata, Prasopchai Tonglairoom, Praneet Opanasopit, and Purin Charoensuksai. Effect of semi-synthetic andrographolide analogue-loaded polymeric micelles on HN22 cell migration. The 10<sup>th</sup> Walailak Research National Conference, 27-28 March 2018, Walailak University Thailand. (Poster presentation)
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- (20) Nareerat Sujarit et al., Modulating effect of Wan-Chak motluk in adipose tissues of ovariectomized (OVX) rats. The 45<sup>th</sup> Physiology Society of Thailand Annual Meeting 2017, 6-8 December 2017, Khonkaen University Thailand

**นักศึกษาระดับปริญญาเอกร่วมโครงการด้วยทุนของ**

☒ โครงการ IRN จำนวน 2 ทุน จบการศึกษาได้แก่

1. นายเกียรติดำรงค์ จันทรพิพัฒน์กุล ภายใต้การดูแลของ รศ.ดร.อาทิตย์ ไชยร้องเตื้อ
2. นางสาวเพ็ญใจ ทองนวลจันทร์ ภายใต้การดูแลของ รศ.ดร. สันมหาส สุตวิสัย

**☑ โครงการอื่นๆที่ร่วมโครงการลงทะเบียนการศึกษา 9 คน จบการศึกษาได้แก่**

**ก.. โครงการ คปก.**

- นักศึกษา คปก. ชื่อ น.ส.นิตยา บุญหมื่น และ น.ส. สมฤดี เรียบร้อย หลักสูตรสรีรวิทยา
- นักศึกษา คปก. ชื่อ น.ส.ศรัญญา กิจดำรงธรรม นักศึกษาหลักสูตรพิษวิทยา
- นักศึกษา คปก. ชื่อ นางสาวศิริมา สุตวิสัย นักศึกษาหลักสูตรเทคโนโลยีเภสัชกรรม
- นักศึกษา คปก. ชื่อ นางสาวศิริมา สุตวิสัย นักศึกษาหลักสูตรเทคโนโลยีเภสัชกรรม
- นักศึกษาคปก. ชื่อ นายธีรทัศน์ กันโสม นักศึกษาหลักสูตรเทคโนโลยีเภสัชกรรม

**ข. โครงการพัฒนากำลังคนด้านวิทยาศาสตร์**

1. นางสาวนริรัตน์ สุจริต นักศึกษาปริญญาเอก หลักสูตรพิษวิทยา
2. นางสาวขวัญชนก อุปการะ นักศึกษาปริญญาเอก หลักสูตรพิษวิทยา
3. นายเอกพจน์ คงกล้า นักศึกษาปริญญาเอก หลักสูตรสรีรวิทยา

**ค. โครงการศรีตรังทอง**

1. นายจาตุรนต์ ขวัญทองดี นักศึกษาปริญญาเอก หลักสูตรสรีรวิทยา

**สรุปผลการดำเนินงานของโครงการโดยย่อ (หากพบอุปสรรคในการดำเนินงาน กรุณาระบุว่ามีอะไรบ้าง และได้แก้ไขอย่างไร)**

การดำเนินงานของโครงการเป็นไปตามที่ได้วางแผนไว้ ไม่มีอุปสรรคใดๆ โดยได้มีความร่วมมืออย่างใกล้ชิดกับนักวิจัยในโครงการทั้งในสถาบันและต่างสถาบันในประเทศ และได้มีความร่วมมือกับนักวิจัยในต่างประเทศ ทั้งในสาธารณรัฐประชาชนจีน และประเทศสหรัฐอเมริกา ความร่วมมือในด้านงานวิจัยเป็นการทำงานที่เสริมกัน ความร่วมมือกับนักวิจัยจีนที่เป็นนักเคมีเป็นหลัก ทีมนักวิจัยไทยได้ทำการทดสอบฤทธิ์ทางเภสัชวิทยาและวิจัยต่อยอดสารจีน ได้พบฤทธิ์ที่น่าสนใจของสารที่พัฒนาโดยนักเคมีชาวจีนหลายชนิด ผลงานดังกล่าวได้รับการเผยแพร่ทั้งในรูปแบบของการตีพิมพ์ในวารสารวิชาการนานาชาติ และการนำเสนอผลงานในที่ประชุมวิชาการ นอกจากนี้นักวิจัยหลักแต่ละท่านของโครงการ ยังมีความร่วมมืออยู่แล้วกับนักวิจัยในประเทศสหรัฐอเมริกา ได้เข้ามามีส่วนร่วมในโครงการ ได้มาเยี่ยมเยียน ได้สร้างความร่วมมือทั้งการวิจัยให้เข้มแข็งและพัฒนาศักยภาพ ได้ส่งนักศึกษาและอาจารย์ไปปฏิบัติงานวิจัยในห้องปฏิบัติการในต่างประเทศ ระยะสั้นเพื่อต่อยอดงานวิจัยที่ใช้เครื่องมือสมัยใหม่ ดังที่ได้ระบุไว้ในรายละเอียดในส่วนที่ 1

โครงการได้มีการจัดกิจกรรมเพื่อพัฒนาเครือข่ายอย่างสม่ำเสมอต่อเนื่อง โดยได้จัดให้มีการประชุมหารือเกี่ยวกับงานวิจัยร่วมกับนักวิจัยในโครงการทั้งในสถาบันและต่างสถาบันเป็นประจำ และมีการจัดสัมมนาวิชาการเพื่อให้นักวิจัยและนักศึกษาในกลุ่มวิจัยนำเสนอผลงาน แลกเปลี่ยนความรู้ และหารือเกี่ยวกับความร่วมมือในงานวิจัย อีกทั้งยังมีการจัดสัมมนาพิเศษให้แก่ักวิจัยในทีมวิจัยรวมทั้งผู้ที่สนใจทั่วไป โดยได้เชิญนักวิจัยในเครือข่ายที่ได้เดินทางมาเจรจาธุรกิจเป็นวิทยากรให้สัมมนาพิเศษ วิทยากรที่มาล้วนเป็นผู้เชี่ยวชาญในสาขาของตนเองทั้งจากประเทศจีน และประเทศสหรัฐอเมริกา นอกจากนี้คณะวิจัยจีนได้เดินทางมาประเทศไทย และนักวิจัยไทยได้มีโอกาสเดินทางไปประเทศจีน เพื่อประชุมปรึกษาหารือวางแผนงานวิจัยร่วมกัน และทำความรู้จักกับสถาบัน กลุ่มสมาชิก และนำเสนอความก้าวหน้าของงานวิจัยในโครงการ



การสร้างเครือข่ายของกลุ่มนักวิจัยในประเทศของโครงการ มีแนวคิดที่จะพัฒนาสารที่ได้ไปเป็นผลิตภัณฑ์ให้ผู้บริโภคในอนาคต โดยดำเนินการวิจัยในแนวทางของ Modern drug discovery and development เป็นเครือข่ายของผู้วิจัยที่มีความเชี่ยวชาญแตกต่างกัน รับช่วงการทำงานต่อเนื่องกัน โดยในโครงการนี้ได้ศึกษาวิจัยพัฒนาสารที่มีประสิทธิภาพดีเด่นเป็นยาต้านมะเร็ง โดยนักเคมีได้ปรับปรุงโครงสร้างสาร จนทดสอบฤทธิ์ได้ว่าดีเด่น ได้ศึกษากลไกการออกฤทธิ์ต่อเป้าหมายจำเพาะในเซลล์มะเร็ง ได้พัฒนาระบบนำส่งยาไปยังเป้าหมาย นับว่ามี readiness ทาง technology ที่ใกล้จะสมบูรณ์

ปัจจุบัน มีผลงานตีพิมพ์ในวารสารนานาชาติ 37 เรื่องมากกว่าเป้าหมาย

#### 4. ความเห็นและข้อเสนอแนะ

ระเบียบและเกณฑ์ต่างๆที่สกว.มีอยู่ในปัจจุบันดีอยู่แล้ว โดยเฉพาะระเบียบการตีพิมพ์ผลงานทำให้นักวิจัยตื่นตัวอยู่ตลอดเวลาถึงความก้าวหน้า นวัตกรรม และความสมบูรณ์ของงาน

ลงนาม..... 

ศาสตราจารย์ภาวิณีปิยะจตุรวัฒน์)

หัวหน้าเครือข่ายวิจัยผู้รับทุน

วันที่ 19 กรกฎาคม 2564

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