THE INHIBITORY EFFECT OF ANTHOCYANINS-RICH THAI BERRIES EXTRACTS ON LIPID DIGESTION AND ABSORPTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Food and Nutrition
Department of Nutrition and Dietetics
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2017

ฤทธิ์ของสารสกัดจากผลไม้ไทยเบอรี่ที่มีแอนโทไซยานินสูง ต่อการยับยั้งกระบวนการย่อยและการดูดซึมไขมัน

นางสาวเนติมา ชำนาญศิลป์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาอาหารและโภชนาการ ภาควิชาโภชนาการและการกำหนดอาหาร
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

Thesis Title	THE INHIBITORY EFFECT OF ANTHOCYANINS-RICH THAI BERRIES EXTRACTS ON LIPID DIGESTION AND ABSORPTION
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Field of Study	Food and Nutrition
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เนติมา ชำนาญศิลป์ : ฤทธิ์ของสารสกัดจากผลไม้ไทยเบอรี่ที่มีแอนโทไซยานินสูงต่อการยับยั้งกระบวนการย่อยและการดูดซึมไขมัน (THE INHIBITORY EFFECT OF ANTHOCYANINS-RICH THAI BERRIES EXTRACTS ON LIPID DIGESTION AND ABSORPTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. สถาพร งามอุโฆษ, 153 หน้า.

ภาวะไขมันในเลือดสูงเป็นหนึ่งในปัจจัยเสี่ยงที่สำคัญของการเกิดโรคเรื้อรังโดยเฉพาะ โรคหัวใจและหลอดเลือด และโรคเบาหวาน งานวิจัยในอดีตพบว่าฤทธิ์ของแอนโทไซยานินในผลไม้ไทยกลุ่มเบอรี่มีคุณสมบัติในการช่วยลดระดับไขมัน แต่อย่างไรก็ตาม ยังไม่มีข้อมูลทางวิทยาศาสตร์ที่ระบุกลไกการช่วยลดระดับไขมันได้ ดังนั้น งานวิจัยชิ้นนี้จึงมีวัตถุประสงค์เพื่อศึกษากลไกของผลไม้ไทยกลุ่มเบอรี่ ได้แก่ ไทยพลัมหรือลูกไหน (*Prunus domestica* L.) มะเม่า (*Antidesma bunius* (L.) Spreng) ลูกหว้า (*Syzygium cumini* (L.) Skeels) มะเกี๋ยง (*Syzygium nervosum* A. Cunn. Ex DC) และตะขบ (*Muntingia calabura* L.) ต่อการลดการย่อยและการดูดซึมไขมันในหลอดทดลอง โดยทำการศึกษาปริมาณโดยรวมของสารประกอบฟีโนลิค ฟลาโวนอยด์ และแอนโทไซยานิน รวมถึงศึกษาฤทธิ์ในการลดไขมัน โดยศึกษาความสามารถของสารสกัดในการจับกับกรดน้ำดี การยับยั้งทำงานของเอนไซม์ไลเปสและคอลเลสเตอรอลเอสเตอเรส และยับยั้งการรวมตัวของคลอเลสเตอรอลเข้าสู่ไมเซลล์ รวมถึงศึกษาการดูดซึมคลอเลสเตอรอลเข้าสู่เซลล์ลำไส้เล็ก (Caco-2 cells) จากผลการทดลองพบว่า ปริมาณโดยรวมของสารประกอบฟีโนลิค ฟลาโวนอยด์ และแอนโทไซยานินของสารสกัดจากผลไม้ไทยกลุ่มเบอรี่ที่มีแอนโทไซยานินสูงทั้ง 5 ชนิด อยู่ในช่วง 222.7-579.5 มิลลิกรัมเทียบเท่ากับกรดแกลลิคต่อกรัมสารสกัด 91.2-184.3 มิลลิกรัมเทียบเท่ากับ คาเทชินต่อกรัมของสารสกัด และ 37.9-49.5 มิลกรัมเทียบเท่ากับของไซยานิดิน 3 กลูโคไซน์ต่อกรัมสารสกัด ตามลำดับ สารสกัดจากผลไม้ไทยกลุ่มเบอรี่ที่มีแอนโทไซยานินสูงทั้ง 5 ชนิดที่ความเข้มข้น 1 มิลลิกรัมต่อมิลลิลิตร แย่งจับกับกรดน้ำดีชนิดปฐมภูมิ (ทอโลคลอลิคแอซิด) และชนิดทุติยภูมิ (ทอโลดีอ๊อกซีคอลิคแอซิดและไกลโคดีอ๊อกซีคอลิคแอซิด) อยู่ในช่วงร้อยละ 20.1-42.3, 16.4-36.2 และ 27.9-39.3 ตามลำดับ นอกจากนี้ยังพบว่าสารสกัดดังกล่าวมีฤทธิ์ในการยับยั้งการทำงานของเอนไซม์ไลเปสและคลอเลสเตอรอลเอสเตอเรส โดยมีค่า IC_{50} อยู่ในช่วง 90.6-336.9 ไมโครกรัมต่อมิลลิลิตร และ 288.7-469.1 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และสารสกัด (ความเข้มข้น 0.25 มิลลิกรัมต่อมิลลิลิตรของสารสกัด) สามารถลดการรวมตัวของคลอเลสเตอรลในไมเซลล์ ในช่วงร้อยละ 53.0-67.3 ทั้งนี้จากผลการวิจัยพบว่า สารสกัดจากผลไทยพลัม (*Prunus domestica* L.) มีปริมาณโดยรวมของฟลาโวนอยด์และแอนโทไซยานินสูงที่สุด รวมถึงมีคุณสมบัติเข้าจับกับกรดน้ำดี ยับยั้งเอนไซม์ ไลเปสและลดการรวมตัวของคอเลสเตอรอลเข้าสู่ไมเซลล์ได้ดีที่สุด ดังนั้นสารสกัดจากผลไทยพลัมจึงถูกนำมาศึกษาฤทธิ์ของสารสกัดดังกล่าวต่อการลดการดูดซึมคลอเลสเตอรอลเข้าสู่เซลล์ลำไส้เล็ก (Caco-2 cells) และพบว่าสารสกัดจากไทยพลัมสามารถลดการดูดซึมของคลอเลสเตอรอลเข้าสู่เซลล์โดยความสามารถในการยั้บยั้งการดูดซึมเพิ่มขึ้นตามความเข้มข้นของสารสกัดที่เพิ่มมากขึ้น นอกจากนี้ การรวมกันของสารสกัดจากผลไทยพลัม (ความเข้มข้น 0.1 มิลลิกรัมต่อมิลลิลิตร) กับยาezetimibe (ความเข้มข้น 0.05 มิลลิกรัมต่อมิลลิลิตร) ให้ผลการยับยั้งการดูดซึมคลอเลสเตอรอลเข้าสู่เซลล์แบบผลบวกสะสมซึ่งสามารถลดการดูดซึมได้ดีกว่า สารสกัด และยา เพียงอย่างเดียวอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) จากงานวิจัยนี้ พบว่า สารสกัดที่มีแอนโทไซยานินสูงจากผลไม้ไทยกลุ่มเบอรี่ ช่วยลดระดับไขมันโดยขัดขวางการทำงานของกรดน้ำดี ยับยั้งการทำงานงานของเอนไซม์ไลเปสและคลอเลสเตอรอลเอสเตอเรส และลดการรวมตัวของคอเลสเตอรอลเข้าสู่ไมเซลล์ โนอกจากนี้ สารสกัดจากไทยพลัมสามารถยับยั้งการดูดซึมคอเลสเตอรอลเข้าสู่เซลล์ลำไส้เล็กได้ จากผลงานวิจัยสามารถใช้เป็นข้อมูลยืนยันทางวิทยาศาสตร์ที่ช่วยสนับสนุนการใช้สารสกัดจากผลไม้ไทยกลุ่มเบอรี่ที่มีแอนโทไซยานินสูงเป็นสารสกัดที่มีฤทธิ์ในการช่วยลดระดับไขมันอย่างมีประสิทธิภาพ

ภาควิชา โภชนาการและการกำหนดอาหาร ลายมือชื่อนิสิต .....

สาขาวิชา อาหารและโภชนาการ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

5876854337 : MAJOR FOOD AND NUTRITION
KEYWORDS: THAI BERRY, ANTHOCYANINS, HYPERLIPIDEMIA, LIPID LOWERING EFFECT, CHOLESTEROL UPTAKE

NETIMA CHAMNANSILPA: THE INHIBITORY EFFECT OF ANTHOCYANINS-RICH THAI BERRIES EXTRACTS ON LIPID DIGESTION AND ABSORPTION. ADVISOR: ASST. PROF. SATHAPORN NGAMUKOTE, Ph.D., 153 pp.

Dyslipidemia is one of the major risk factor that contributes to chronic diseases such as cardiovascular disease and diabetic mellitus. Anthocyanin-rich Thai berries have been documented to have hypolipidemic activity. However, no scientific data is available regarding the mechanism of lipid lowering property. Thus, the objective of this study was to elucidate the mechanism of action of Thai berries including *Prunus domestica* L., *Antidesma bunius* (L.) *Spreng, Syzygium cumini* (L.) Skeels, *Syzygium nervosum* A. Cunn. Ex DC, and *Muntingia calabura* L. on a reduction of lipid digestion and absorption in vitro. Phytochemical compounds of Thai berries, including total phenolic (TP), total flavonoids (TF) and total anthocyanins (TA) were determined. The lipid lowering activities were evaluated via bile acid binding, pancreatic lipase and cholesterol esterase activities and cholesterol micellization. Furthermore, cholesterol uptake into intestinal Caco-2 cells was also observed. Total phenolic, total flavonoids and total anthocyanins contents of five Thai berries were range form 222.7-579.5 mg gallic acid equivalents/g extract, 91.2-184.3 mg catechin equivalents/g extract and 37.9-49.5 mg cyanindin-3-glucoside equivalent/g extract, respectively. All five Thai-berries extracts (1 mg/mL) bound to taurocholic acid (primary bile acid), taurodeoxycholic acid and glycodeoxycholic acid (secondary bile acids) ranged from 20.1-42.3%, 16.4-36.2%, and 27.9-39.3%, respectively. In addition, Thai berries extracts showed inhibitory activities against pancreatic lipase and cholesterol esterase with IC_{50} values ranged from 90.6-336.9 µg/mL and 288.7-469.1 µg/mL, respectively. The extracts (0.25 mg/mL) also reduced the solubility of cholesterol in mixed micelles with inhibitory value of 53.0-67.3%. Our finding indicated that *Prunus domestica* L. had the highest TF, TA and also demonstrated the strongest potent to bind to bile acid, inhibit pancreatic lipase, and reduce cholesterol micellization. Therefore, *Prunus domestica* L. was then further subjected to investigate cholesterol absorption in Caco-2 cells. It was found that the extract reduced cholesterol absorption in Caco-2 cells in a concentration-dependent manner. Moreover, the combination of *Prunus domestica* L. (0.1 mg/mL) with ezetimibe (0.05 mg/mL) exhibited additive effect on the reduction of cholesterol uptake into the cells which had more ability than *Prunus domestica* L. and ezetimibe alone ($p< 0.05$). These results illustrate that anthocyanins-rich Thai berries are able to contribute lipid lowering effects via binding to bile acids, decreasing pancreatic lipase and cholesterol esterase activities and also reducing cholesterol incorporation into mix micelle. Moreover, Prunus domestica L. decreases cholesterol absorption into the intestinal cells. Taken together, our finding provides scientific evidence supporting the potential of anthocyanins-rich Thai berries extracts can be used as a new and efficient lipid lowering agents.

Department:	Nutrition and Dietetics	Student's Signature	.....
Field of Study:	Food and Nutrition	Advisor's Signature	.....
Academic Year:	2017		

ACKNOWLEDGEMENTS

Foremost expression, I would like to give my sincere thanks to my thesis advisor, Asst. Prof. Sathaporn Ngamukote, Ph.D. for her precise care and steady help along with the good encouragement and patience. I am very appreciated for her teaching and monitoring all directions throughout my thesis. Without her guideline, I would never be able to finish my project of the master's degree study

My sincere is also express thankfully to my thesis committee: Assoc. Prof. Sirichai adisakwattana, Ph.D., Kittana Makynen, Ph.D., Assoc. Prof. Somsri Charoenkiatkul, D.Sc. and Thavaree Thilavech, PhD. for their guidance, suggestions, and the available time for proposal and thesis defends

I would like to thanks my colleagues, Miss Pattamaporn Aksornchu (Ph. D students) and Miss Sakawrut Poosri (Ph. D students), Mister Weerachat Sompong (Postgraduate student), and all postgraduate students in Department of Nutrition and Dietetics in the Faculty of Allied Health sciences for their kind incorporation of my work together with our good relationship. I also would like to thanks Department of Nutrition and Dietetics in the Faculty of Allied Health sciences, Chulalongkorn University for all instrument support.

In addition, I have taken the opportunity for scholarship supports from grant for international research Integration: the 90th Anniversary of Chulalongkorn University Scholarship, Chulalongkorn University, and the Research Group of Herbal Medicine for Prevention and Therapeutic of Metabolic disease.

The last but not meaning least, I totally appreciated to my dearest family: my parents, my sister, and my husband for always take care and support me in every step of my life. The success and many more meaningful goals will be always provided by their idea, suggestion and love.

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CHAPTER I
INTRODUCTION

1. Background

The World Health Organization (WHO) has been monitoring both incidence and prevalence of cardiovascular disease (CVD), which are the prime cause of worldwide death in many developed and developing countries (Kubo et al., 2003; Roth et al., 2015). In 2015, an estimated 17.7 million people worldwide died from CVD (31% of all global deaths). In addition, CVD is the top cause of death in Thailand. The CVD prevalence data, based on coronary heart disease (CHD), is high (27% of the mortality burden) (Hoffman & Kaplan, 2002). Patients with CVD generally end up with heart attack, angina, onset of atherosclerosis, myocardial infarction, and discovery of a vessel dysfunction (Libby et al., 1998). The strategic priorities to prevent and manage CVD are focus on the reduction of CVD risk factors (Doroodchi et al., 2008). High consumption of high fat food together with less physical activities are powerful increasing the CVD risks, known as dyslipidemia and hyperlipidemia (Ness & Powles, 1997). Dyslipidemia, known as low level of high-density lipoprotein cholesterol (HDL-cholesterol), high level of low-density lipoprotein cholesterol (LDL-cholesterol), or triglyceride (TG), accelerates lipid oxidation, particularly LDL-cholesterol in arterial wall which induces inflammation. (Tietge, 2014). The oxidized LDL-cholesterol eventually develop plaque contains necrotic lipids. The plaque generally narrows the blood vessel or artery and, after rupturing, it causes vascular damage, leading to a result of coronary dysfunction (Barter et al., 2007). Nowadays, multiple therapies are using drugs that also play a prominent role in lowering or maintaining blood lipid profile in the normal level (Zhou et al., 2012). The available drugs such as statins, orlistat, cholestyramine, and ezetimibe are effectively used among hyperlipidemia-patients

(Rouhi-Boroujeni et al., 2015). For example, orlistat function by making covalent bond to the serine residue at the active site of pancreatic lipases, leading to inhibit digestion of triglycerides (Reis et al., 2009). Ezetimibe is selectively blocking the Niemann-Pick C1-Like 1 (NPC1L1) protein in the jejunal brush border membrane, causing a decrease lipid micelles uptake by intestinal lumen into the enterocytes (Phan et al., 2012). However, serious reports of adverse events have been recorded myopathy and mild elevations of liver transaminases (Phillips, 2004; Havranek et al., 2006; Hollingworth et al., 2017).

Hence, many studies have focused on phytochemicals from vegetables and fruits as an alternative therapeutic strategy for preventing and ameliorating cardiac dysfunction (Christen & Cuendet, 2012). Phenolic compounds, major phytochemicals, have been documented to improve endothelial function, called as vaso-protection for microvasculature. In addition, antioxidant activity of this compounds play a vital role to prevent chronic disease, especially cardiovascular disease, cancer and diabetes (Borek, 2004). There are many sources of food containing phenolic compounds that demonstrate health benefits including tea, red wine, turmeric, capers, tomatoes, and berries. (Ness & Powles, 1997; Kuriyama et al., 2006; Pandey & Rizvi, 2009).

Berries are now reported as an emerging potential for lowering high blood lipid profiles because of their flavonoids and anthocyanins contents (Basu et al., 2010). Flavonoids, a large group of phenolic compound, are vastly distributed as secondary metabolites with different metabolic functions in fruits and vegetables. They can be divided into six groups (anthocyanins, flavonols, flavanols, flavanons, chalcones, isoflavones). Anthocyanins are the main part of flavonoids containing in berries. Blueberries, strawberries, blackberries, cranberries and red-skinned grapes are the excellent source of anthocyanins since they provided anthocyanins around 86 mg per

100 g of fruit. In the same way, the average anthocyanin content in others fruits (apple, banana, grapes, melons) was around 8 mg per 100 g portion (Wang & Lin, 2000). The well documents for human health benefits related to consumption of dietary anthocyanins has been revealed (Lila, 2004). Recently, many previously studies demonstrated that anthocyanins play beyond their antioxidant property. The pharmacological actions of anthocyanins have been proved *in vitro*, *in vivo*, and clinical researches. Based on a systematic review, consumption of strawberries and blueberries showed a significant decrease in relative risk of CVD with the mean consumption of anthocyanin by intake of 0.2 mg/day (Seeram, 2008). In addition, blueberries and strawberries juices from purified anthocyanins decreased the onset of dyslipidemia in mice fed a high-fat diet for 90 days (Zafra-Stone et al., 2007). *In vitro* study, polyphenol-rich berry extracts inhibited pancreatic lipase (McDougall et al., 2009). It was found that reactive groups (hydroxyl and methyl groups) in anthocyanins structure have ability to form oxygen bridges with the substrate and thus inhibit hexokinase or other enzyme systems (Carpenter et al., 1967). Yao and his colleagues recently suggested that black rice anthocyanins may be partly active for the inhibitory effects on cholesterol absorption, so they may benefit for prevention and treatment of hypercholesterolemia (Yao et al., 2013). Moreover, blueberries extract had highest ability to bind to bile acids and when compared with other tested fruits (pear, prune, banana or apple) (Kahlon & Smith, 2007). It was suggested that this binding ability was related to their phytonutrients such as polyphenols, catechins, flavonoids, hydroxycinnamic acids, anthocyanins, flavonols and proanthocyanidins (McDougall et al., 2005).

In Thailand, Thai plum or Luknhai (*Prunus domestica* L. (Swain & Hillis, 1959; Usenik et al., 2009), Mamao (*Antidesma bunius* (L.) *Spreng*) (Chaikham, 2015; Chaikham et al., 2016), Lukwha (*Syzygium cumini (*L.*) Skeels*) (Ayyanar & Subash-Babu, 2012; Banerjee et al., 2005), Makiang (*Syzygium nervosum* A. Cunn. Ex DC) (Jansom et al, 2008; Swami et al., 2012), and Ta-kob or Jamaican cherry (*Muntingia calabura* L. (Gomathi et al.,

2013; Jansom et al., 2008), known as Thai berries, are good source of phenolic compounds, mainly flavonoids and anthocyanins. Although the pharmacologic actions of Thai-berries have been illustrated in many studies including antioxidant capacity against the mutagenic events, as well as, anti-diabetic and anti-inflammatory effects, as far as we know, the mechanisms of Thai-berries on anti-hyperlipidemic property have never been conducted. Thus, the exploration of Thai-berries on lowering lipid digestion and absorption would exert the potential of Thai-berries use as a novel lipid-lowering therapeutic agent in the future.

2. The objectives of this study

The aims of this study were to investigate properties of Thai-berries including Thai plum or Luknhai (*Prunus domestica* L.), Mamao (*Antidesma bunius (*L.*) Spreng*), Lukwha *(Syzygium cumini (*L.*) Skeels*), Makiang (*Syzygium nervosum* A. Cunn. Ex DC), and Takob or Jamaican cherry (*Muntingia calabura* L.) through lipid digestion and absorption mechanism as following to

2.1 To quantify phytochemical contents of anthocyanins-rich Thai berries extracts including

2.1.1 total phenolic

2.1.2 total flavonoids

2.1.3 total anthocyanins

2.2 To determine anthocyanins-rich Thai berries extracts on

2.2.1 bile acids binding

2.2.2 pancreatic lipase activity

2.2.3 cholesterol esterase activity

2.2.4 cholesterol micellization

2.3 To investigate anthocyanins-rich Thai berry extract that was selected based on the following criteria on cholesterol uptake by Caco-2 cells.

2.3.1 highest amount of anthocyanins contents

2.3.2 highest binding percentage of bile acids

2.3.3 lowest IC_{50} values of the inhibitory activity on pancreatic lipase, cholesterol esterase

2.3.4 highest inhibitory percentage of cholesterol micellization

3. Hypotheses of the study

3.1 Anthocyanins-rich Thai berries extracts contained a high amount of total phenolic compound, total flavonoids, and total anthocyanins.

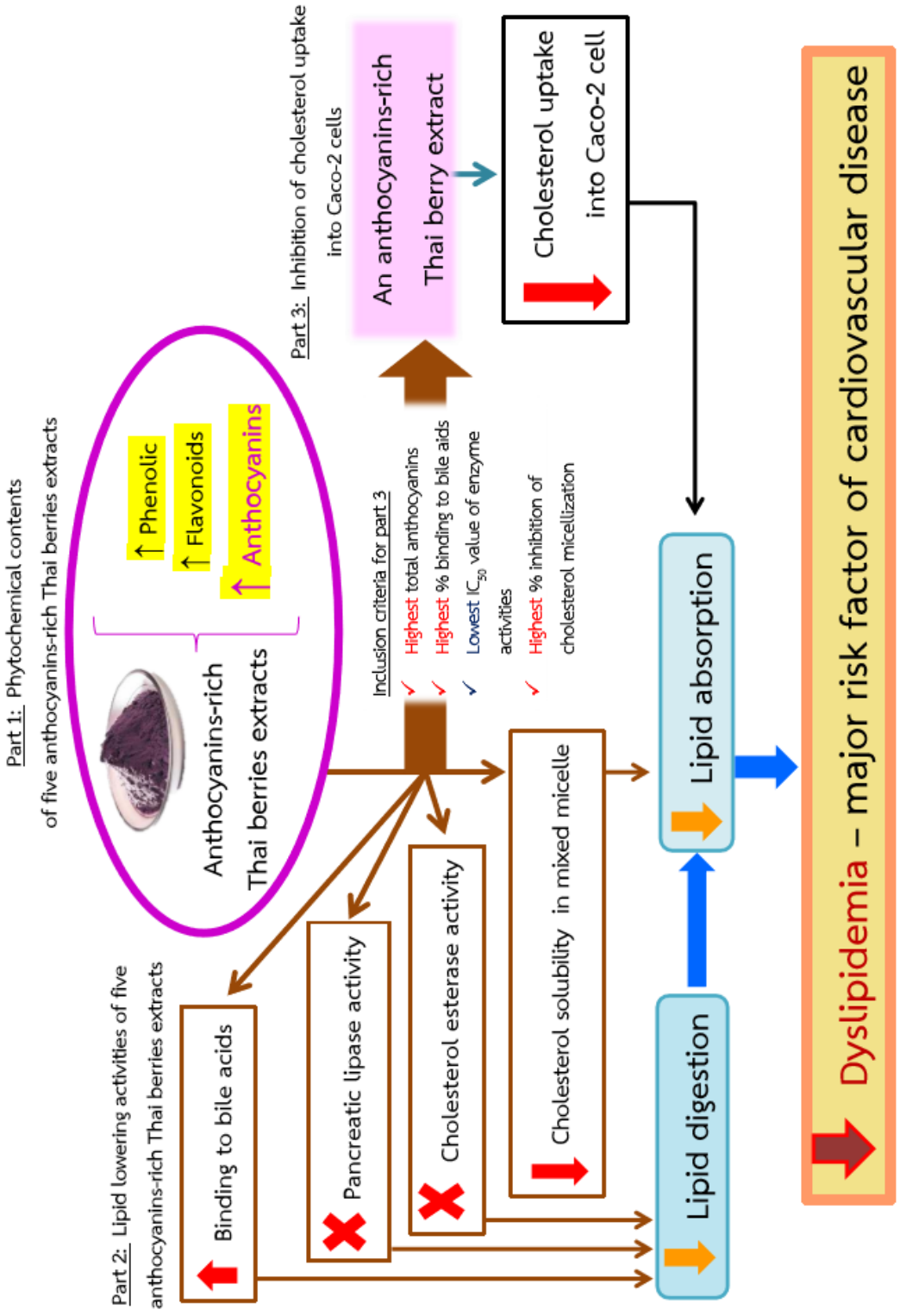
3.2 Anthocyanins-rich Thai berries extracts were able to directly bind to bile acids, to inhibit pancreatic lipase and cholesterol esterase activities, and to decrease cholesterol solubility in mixed micelle.

3.3 Anthocyanins-rich Thai berry extract with the number of anthocyanins content, and having the greatest ability on enzymatic activities (lowest IC_{50} value) decreased cholesterol uptake into Caco2 cell.

4. The advantages of the study

This study provides a scientific evidence of Thai berries related to decrease lipid digestion and absorption. In addition, it also demonstrates the ability of using Thai berries as materials for development of function food or dietary supplement regarding attenuation or prevention of dyslipidemia. Furthermore, the anti-hyperlipidemia property of Thai berries may potentially promote their consumption with both applied products and fresh fruits, leading to raise values of Thai berries agriculture and food industry.

5. Conceptual framework of the study



CHAPTER II

LITERATURE REVIEW

1. Cardiovascular diseases (CVD)

Cardiovascular disease is a group of impairment of heart and blood vessel, in which is attack by plaque, a substance with cholesterol content. Cholesterol can be divided in to many subgroups, and one type, known as LDL-cholesterol is a key substance in plaques attached on the inner walls of blood vessels. The cholesterol plaque leads to blood clot formation in the blood vessel, vascular dysfunction, and vascular inflammation, causing the high risk of cardiovascular condition (Table 1) like heart attack of stroke. Mortality of cardiovascular disease is increasing globally (Santulli, 2013).

Table 1 Types of cardiovascular disease (CVD)

Types	Damaged tissue/organ	Cause	Outcome
1. Coronary heart disease	Blood vessels of the heart muscle	Plaque	Heart attacks
2. Cerebrovascular disease	Blood vessels of the brain	Plaque	Strokes
3. Peripheral arterial disease	Blood vessels of the arms and legs	Plaque	Paralytic appendage
4. Deep vein thrombosis	Blood clots in the leg veins, potentially moving to the heart	Plaque	Heart attacks Heart failure

Generally, people with CVD condition likely end up with heart attack: having blocked the blood flow to heart, and stroke: having blocked the blood vessel feeding

to the brain. Because of plaque, the blockage has necrotic lipids, called as a fibrous cap with immune cells.

2. The process of cardiovascular disease (CVD)

Plaque is normally made up of deposits of cholesterol and other lipid substances in the blood vessel, artery. When plaque has located inside of arteries, which are narrow over time, it could partly or completely block the blood flow. In case of too much plaque full up and narrowed artery walls, this event is able to make it harder for blood flow through the whole body. For example, over the time of plaque in the heart blood vessel can cause heart failure, which that heart dysfunction to pump blood as usual. Many studies suggest that having low level of HDL-cholesterol together with having high level of LDL-cholesterol or triglyceride strongly associated to build up the plaque. Total cholesterol (LDL-cholesterol, HDL-cholesterol and non-HDL cholesterol, which are counted as differently from total cholesterol and HDL cholesterol) display robust log-linear associations with CVD. Thus, they all are considered classic predictors (Bass et al., 1993).

In accordance with the centers for disease control data from a survey of a thousand physicians was reported people with high blood total cholesterol and/or triglycerides together with low blood HDL-cholesterol are diagnosed as dyslipidemia, which is one list of top 10 common chronic criteria related to cardiovascular disease.

3. Dyslipidemia

Dyslipidemia can be diagnosed by high levels of either total cholesterol or triglycerides together with low level of HDL-cholesterol, and it has a strong risk factor of CVD, based on well established (Fakhrzadeh & Tabatabaei-Malazy, 2012). It was

presented in 90.1% of patients with coronary heart disease (CHD) (Wilson et al., 1998). Meta-analysis from randomized controlled trials (RCTs) concluded that a 40% reduce level of LDL-cholesterol with a 30% increase in HDL-cholesterol could lower 70% the risk of cardiovascular disease (Law et al., 2003). Because of the LDL-cholesterol, very high level of LDL-cholesterol can lead the oxidation of lipoprotein in the damaged vessels due to an increase in oxidized LDL-cholesterol level (Frostegard, 2002). The LDL-cholesterol then makes up more than half of the total lipoprotein in plasma. It is well understood that accumulation of LDL-cholesterol over time could lead to have lower other lipid particles, especially HDL-cholesterol, so this stage of dyslipidemia may cause then vascular dysfunction (Holvoet et al., 2004). Moreover, this situation may be due solely to hereditary factors, but there are many more acquired conditions. One of the conditions is relatively dietary habits, especially having high fat food. Having high fat food daily was alternatively elevated levels of some or all lipid particles and/or lipoproteins in the blood circulation (Azadbakht & Esmaillzadeh, 2008). The accumulation of lipid particles would also cause the initial stage of dyslipidemia, known as hyperlipidemia (having high cholesterol or high triglycerides levels in the blood). Two studies demonstrate that hyperlipidemia, induced by a high-fat diet, causes heart-transplant rejection in mice (Blessing et al., 2002; Sullivan et al.,2014). By using models that mimic the health conditions found in human transplant recipients, It was found that transplant rejection was accelerated whether the hyperlipidemia was caused by genetics or solely by a high-fat diet (Freigang et al.,1998).

Hyperlipidemia normally refers to have either high of total cholesterol or triglycerides, and the other term, it can be named as hypertriglyceridemia and hypercholesterolemia, respectively.

3.1 Hypertriglyceridemia

Hypertriglyceridemia is considered to have serum triglyceride levels over 150 mg/dL (1.7 mmol/L). The factors include obesity, unhealthy diet, stress, and less physical activity lifestyles (Fletcher et al., 2005; Weinsier et al., 1998). The entire factor is typical as a combination of genetic and environmental factors.

3.2 Hypercholesterolemia

Hypercholesterolemia is characterized by elevated cholesterol level due to an increase of non-HDL-C cholesterol. It refers to a blood cholesterol measurement of more than 200 mg/dL.

4. Lipid particles: mediator of cardiovascular risk

4.1 Triglyceride (TG) or Triacylglycerol (TAG)

Triglyceride is the most common type of fat that can be found in food, and it is also the major components fat in humans. Having high level of triglyceride can be both gained from dietary fat and synthesized in the body. The components of triglyceride are ester derived from glycerol and three fatty acids. In addition, there are two types of triglyceride by the major division between saturated and unsaturated fats. The first type is saturated fats that are full with hydrogen that could be bonded to carbon atoms. The other type, unsaturated fats, has double bonds between carbon atoms that can be reducing the bond of hydrogen atoms. Plasma triglyceride levels can be used to predict cardiovascular disease as the significant relationship between triglyceride and congenital heart disease, observed in the general population. Moreover, in patients with chronic disease such as metabolic syndrome and diabetes, the plasma triglyceride appeared as independent induced cardiovascular risks together with a strong opposite relationship between HDL-cholesterol and triglyceride.

4.2 Cholesterol

Cholesterol is a type of lipid molecule that can be biosynthesized naturally by all animal cells. It generally plays a key role in body including a structure of both membrane structural integrity and fluidity, a function of the central nervous system and an initiator for production of steroid hormones, bile acids and vitamin D. In order to perform its vital roles, cholesterol has to be transported to the cells via the bloodstream. People needs per day approximately 900 mg of cholesterol of which about 200 - 500 mg comes from the diet, whereas the rest is synthesized in the liver (Howell et al., 1997). In addition, cholesterol presents as free form when first synthesized in human hepatic cells whereas it normally presents as cholesterol ester in food. In the blood circulation, free cholesterol, in form cholesterol esters, appear as a major component of lipoproteins. The cholesterol esters are the form of free cholesterol binding with an ester group between the cholesterol structure and fatty acid molecules and have a lower solubility in water than the other form. Furthermore, in the blood stream, total cholesterol are composed of

4.2.1 LDL-cholesterol (Low Density Lipoprotein) contains 20 % protein, 15% triglycerides, 25% phospholipids, 45% cholesteryl esters, and 10% cholesterol. It is common to know as "Bad" Cholesterol. In addition, apolipoprotein B100 is a protein components of LDL-cholesterol for serving to bind lipoprotein particles to LDL-cholesterol-specific receptors on the cell surfaces (Dietschy & Wilson, 1970). Except high density lipoprotein (HDL-cholesterol), the apoprotein B is the major protein in all lipoproteins. The LDL-cholesterol particles bound to the cell surface are then engulfed, so the cholesterol in the LDL-cholesterol particles become a structure of cell membranes or made into steroid hormones.

4.2.2 HDL-cholesterol (High Density Lipoprotein) is the smallest of the lipoproteins. It contains approximately 55% protein, 15% triglycerides, 45% phospholipids, 30% cholesteryl esters, and 10% cholesterol. The HDL-cholesterol proteins operate in complement of cell regulation, lipid metabolism, and connect to both proteinase inhibitors and acute state that support the immunity against inflammation.

4.2.3 IDL-cholesterol (Intermediate Density Lipoproteins) is roughly composed of 12% protein, 30% triglycerides, 25% phospholipids, 35% cholesteryl esters and 10% cholesterol. It is derived from VLDL-cholesterol by triglyceride depletion. Therefore, it contains the same apolipoproteins as VLDL-cholesterol, which then becomes LDL-cholesterol once its triglycerides are reached to the cells.

4.2.4 VLDL-cholesterol (Very Low Density Lipoprotein) contains roughly 12% protein, 55% triglycerides, 20% phospholipids, 15% cholesteryl esters and 10% cholesterol. It seems to be a source of triglycerides for the cells that result to acquire several apolipoproteins from plasma HDL-cholesterol.

5. Blood lipid profile and cardiovascular risk

The lipids and lipoproteins family are purposed as the main plasma metabolic changes that associated with cardiovascular disease, particularly LDL-cholesterol and VLDL cholesterol (Banel & Hu, 2009). Studies carried out by the sensitive nuclear magnetic resonance (NMR) approach were noted that several serum spectral regions distinguished the metabolome, which a complete set of small-molecule in human, of healthy and disease groups (Freedman et al., 1998). It was also reflecting differences in a decrease in HDL-cholesterol and an increase in LDL-cholesterol and VLDL-cholesterol in subjects of the severe atherosclerotic group (Freedman et al., 1998).

High blood cholesterol is a condition, having cholesterol in the bloodstream higher than the normal level (200 – 240 mg/dL). It leads to a greater chance of coronary heart disease since dietary cholesterol increased both serum total cholesterol and LDL-cholesterol with statistical significance, but had no statistically change in serum triglycerides or VLDL-cholesterol concentrations (Derosa et al., 2004). Many previous studies have shown a link between having saturated fatty acids, mono- and poly-unsaturated fatty acids and cholesterol since the investigations have purposed that dyslipidemia can be built up mainly to improper dietary habits, especially having fast foods and snacks containing high saturated and trans fatty acids (Stender et al., 2006). Therefore, understanding of how dietary fat go to present partially their molecules in the blood stream could be a crucial key to manage the abnormal lipid profile and prevent the cardiovascular risks.

6. Process of lipid digestion and absorption in human

Dietary fat normally refers to cholesterol and triglycerides. They influence the lipid serum in human body. Triglyceride is the major fat in the diet, gaining 90–95% of the total energy following by phospholipids (PLs), and cholesterol. They are usually transported in the blood stream as the lipoproteins. Some previous studies have been reported various steps involved in lipid digestion and absorption that normally serve as targets of drugs treament for reducing the cardiovascular risk, including dyslipidemia or hyperlipidemia (Pahan, 2006).

6.1 Lipid digestion

In critical steps, the researchers have explored not only key aspects of lipid digestion, but also key issues of absorption including uptake of the lipid particles by enterocytes at the epithelial cells of the intestinal lumen. Fatty acids and cholesterol are transformed into neutral lipids after being transported to the

intracellular compartments. Since lipid digestion begins firstly in the oral cavity with lingual lipases, secreted by glands in the tongue, the digestion of triglycerides continues in the stomach by gastric enzymes. Figure 1 is showed a successive and coordinating step in the process of intestinal lipid digestion and absorption. Because fats are insoluble in water, they could not easily diffuse across cell membranes by themselves. Thus, the body has found an alternated way to transport fat droplets into enterocytes (Friedman & Nylund, 1980). First, lipids needs to be formed emulsion droplets by combining with bile salts. These droplets

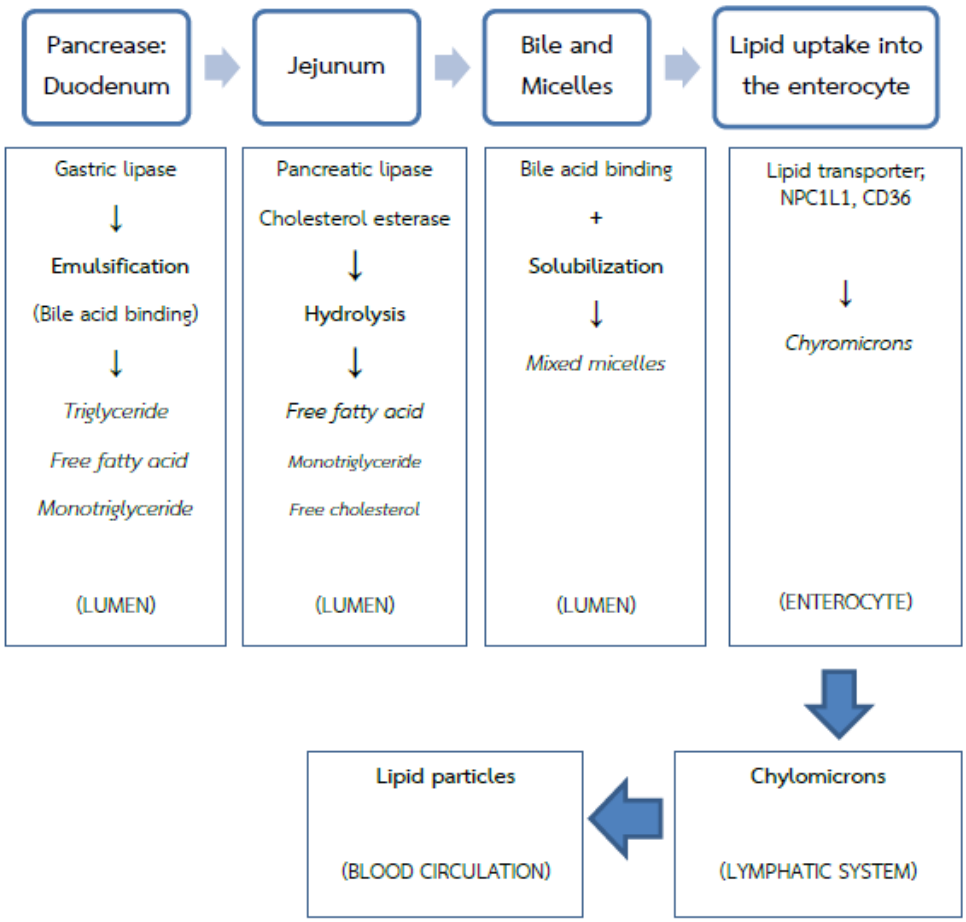


Figure 1 Overview of lipid digestion and absorption in human body system (Friedman & Nylund, 1980)

are then digested by pancreatic lipases and cholesterol esterase. Dietary fats are eventually absorbed as smaller particles such as free fatty acids (FFAs).

6.2 Lipid emulsification

The lipid digestion needs bile acids, which is an ideal detergent for the solubilizing all lipid products (Friedman & Nylund, 1980). The emulsification is an initial phase, in which fat globules are broken down into much smaller emulsion droplets, but they are not digested. This process likely increases the fat molecules surface, making it free for digestion by enzymes. Because pancreatic lipase could not itself access the inside of lipid droplets that have an amphipathic structure, the bile acids molecules with hydrophobic and hydrophilic regions are equilibrated (Dietschy, 1968).

6.3 Bile acid and its role in lipid digestion and absorption

Bile acids are derivatives from cholesterol that synthesized in the liver cell (hepatocyte), by which cholesterol is ingested partly from diet or obtained from hepatic synthesis (Dietschy, 1968). The cholesterol is finally converted into the bile acids. Cholic acid (Figure 2) and chenodeoxycholic acids are identified as primary bile acids, which are then conjugated to glycine or taurine (amino acid groups). After the conjugation, conjugated bile or secondary bile acids, are actively secreted to function in the small intestine including the emulsification of lipid aggregation and the solubilization and transportation of lipids in an aqueous condition.

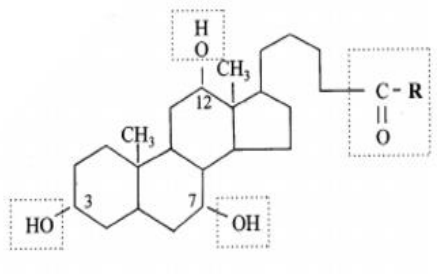


Figure 2 Bile acid structure (Cholic acid) (Mukhopadhyay & Maitra, 2004)

The bile acids play another role as lipid carriers, which are able to solubilize much lipids for forming mixed micelles. The mixed micelles aggregate neutral lipids such as free fatty acid, cholesterol and mono-glycerides (Dietschy, 1968). In addition, a study indicated that rapid and single-step analyses of individual conjugated bile acids on 16 human gallbladder bile specimens and reported the quantities of six secondary bile acids (taurocholic acid, glycocholic acid, taurodeoxycholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid and taurochenodeoxycholic acid). The result was also reported the recovering of the secondary bile acids appears in colon. They are absorbed to the liver for being in binary bile salt-eater system while a little of them is excreted in feces.

6.4 Intestinal digestion of triglyceride

Triglycerides are hydrolyzed firstly by pancreatic lipase in the upper part of jejunum. This process produce a liquid-crystalline, which interfaces at the surface of the lipid emulsion (Hofmann & Borgstrom, 1964). Pancreatic lipase activity on the sn-1 and sn-3 positions of triglycerides (Figure 3) results in the release of 2-monoglycerides (2-MAG) and free fatty acids. The 2-MAG is the predominant form of mono-glyceride (MAG) that is absorbable in the small intestine. The formations of 2-MAG and MAG through isomerization in aqueous medium appears very slowly than the 2-MAG uptake in the small intestine.

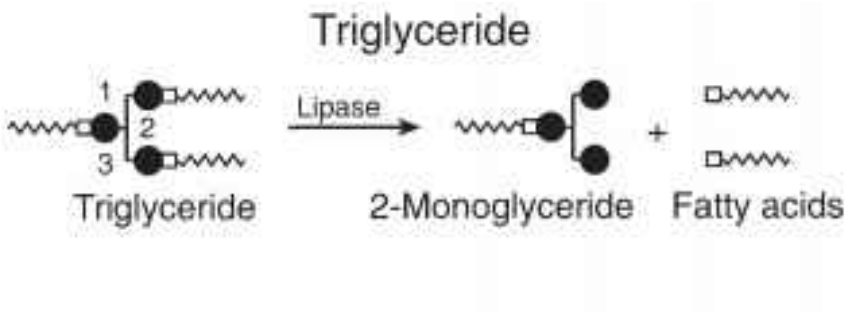


Figure 3 Digestion of triglycerides: first to di-monoglycerides and then to monoglycerides and free fatty acids (Ros, 2000)

6.5 Intestinal digestion of cholesterol

Dietary fat provides around 400 mg of cholesterol daily, and the liver secretes about 1 g daily in the body (Carey et al, 1983). About half of the dietary cholesterol in the small intestine is absorbed, while the other is excreted through feces (Borgstrom, 1960). Cholesterol esterase is ascribed to the only enzyme that hydrolyzes cholesterol esters from dietary fat to un-esterified cholesterol or free-cholesterol and fatty acids (Figure 4). It hydrolyzes the acyl group at the sn-2 position to then form glycerol and free fatty acids.

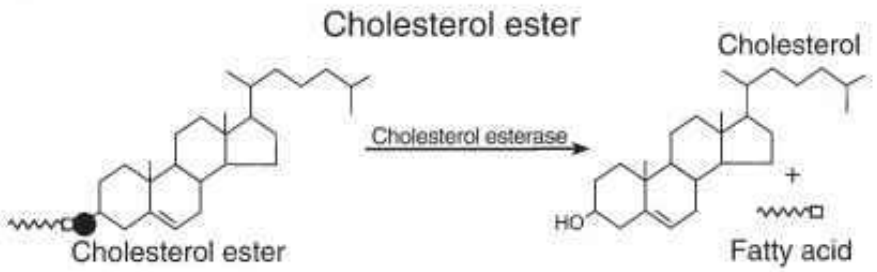


Figure 4 Digestion of cholesterol ester: to free cholesterol and free fatty acids (Wilson & Rudel, 1994)

6.6 Intestinal lipid absorption

Lipid absorption is slightly complicated processes. Small molecules, glycerol and short chain fatty acids (SCFA), from triglycerides can be absorbed by simple diffusion across the cell membranes in the enterocytes (Iqbal & Hussain, 2009). On the other hand, cholesterol and long chain fatty acids are unable to diffuse through the cell membranes, so they must be handled differently. The lipid absorption starts in duodenum. Bile salts commonly combine with the digested fats to form small spheres called as mixed micelles (Figure 5). By the time fats leave the lining cells of the enterocyte, it has been joined with cell proteins, known as chylomicrons. The chylomicrons carry fats lymphatic system to blood stream. The

chylomicrons naturally form inside of the epithelial cells. They would generally circulate in blood to drop off lipid molecules for body tissues. Finally, the chylomicron remnants would be picked up by the liver, and prepare for another cycle of lipid absorption.

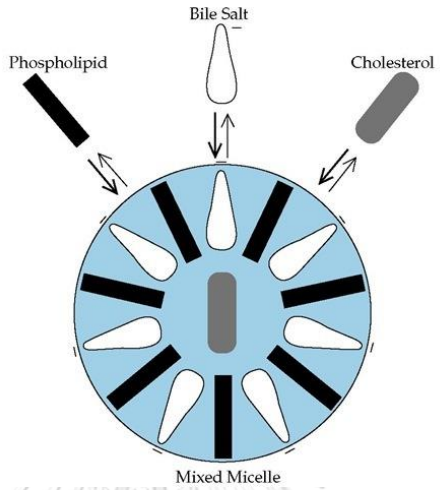


Figure 5 Mixed micelle structure (Hofmann & Borgstrom, 1964)

6.7 Cholesterol micellization

Mixed micelles, which plays as vehicles taking digested lipid (free fatty acids, free cholesterol, monoglyceride and phospholipids) to be absorbed by the enterocyte, are greatly water-soluble polymolecular spheres (Westergaard & Dietschy, 1976). They aggregate with a discoid configuration, where the polar heads of the lipid particles are on the surface protruding into the aqueous solution. Whereas their polar-hydrocarbon parts are at the core, as shown in Figure 5, away from the water. These mixed micelles can pass through the cell membranes of endothelial cells and drop off the digested fats into the enterocytes. The digested lipids both diffuse by themselves and pass through lipid transporters (Westergaard & Dietschy, 1976).

In addition, the cholesterol solubility in mixed micelles likely depends on part of hydrophobicity of bile salts (Hayter & Penfold, 1983), so decrease in the

cholesterol solubility are now become a target of lipid lowering agents (Emukowhate & Wierzbicki, 2013).

6.8 Uptake of free fatty acids (FFAs) into enterocyte

Free fatty acids are normally taken up passively from the intestinal epithelium cells into the enterocytes. A protein-dependent mechanisms have been revealed for uptake across the apical membrane of the enterocyte (Iqbal & Hussain, 2009). Several candidate proteins have been reported to role in part of protein-dependent uptake mechanisms, and there is still inconstantly conclusive information (Potter et al., 1989). For instance, FAT or CD36 has been highly found to play a key role in the uptake of fatty acids (Goldberg et al., 2009). Later on, a study in CD36-null mice showed the result that was intimately involved in uptake and transportation of free fatty acids via members in the assembly of chylomicrons (Drover et al., 2008). Thus, the uptake of free fatty acids was not impaired in CD36-null animals. It can be totally inferred that free fatty acids may be uptake via other channels.

6.9 Cholesterol absorption and cholesterol uptake into enterocyte

To absorb cholesterol, only free cholesterol (non-esterified cholesterol) is incorporated with bile acids in mixed micelles, which then be absorbed by the enterocytes. Free cholesterol enters mixed micelles along with phospholipids, ionized and non-ionized free fatty acids, mono-glycerides, and lysophospholipids (Westergaard & Dietschy, 1976). The mixed micelles are transported to the brush border membrane of enterocyte depending on presence of bile acids in the small intestine. The next step involve to protein-facilitated selective cholesterol uptake, called NPC1L1 (Niemann-Pick C1-like 1). The NPC1L1 facilitates cholesterol molecules into the enterocytes (Yu et al., 2006) since this transporter protein has been revealed to play a major role in cholesterol uptake by enterocyte (Garcia-

Calvo et al., 2005). It is concentrated in the plasma membrane at micro-domains called rafts. In general condition, once N-terminal domain of NPC1L1 that harbors cholesterol binding site is exposed (Betters & Yu, 2010), the C-terminal domain immediately interacts with the inner leaflet of plasma membrane. Matching between cholesterol and NPC1L1 at the N-terminus exposes the C-terminal domain, leading to contact with numb to facilitate recruitment of clathrin. The clathrin is a protein helping to form coated vesicles. The micro-domains are then internalized and transported by endocytic recycling accompany where cholesterol is secreted and transported to other organelles. This cholesterol transporter, NPC1L, is critically indicated as a target of cholesterol lowering medicine, called ezetimibe, as shown in Figure 6 (Landmesser et al., 2005).

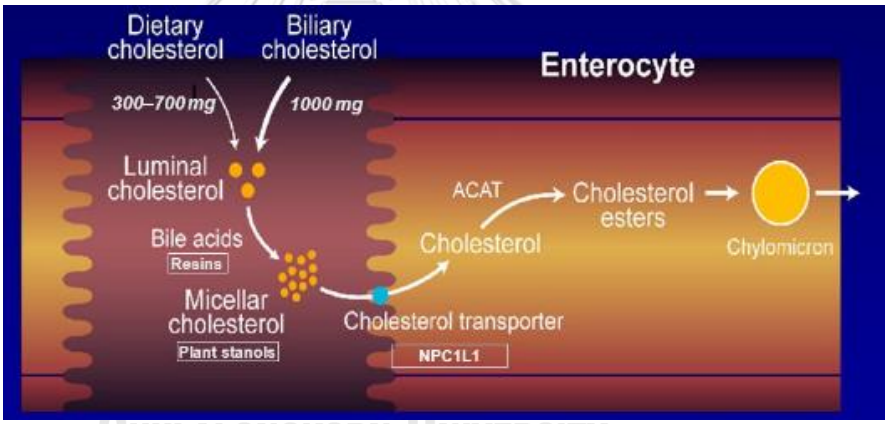


Figure 6 Colesterol absorption and cholesterol uptake into enterocyte (Investigators, 2014)

7. Controlling blood lipid profile and cardiovascular risk

The emphasis on lowering lipid level, in particular LDL-cholesterol, has resulted in important improvement in prevalence and incidence of CVD (Gordon et al., 1989; Nissen et al., 2004). However, they were report of cardiac symptoms occur in people without clinically abnormal LDL-cholesterol contents in spites of the emphasis on control of LDL-cholesterol. One way for improvement of predicting risks and treatment

of cardiovascular disease has been focused on non-HDL cholesterol levels rather than on just LDL- cholesterol. In a meta-analysis related to statin, a lipid lowering drug, treated subjects, makers including non-HDL-cholesterol, both Apo-B and LDL-cholesterol were compared to correlate with cardiovascular disease. The result was found that non-HDL-cholesterol slightly showed greater association than others (Boekholdt et al., 2012). The explanation for this event was that other lipoprotein fractions are substance from dietary fat and they have been played a role of lipid oxidation that may influence to formation of plaque. In addition, non-HDL cholesterol are either intermediate or end products of triglyceride rich lipoprotein (TGRL) catabolism, especially chylomicrons and VLDL-cholesterol. Since triglycerides are removed from TGRLs by lipases, the small particles become denser and have a bigger portion of VLDL-cholesterol, having more complicated catabolism. Moreover, in a simplified pattern, the catabolic processes can be emerged as a conversion of VLDL-cholesterol to IDL-cholesterol, which are then changed to LDL-cholesterol. The particles can be defined by their different densities (Potter et al., 1999). Another discussion was also conferred to apoB-100, which are remnant particles belonging to all type of non-HDL cholesterol. The researcher believed that the remnant particles were significant contributors to CVD as those particles would be not taken out by the liver, the preferred disposal site, in the fact that, they might be taken up in arterial walls and finally become lipid plaque (Rudijanto, 2007).

Regard to the previous studies associated to cardiovascular disease, bio-fluid samples including blood plasma and serum were the most widely used as biological sample. In a reviewed research of application of metabolomics, as a powerful strategy, obtained new cardiovascular biomarkers, there was no single biomarker that is specific enough to be used on the only marker of cardiovascular disease. Thus, the clinically useful biomarkers for cardiovascular diagnosis were well-established relationship between levels of plasma triglycerides and total cholesterol. The management of

blood lipid level was primarily purposed to control for management of hyperlipidemia and dyslipidemia, as well as, be used as prognosticator of cardiovascular risks (Hokanson & Austin, 1996; Wilson et al., 1998). The lipid biomarkers that have been investigated for last two decades examined in a great potential utility for cardiovascular health. To identify a stage of cardiovascular risk, the National Cholesterol Education Programme (NCEP) and Adult Treatment Panel (ATP)-III has defines levels of blood lipid profile related to cardiovascular risks, as following to Table 2.

Table 2 Classified risk levels of cardiovascular disease by lipidvalues including LDL-cholesterol, total cholesterol, HDL-cholesterol, and triglycerides

Lipid values	Classification of cardiovascular risk
LDL-cholesterol (mg/dL)	
< 100	Optimal
100 – 129	Near or above optimal
130 – 159	Borderline high
160 – 189	High
≥ 190	Very high
Total cholesterol (mg/dL)	
< 200	Desirable
200 – 239	Borderline high
≥ 240	High
HDL-cholesterol (mg/dL)	
< 40	Low
≥ 60	High
Triglycerides (mg/dL)	
< 150	Normal
150 – 199	Borderline high
200 – 499	High
≥ 500	Very high

Regardless of recommendations by the NCEP/ATP-III, the current treatment goals are mostly accepted by medical specialty organizations. A national survey conducted in 2003 (NEPTUNE II) reported 67% of almost 5,000 patients with high cholesterol completed their LDL-cholesterol treatment goal, based on the recommendation

(Pearson et al., 2005). Life style change is the first recommendation to reduce blood lipid level. To change in diet, control maintaining optimal weight and increasing exercise are well known to be very effective for practical management of high blood lipid profiles. However, a recent article involved to weight loss diets, it was concluded that type of food was less important than its palatability, so the long term changes was seemed to easily occur (Ignarro et al., 2007). More than 50% of participants in weight loss programs showed their weight reduction reduced related to both total cholesterol and triglycerides, but in the long term, the initial weight loss was regained after one year (Brownell & Rodin, 1994). The drawbacks of lifestyle change may be difficult to success the lipid lowering goals. If life style change into healthy living are completed, the need for using lipid lowering drugs can be declined.

8. Treatment of hyperlipidemia and dyslipidemia by lipid lowering drugs

Even a change in life-style is the first way for lipid lowering goal though, in usual pattern, lipid-lowering drugs help to manage in the different forms of lipids in hyperlipidemia patients. Available lipid lowering drugs, such as statins, fibrates, are known to decrease of free fatty acids and triglycerides. Others, such as ezetimibe and cholestyramine are also used to manage hyperlipidemia. As high blood lipid levels are very initial for cardiovascular disease, these drugs reduce the cardiovascular abnormalities in the general population. Thus, using lipid lowering drugs such as statins, cholestyramine, and other cholesterol absorption inhibitors shows highly effective to lower LDL-cholesterol, affecting a reduction of total cholesterol, and triglycerides serum level as they have been discovered the mode of action as following to

8.1 Orlistat

An inhibitor of pancreatic lipase activity that generally use for hyperlipidemia patients (Guerciolini, 1997). It is a selective inhibitor of the enzyme

without activity against chymotrypsin, phospholipases, amylase and trypsin. Its mechanism functions by binding covalent bond to the serine residue at the active site of gastric and pancreatic lipases, leading to reduce the subsequent absorption of mono-glycerides and free fatty acids.

8.2 Statins

An agent acting an inhibitory activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is drug to lower a production of cholesterol (Ward et al., 2007). HMG-CoA reductase, which is a rate-limiting process in cholesterol synthesis and other isoprenoids, can be competitive bound to substrate by statins with an affinity around 1000–10,000 times higher than that of the real substrate. The action results in a decrease in levels of plasma cholesterol indirectly due to up-regulation of the LDL-cholesterol receptor (Stancu & Sima, 2001). Furthermore, statins have been discovered their activities including inhibition of small G-protein activation, which suppresses isoprenoids (initial intermediate of cholesterol synthesis. Several epidemiological articles have established statins as the highest potent class of drugs for cardiovascular disease because of their cholesterol-lowering and anti-inflammatory effects, leading to improve vascular function and plaque stabilization.

8.3 Simvastatin or Lovastatin

They are drugs like statins. Figure 7 is shown the portion of lovastatins at the top resembles the HMG portion of HMG-CoA reductase. Both of them are chemical derivatives, which are used for the anti-hyperlipidemic medication trough the inhibition of HMG-CoA reductase. Animal and clinical studies were suggested that simvastatin was twice as ability as lovastatin (Jones et al., 1998). It lowered serum cholesterol by inhibition of hepatic cholesterol synthesis that was more importantly increased the concentration of LDL-cholesterol receptors present on hepatic cellular membranes. In clinical studies, taking simvastatin (40 mg/day) in

heterozygous-familial hypercholesterolemia patients showed the significant reduction of total cholesterol and LDL-cholesterol, as well as, had trends to reduce triglycerides, leading to increase HDL-cholesterol.

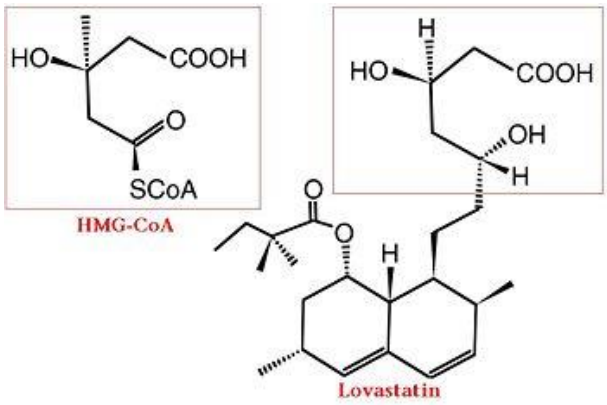


Figure 7 Structure of lovastatin and HMG-CoA reductase (Smith & Burtner, 1994)

8.4 Cholestyramine

It is known as a quaternary ammonium anion exchange resin with a very strong affinity for bile salts. Administered by cholestyramine as a chloride salt, exchanged the chloride for glycocholate and taurocholate that were related to secondary bile salts in the gastrointestinal tract (Ekelund et al., 1988). Thus, decreases in reabsorption of bile salts in the ileum occurred. This results alternatively depleted in bile salts in the blood circulation, leading to produce bile salts feeding to their pool. Because bile salts are synthesized in from cholesterol containing liver, cholesterol was likely declined.

8.5 Ezetimibe

It is an available cholesterol lowering drug that inhibits cholesterol absorption by selectively blocking the NPC1L1 protein, located in the jejuna brush border (Betters & Yu, 2010). Many studies had much attempt to find out the crucial mechanism of ezetimibe. The most possible mechanism involved with

interaction of NPC1L1/sterol complex and AP2 in clathrin coated vesicles, as shown in Figure 8. It was suggested that ezetimibe has been distorted the complex, causing to render of binding to sterols, or interfering with the free cholesterol binding at the cell membrane (Garcia-Calvo et al., 2005). Another finding indicated that ezetimibe powerfully disrupted the CAV1–annexin 2 heterocomplex, which was a small protein that forms at least two distinct chaperone complexes of both total cellular cholesterol regulation (Investigators, 2014), *in vivo*. In addition, the reduction of cholesterol uptake into the enterocyte, ezetimibe decrease cholesterol in the hepatic pools and increases the expression of the LDL receptor on hepatocytes surface, leading to decrease of LDL-cholesterol (Nutescu & Shapiro, 2003). Unlike total cholesterol, ezetimibe has no affect triglycerides and fat-soluble vitamins. In the same way, ezetimibe interacted with hepatic NPC1L1, as well as, a reduction of biliary cholesterol absorption, leading to lower serum cholesterol levels (Phan et al., 2012). In animal models, treatment of mono-therapy with ezetimibe has been reported to induce HMG-CoA reductase expression (Langone & Chuang, 2006), so the researchers found the relative treatment of hypercholesterolemia by using inhibitors ezetimibe mixed with statins. The result was discovered yields an additive effect on lowering serum cholesterol levels (Pearson et al., 2005). Ezetimibe has a long half-life of enterohepatic circuit for 22 h (Hollingworth et al., 2017) that not undergo metabolism through the cytochrome P450 pathway. However, the true mechanism where ezetimibe reduces the cholesterol into both intestinal cells and hepatocytes is still not completely concluded.

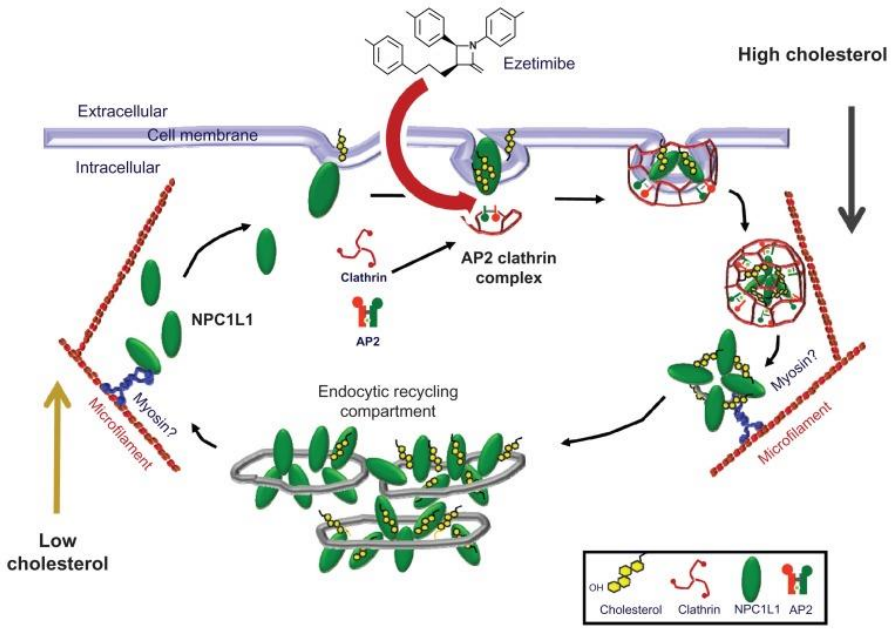


Figure 8 Interaction of ezetimibe in hypercholesterolemia situation

9. Type of inhibitory action on catalyzed enzymes

When an inhibitor binds to the catalyzed enzyme, it commonly makes non-covalent bonds, similar to hydrophobic interactions, or hydrogen bonds or ionic bonds. The enzyme would be inhibited as a reversible inhibition (Figure 9) (Williams & Morrison, 1979), which can be categorized into

9.1 Competitive inhibition

It proposes to an inhibitor that has a similar structure to the actual substrate. The inhibitor has a higher affinity than the substrate binding to the active site of the enzyme. The inhibitor and the substrate could not bind at the same period at the active site. However, the high accumulation of substrate can overcome the number of inhibitions.

9.2 Non-competitive inhibition

It involves binding with enzymes by the time of the substrate have bound to the enzyme, so it would not affect the active site. The binding complex between

enzyme and substrate has no function after that. In addition, the extent of this inhibition depends on the number of the inhibitors.

9.3 Uncompetitive inhibition

An inhibitor binds with complex of substrate-enzyme, which causes maximum speed to decrease because of the removal of activated complex. According to inhibitor bonds with the complex, the kinetic of uncompetitive could be decreased. As a result, products of enzyme will be released a smaller amount.

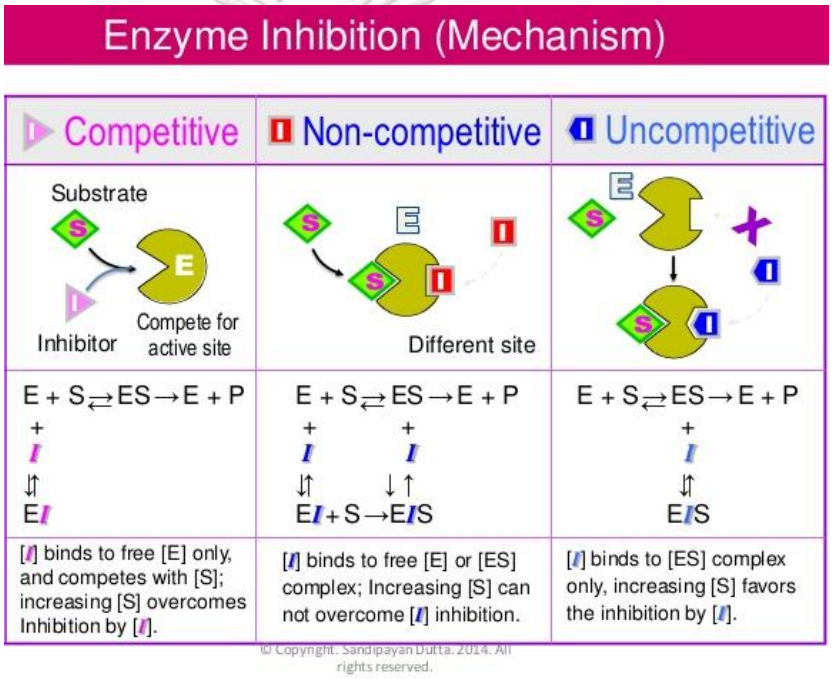


Figure 9 Category of catalyzed enzyme inhibition (Morrison, 1969)

9.4 Mixed inhibition

It is an intermediate stage between the competitive and uncompetitive inhibition. For instance, an inhibitor could bind either to enzyme or to substrate. The mixed inhibition could be reduced by increase in the number of substrate, but not completely.

10. Adverse effects of the lipid lowering drug

Along with sufficient treatment of lipid lowering drugs, unpredictable effects would be observed. For example, statins were recorded their serious adverse effects such as inflammation, dermatomyositis or polymyositis, and necrotizing myopathies (Godlee, 2014). Up to June 2007, reviewed articles were showed that adverse effects associated with ezetimibe usage had no serious clinical outcomes, except a few and mild levels of myopathy (Knopp et al., 2003). Either used alone or in combined with statins, ezetimibe has been some case reports of with mild elevations of liver transaminases. Moreover, mixing cholestyramine with simvastatin enhanced the LDL-cholesterol lowering (Molgaard et al., 1989). The clinical adverse effects were reported along with simvastatin including gastrointestinal complaints and headaches. The side effects may be severity as long term using drugs, and in fact, individual symptoms are various depending on drug type, dose of use and time, or even the health status of user.

Due to the concerns, increasing attempts was focused on using natural products as alternative strategies to the conventional lipid-modulating treatments (Graham et al., 2004). Processes of triglyceride and cholesterol digestion and absorption have been often proposed as targets for prevention of cardiovascular risks (Programme, Organization et al., 2002). Recently, scientific nutrition has been a key to apply in alternative medicine. Studied research of polyphenols containing in fruit and vegetables, especially flavonoid and anthocyanins, have become bioactive compounds to slow down the early stage of cardiovascular disease.

11. Polyphenol and health benefits

Polyphenols naturally occur compounds found highly in fruits and vegetables (Cieslik et al., 2006). They are presence in large multiples of phenol structure (Figure 10). The most common class of polyphenols is phenolic compound, which is the

secondary metabolized molecules of plants. The term ‘phenolic’ refers to compounds that have an aromatic ring bearing one hydroxyl group, while the term ‘polyphenol’ refers to having one or more aromatic rings bearing more than one hydroxyl groups.

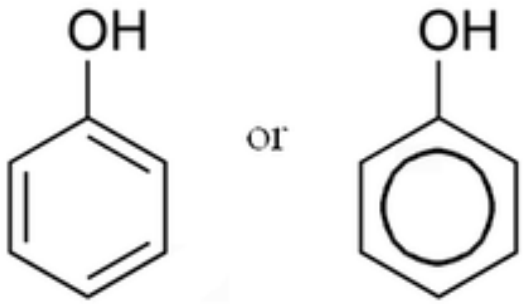


Figure 10 Phenol structure (Perron & Brumaghim, 2009)

Previous research have found these phenolic compound potentially neutralize the destructive action of reactive nitrogen/oxygen from metabolic processes in the body (Chong et al., 2010; Paganga et al., 1999). Thus, besides having a critical role in pigmentation of plants to their environment, phenolic compounds are pharmaceuticals molecules from natural sources (Haslam, 1996). Furthermore, phenolic compounds can be divided into five main groups, as shown in Figure 11.

Epidemiological studies have shown that polyphenols or phenolic compound have had a crucial protection against risks of several chronic diseases such as cardiovascular diseases, diabetes and cancer (Arts & Hollman, 2005). Specifically, flavonoids and anthocyanins were found often as doers for those benefits (Bohm et al., 1998; Wallace, 2011).

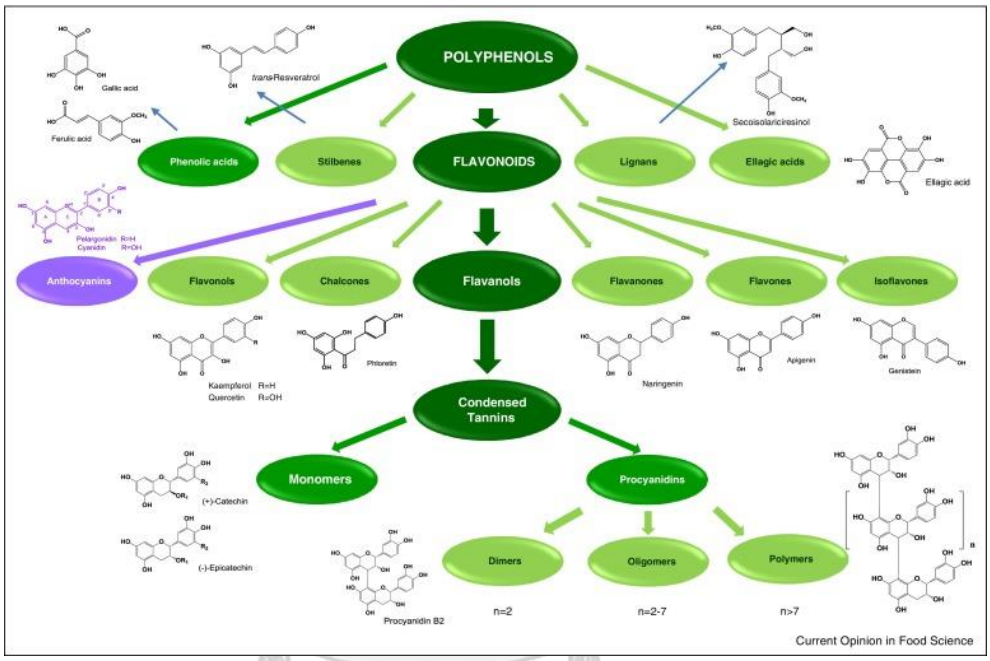


Figure 11 Polyphenol classification (Tsao, 2010)

12. Flavonoids and health benefits

Flavonoid, which have many phenolic structures, can be divided into different subgroups based on their carbon of the C ring on which the B ring is attached and the number of unsaturation and oxidation of the C ring (Figure 12). Flavones, flavonols, flavanols, flavanonols, flavanones or catechins, chalcones and anthocyanins are widely found as component of flavonoids in dietary plant. Flavonoids have been shown protective effects on many diseases such as Alzheimer's disease (AD) and atherosclerosis or CVD, and they were noted to be an agent for anti-carcinogenic, anti-

mutagenic, anti-inflammatory and antioxidant effects (Agrawal, 2011; Patel, 2008). Some studies revealed their benefits that may result from interaction with key cellular enzyme functions, and flavonoids were also recognized as effective inhibitors for many enzymes, such as xanthine oxidase (XO), cyclooxygenase (COX), phosphoinositide 3-kinase and lipoxygenase (Nileet et al., 2016).

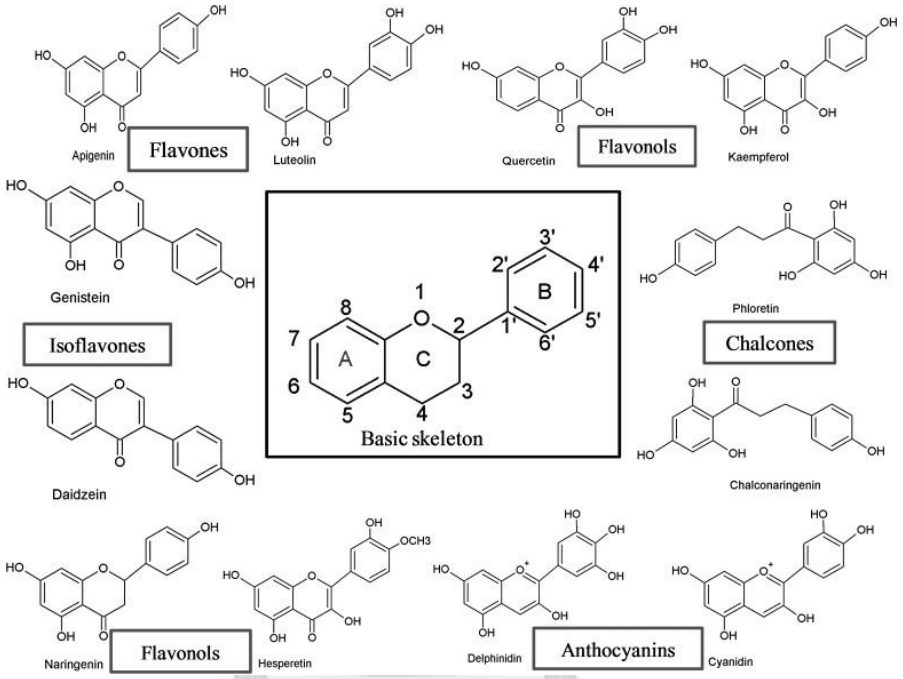


Figure 12 Flavonoids classification (Brodowska, 2017)

Flavonoids in fruits like berries are mainly anthocyanins acting as pigments determining the colors of plants, flowers and fruits.

13. Anthocyanins and health benefits

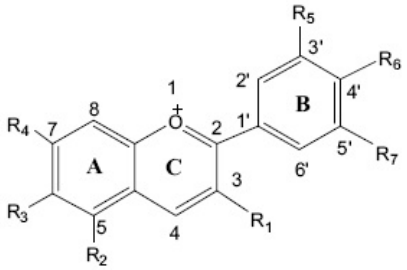
Anthocyanins are aqueous-soluble pigments contributing red to blue colors in fruits and other vegetal tissues (Castaneda-Ovando et al., 2009). They have a complex structure of glycosides of flavylium (2-phenylbenzopyrylium) salts (Figure 13). The structure differs from their derivatives by number of hydroxyl groups in their structure,

the form and number of sugar moieties of the molecule, the degree of methylation of these hydroxyl groups and the characteristic and amount of aliphatic or aromatic acids that attached to the sugars (Jackman et al., 1987).

Anthocyanins are likely stable in acidic environment (pH 1–3) because they stay in flavylium cations. At pH > 4, the anthocyanins are in the forms of the carbinol and chalcone, which could be degraded into phenolic acids or polyphenol. pH controls the expression of anthocyanin color as the pH affect the acylation and methylation of hydroxyl groups in the anthocyanin structure (Iacobucci & Sweeny, 1983).

In previous studies, anthocyanin absorption into Caco-2 cells were found that anthocyanins are able to be transported through Caco-2 monolayers with their intact glycone forms (Yi et al., 2006). Pelargonidine-3-glucoside found mainly in strawberry extract was revealed that its trace amount can be detected on the basolateral side of the epithelium, as well as, anthocyanins from blueberry extracts was found averaged 3%–4% after being transported (Kamiloglu et al., 2015). In the same way, anthocyanin found in açaí fruit were transported with 0.5% to 4.9% (Kamiloglu et al., 2015). Based on *in vivo* studies, it can be summed up that anthocyanins has a very low bioavailability (less than 1% of the ingested amount)

In addition, anthocyanins have gained relevant to the food industry due to their widespread use as natural alternatives for artificial colors along with knowledge of their health benefits.



General anthocyanins structure

Name	Abbreviation	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH

Figure 13 General anthocyanins structure (Castañeda-Ovando et al., 2012)

Cyanidin, pelargonidin, malvidin, peonidin and delphinidin can be defined as the common anthocyanins found in plant, so they all usually studied and reported their ability for health. In addition, their stability together with their health benefits of those subgroup of anthocyanins can facilitate themselves to be applied in the food industry and food application, recently (Nestle, 2013). The properties of anthocyanins have been evident as various researches have reported the following benefits.

13.1 Anti-oxidant activity

The free-radical scavenging event, known as antioxidant, of anthocyanin are clearly suggested including the mechanisms such as prevention of DNA cleavage, boosting production of cytokines, enzyme inhibition, lipid peroxidation, membrane strengthening and a decrease in capillary permeability and fragility (Kong et al. ,2003). A previous study has shown and confirmed that the effects of the different active groups in the B ring and different glycosylation forms of anthocyanin may significantly change the antioxidant and antiradical activities, thus direction of the change could depend on the aglycones. Glycosylation of cyanidin to cyaniding-3-

glucoside increased the activities, but glycosylation of malvidin to the corresponding 3-glucoside lower the activities. Pelargonin and its 3-glucoside also showed similar responses. Another investigation reported the DPPH radical scavenging activities of the mono-glucosides of pelargonidin, cyanidin and peonidin were less than activities of their aglycons (Wang et al., 1999). However, another influence which is of the absorption and metabolism of anthocyanin-glycosides has been continuously studied and reported.

13.2 Anti-cancer activity

Anthocyanins were responsible at least in part of anti-cancer activity. Their abilities may result in the additive effect in multiple mechanisms (Wang & Stoner, 2008). The results from *in vitro* studies showed different anti-proliferative effects in different anthocyanins including types of aglycone, glycosylation patterns as well as acylation (Bagchi et al., 2004) on the colon cancer cells. The possible mechanisms were found that have been indicated as inhibition of oxidative DNA damage, COX-2 enzymes and carcinogen activation, and induction of phase II enzymes for detoxification (Lazze et al., 2003). Chokeberry anthocyanins (25 μg/mL) provided haft inhibition of the carcinoma cell line with no effect on the growth of normal colonic cells (Jing et al., 2008). Another study was proposed that the inhibitory result of dietary anthocyanins in an azoxymethane (AOM)-induced rat colon cancer model was firstly emerged to the direct effect of improving colon luminal condition (Harris et al., 2001). Research results also reported a high correlation between the growth of colon cancer and the total anthocyanin concentration in the colonic lumen as evaluated by fecal anthocyanin level, increasing fecal moisture content and excretion of bile acids via feces.

13.3 Anti-inflammatory activity

Inflammation is a multiple biological response of tissue injury caused by cyclooxygenases (COXs). As COX can convert arachidonic acid to prostaglandins that stimulate the inflammation, an inhibitory activity on COX enzymes is highly desirable (Jung et al., 2015). COX activities assays reported that anthocyanins, specifically its aglycone, have more effective anti-inflammatory activity when compared to aspirin (Andersen et al., 2004). The research studies on strawberries, blackberries, and raspberries reported that anthocyanin fractions presented inhibitory effect on COX-1 and COX-2 enzymes with the highest activity when compared to positive controls (ibuprofen and naproxen). Moreover, *in vivo* studies on pharmacological activity of blackberry anthocyanins, mainly cyanidin-3-glucosides, investigated in rats with carrageenan-induced lung inflammation (Rossi et al., 2003) showed that inflammatory parameters were decreased in a dose-dependent manner by anthocyanin.

13.4 Prevention of obesity

Consumption of anthocyanins may prevent obesity and metabolic syndrome. The process of down regulation of the mRNA levels of enzymes of fatty acid and triacylglycerol synthesis was noted to contribute to this anti-obesity aibility (Kwon et al., 2007). The researchers also previously reported that the contents of RNA isolated from adipocytes was treated with cyanidins or cyanidin-3-glucoside, 633 and 427 genes, respectively. These were upregulated by higher than five-fold (Tsuda et al., 2004). Due to the gene expression profile, the enhancement of the lipolytic activity and up-regulation of hormone-sensitive lipase were eventually summarized to be influenced by anthocyanin treatment on adipocytes (Tsuda et al., 2005).

13.5 Control of type 2 diabetes

Insulin is secreted from the β-cells of the pancreas and is responsible for activation of blood glucose transport into adipose tissue and skeletal muscle. Experimental data elucidating anthocyanin were useful for diabetes and pancreatic disorders (Ghosh & Konishi, 2007). Its efficacy was affected the multiple, simultaneous biological results by component of anthocyanins including a decrease of blood sugar concentrations in blood serum and urine (Van Dam et al., 2013). Moreover, anthocyanins potentially protect β-cells from glucose-induced oxidative stress with its antioxidant effect (Sun et al., 2012). Another examination showed that boysenberry anthocyanins could control oxidative stress in streptozotocin-induced diabetic rats as the result showed a decrease the onset of diabetic complications (Ghosh et al., 2006).

13.6 Decrease of cardiovascular disease risk

Since dietary anthocyanins have antioxidant effect by increase in serum antioxidant capacity. This property could indirectly work against oxidation of LDL-cholesterol, which potently lead to prevention of cardiovascular diseases (Wallace et al., 2016). Using a chemiluminescent assay of serum antioxidant capacity (SAOC) with normal subjects that have ingested of anthocyanin-rich red wine (300 ml) together with vitamin C (1000 mg), the result was found an increase in the mean SAOC when compared to white wine (He & Giusti, 2010). However, the effects may be following to a prolongation in peroxyl-radical-driven of oxidized LDL-cholesterol leading to a decrease in maximum oxidation rate. In addition, pelagonidin-3-glucosides, cyanidin-3-glucocides, and delphinidin-3-glucosides demonstrated to strongly inhibit lipid peroxidation through neutralizing an active oxygen radical scavenging agents (Narayan et al., 1999).

13.7 Downregulation of lipidemic gene expression

In animal models and clinical trials, diestary anthocyanins had effects to decrease LDL-cholesterol and increase HDL-cholesterol concentrations as well as enhance cellular cholesterol efflux to blood circulation after the inhibition of Cholesteryl Ester Transfer Protein (CETP) (Riaz et al., 2016). A previous research suggested that increase of HDL-cholesterol were probably influenced by dietary anthocyanins by excreting the excess cholesterol from adipocyte through an active process mediated by ABCA1 (ATP-binding cassette transporter number 1) (Xia et al., 2005).

14. Toxicity of anthocyanin

Animal and human trials have been done in order to study the effects of oral consumption of anthocyanins for a few years. It was found the no adverse event form the oral intake of anthocyanins on those model (Pojer et al. , 2013). Moreover, Natural anthocyanins have been used broadly as food colorants in food products and beverages with permission in many big country such as Japan, USA, Europe (E163) (Wissgott & Bortlik, 1996). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) also summed up that anthocyanin-rich extracts showed a little toxicity in awareness on early toxicological reports with reproductive toxicity, mutagenicity, teratogenicity, a short phase of toxicity evaluations (Bkowska-Barczak, 2005). Previous observation was reported the dose of anthocyanins intake in young rats (averaged 225 mg/kg body weight) had no toxic in a two-generation reproduction. Thus, the mean acceptable daily intake (ADI) for human was proposed at 2.5 mg/kg body weight/day, using the equation of ADI = NOEL/100 (He & Giusti, 2010).

15. Bio-accessibility of anthocyanins

Nutrient and components for their health benefits depend mostly on their bioactivity. The bioactivity occur after the components has reached systemic circulation in the body including uptake to tissues and their metabolism, interaction with biomolecules, and individuals physiological effects (Cardoso et al., 2015).

Anthocyanins have been previously observed by using Caco-2 cells model for their accessibility in intestinal situation. The cells were treated with pure standard and anthocyanin-rich extracts from plants was showed that those anthocyanins had very low bioactivity and the derivatives of anthocyanins were also suggested to vary in many different levels depend on the natural sources (Kamiloglu et al., 2015). The metabolism of anthocyanins found in Caco-2 cells model could be inferred from phase I and II transformations, simulating within the human body. In detail, phase I transformations, which occur less frequently, include oxidation, reduction and hydrolysis. Another phase, namely biotransformations, occurs more intensively and may take place in the liver and the small intestine. This phase II composes of conjugated reactions where different derivatives including methyl, glucuronic and sulfate derivatives were formed (Cardona et al., 2013). Anthocyanins was methylation in phase II, leading to alter the changes in hydroxyl and methoxyl groups of B ring when compared to the native compound. Previous investigation of blueberry anthocyanins was found total anthocyanin (approximately 42%) and their antioxidant property with 29% were declined during intestinal digestion (Liu et al., 2014). The researchers also discovered some free hydroxyl groups and a number of methoxy groups in the B ring, leading to improve anthocyanin stability after consumption. In addition, it was found that more hydrophobic anthocyanins were better absorbable than more hydrophilic anthocyanins.

The excretion of anthocyanins was also discussed. Total ingested anthocyanins (0.03 and 4%) were found in urinary excretion with the maximum excretion time of 1–4 h (Manach et al., 2005). Another study also suggested that their basic anthocyanin skeleton in the urine found up to 24 h (Kay et al., 2005), thus the biological activities change with metabolic transformation including glycosylation and acylation patterns. Moreover, a glucose moiety may protect the stability of indigested anthocyanins based on the finding of an increase in bioavailability of cyanidin and peonidin anthocyanidins found in cranberry juice (Milbury et al., 2010). Fifteen anthocyanins containing different aglycons and conjugated group of sugars in blueberries, boysenberries, black raspberries, and blackcurrants were indicated that their intact and not metabolized anthocyanins were detected in both humans and rats (McGhie et al., 2003).

16. Definition of berry

Berry is an edible fruit produced from a single ovary. Its pulp is juicy and fleshy with many seeds in the fleshy pulp. The seeds are usually embedded in the fleshy interior of the ovary. Fruits like grapes, bananas, tomatoes, and blueberries are categorized as true berries (Jordan, 1988).

17. Berries anthocyanins and lipid lowering effects

Berry pulp is a rich source of polyphenols, especially flavonoids and anthocyanins. Anthocyanins were major compound to express the color intensity of the berry pulp (Bridle & Timberlake, 1997). In several clinical studies demonstrated that intake of different forms such as fresh berries, berry juices, freeze-dried berries, or purified anthocyanin extracts from strawberries blueberries, and chokeberries resulted in the significant improvements in oxidation of LDL-cholesterol, total plasma antioxidant capacity, and dyslipidemia (Narayan et al., 1999). A research on high fat fed to albino

rats was reported that intake of anthocyanin (0.04 g/day) decresed total cholesterol decreased by 19.7% and the taking of anthocyanin (0.05 g/day) lower triglyceride by 34.4% with (Prior et al., 2009). Another result reported that the decrease of cholesterol was influenced by increase in excretion of fecal neutral and acidic sterols with 22–29% and 41–74%, respectively (Liang et al., 2013). On Fischer rats fed with anthocyanin-rich grape-bilberry juice or an anthocyanin-depleted control juice for 10 weeks had decreased both total serum cholesterol and triglycerides, but it had no effects on serum non-esterified fatty acids, insulin and glucose (Graf et al., 2013). Furthermore, mice fed with HF60 diet plus juice of purified anthocyanins from blueberries for over 8 weeks had lower body fat than controls (60% high fat-fed) (Prior et al., 2010). The mechanism influencing these effects may be indicated by previous investigation on qualitative–quantitative evaluation of anthocyanin pigment that *in vitro* bioactivity is related to inhibit pancreatic lipase activity. Results from thirteen extracts showed a strong relationship between number of total anthocyanin content and pancreatic lipase inhibition (Fabroni et al. ,2016). In addition, the beneficial effect of the decrease in LDL-cholesterol and increase in HDL-cholesterol levels may be caused by dietary antocyanins on a suppression of cholesteryl ester transfer protein (CETP) activity (Fabroni et al., 2016). In the same way, anthocyanin-rich purple corn supplementation could suppress the mRNA levels of enzymes, and the result was involved in free fatty and triglycerides synthesis in high-fat fed mice (Chang et al., 2013). In the case with increase in HDL-cholesterol concentration, the researchers found a decrease value in rats fed with high fat diet containing 0.037% black soybean anthocyanins (Kwon et al., 2007). The result of dietary blueberry anthocyanins fed to hamsters was accompanied by increase in bile acid excretion and lowering the sterol retention through down-regulation of intestinal NPC1L1, ACAT, MTP, and ABCG8, leading to lower plasma cholesterol when compared with control (Chen et al., 2011). Recently, there were investigations on lipid lowering mechanism by anthocyanins in black rice, and the

results showed a potential mechanism of lowering cholesterol level by precipitation between anthocyanins and cholesterol (Yao et al., 2013). The anthocyanins led to cholesterol precipitation in micelle solution, as well as, reduction in cholesterol uptake in Caco-2 cells.

Berries in the tropical countries, like Thailand, should have their capacity for the anti-hyperlipidemia, similar to those investigated berries. Thai berries such as Thai plums or Luknhai (*Prunus domestica* L.) (Chaiyasut et al., 2016; Slimestad et al.,2009), Mamao (*Antidesma bunius* (L.) *Spreng*) (Belmi et al., 2014; Islary et al., 2017; Jorjong et al., 2015), Lukwha (*Syzygium cumini (*L.*) Skeels*) (Kubola et al., 2011; Swami et al., 2012), Makiang (*Syzygium nervosum* A. Cunn. Ex DC) (Charoensin et al.,2012; Sultanbawa & Sultanbawa, 2016), and Ta-kob or Jamaican cherries (*Muntingia calabura* L.) (Kubola et al., 2011; Sibi et al., 2012) have red, dark purple and dark blue pulp pigment. The studies of Thai berries have already revealed their phytochemicals contents related to antioxidant properties.

Prunus domestica L. have appearance similar to prune and have already been reported to contain average total phenolic compound (1.8 g/kg) in the edible part (Usenik et al.,2008). Cyanidin-3-rutinoside was found to be the main anthocyanin in *Prunus domestica* L., although there are no investigations regarding its effects on lipid digestion and absorption (Usenik et al., 2009). Many studies have documented the effectiveness of western plums or prunes to collect oxygen free radicals such as superoxide ($O2^{-}$) and per-oxy radicals ($ROO^{\cdot}$) compared to dried counterparts (Najafabad & Jamei, 2014). *In vitro* investigation showed that plum or prune extracts have anticancer properties by cultured macrophages and blocking of tumor cell growth (Fujii et al., 2006). Plum extracts also showed capabilities to improve learning and memory in mice evaluated by using passive avoidance test, improve cognitive function in mice assessed by Morris Water Maze, as well as, acted as antioxidant and anti-

ulcerogenic activity (Chang et al., 2016). Recently, Kuo and his colleagues reported the effects of plum powders on ameliorative symptoms of neurodegenerative conditions (increase in cholesterol and β-amyloid) (Kuo et al., 2015).

Antidesma bunius (L.) *Spreng* are rich in cyaindin, while also containing malvidin, pelargonidin, delphinidin (Jorjong et al., 2015). The phenolic compounds in *Antidesma bunius* (L.) *Spreng* showed great potential of antioxidant assay including ABTS+, DPPH, and FRAP. Chowtivannakul and his team explored the ethanol seed extract from *Antidesma bunius* (L.) *Spreng* and found hypoglycemic and hypolipidemic effects through the recovering pathology of hematology resulting from its antioxidant and insulin secretion activities (Chowtivannakul et al., 2016).

Syzygium cumini (L.) *Skeels* has been studied and reported to be an anti-diabetic agent as it was used as an alternative to treat hyperglycemia (Bopp et al., 2009; Teixeira et al., 1997). A previous study found *Syzygium cumini* (L.) *Skeels* has high total phenolic compound related to antioxidant activity (Banerjee et al., 2005), while another study using fresh fruits showed *Syzygium cumini* (L.) *Skeels* contains glucose, mallic acid, raffinose, fructose, citric acid, gallic acid and anthocyanins (malvidin-3-laminaribioside, petunidin-3-gentiobioside, delphinidin-3-gentiobioside, malvidin, cyanidin, petunidin and diglycoside) (Ayyanar & Subash-Babu, 2012). Study results showed rich content of flavonoids having the capabilities to scavenge free radicals and having protective effect on antioxidant enzymes (Ayyanar & Subash-Babu, 2012; Banerjee et al., 2005). Flavonoids in *Syzygium cumini* (L.) *Skeel* have been found to have hypolipidemic activity likely achieved through inhibition of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (Malik et al., 2016). Flavonoid-rich extract from *Syzygium cumini* (L.) *Skeel* seeds was found to improve hyperglycemia by restoring peripheral glucose tolerance in the same animal model along with finding of activating adipocyte via peroxisome proliferator-activated receptors (PPAR) alpha and gamma

(Chagas et al., 2015). In addition, quercetin, an important flavonoid found in jambolan berries, has been revealed to improve adipocyte glucose uptake via PPAR gamma upregulation. Additionally, quercetin-3-rutinoside has the ability to improve insulin release via stimulation of calcium influx (Malik et al., 2016).

Thai researchers have recently reported finding of cyanidin 3-glucoside, a major anthocyanin in ripe *Cleistocalyx nervosum* var. *paniala,* fruits like *Syzygium nervosum* A. Cunn. Ex DC (Jansom et al., 2008). Another study has also found that *Cleistocalyx nervosum var. paniala* extract has beneficial effect in preventing against Cd-induced oxidative damage in renal tissue, and antioxidant property (Charoensin et al., 2012).

Muntingia calabura L. has been studied and found to contain polyphenol content as well as other properties including anti-inflammatory (Zakaria et al., 2007), anti-oxidant (Nivethetha et al., 2009), and inhibition of lipid-peroxidation (Preethi et al., 2010). The total phenolic contained in *Muntingia calabura* L. has been evaluated ranging from 1486 mg GAE /100 of fresh mass to 358 mg GAE/100 of fresh mass (Preethi et al., 2010). The researcher also reported high levels of antioxidant contributing to correlation between antioxidant activities and total phenolic or flavonoid contents. However, these properties may be influenced by its phenolic compound with a lesser focus on lipid lowering effects. These bioactive compounds found in the aforementioned five Thai berries have potentials for other beneficial effects, including lipid lowering properties.

CHAPTER III

METHODOLOGY

1. Experiment 1

Phytochemical compositions of aqueous anthocyanins-rich Thai-berries extracts from *Muntingia calabura* L. (Takob), *Prunus domestica* L. (Thai plum), *Syzygium nervosum* A. Cunn. Ex DC. (Makiang), *Antidesma bunius* (L.) *Spreng.* (Mamao), *Syzygium cumini* (L.) *Skeels.* (Lukwha)

1.1 Aims of experiment 1:

To quantify total phenolic, flavonoids and anthocyanins contents of aqueous anthocyanins-rich Thai-berries extracts

1.2 Methods of experiment 1:

1.2.1 Sample preparation:

Thai berries were obtained from various locations of Thailand; *Muntingia calabura* L. from Uthaitanee province, *Prunus domestica* L. from Maehongson province, *Syzygium nervosum* A. Cunn. Ex DC. from Lumpang province, *Antidesma bunius* (L.) *Spreng.* from Sakonnakhon province, and *Syzygium cumini* (L.) *Skeels.* from Phrae province. The collected fruits were authenticated by Taxonomist at Department of Botany, Faculty of Science, Chulalongkorn University, Thailand (Appendix). Ripen Thai berries were picked up during the seasonal period: *Muntingia calabura* L. during July to September, *Prunus domestica* L. during October to Decembe, *Syzygium nervosum* A. Cunn. Ex DC. during May to July, *Antidesma bunius* (L.) *Spreng.* during August to October, and *Syzygium cumini* (L.) *Skeels.* during May to July. Then, they were selected as only trials, presented with dark red and purple pigments. For each Thai berry,

its stock (≥10 kg) was divided into 5 pouches (2 kg/pouch) and stored at -20 °C for further analysis.

1.2.2 Berries extraction:

The berries were defrosted overnight and kept only pulps to be mashed for the extraction. Berries extraction process was performed according to the described method in McDougall, et al. (McDougall et.al, 2011) with some minor modifications. For each Thai berry, its pulp (100 g) was extracted by 200 mL distilled water (DW) in a blender. Blending the pulp was carried at the lowest speed (level 1) for 10 sec. The blend was then filtered with cheesecloth to keep aqueous solution. The remaining residues was repeated for extraction (DW: residues = 1:2) and filtered for two times. The total aqueous solution was centrifuged at 4000 rpm for 5 min at 4°C before being filtered through No.1 Whatman filter-papers under vacuum. pH value of the clear solution was also checked. Solution having pH ranged between 1 and 3 was accepted for the investigation since this pH range related to be suitable for the stabilized anthocyanins-structure, known as flavylium cation (Rahman et al., 2006). The total crude-extracted solution was finally freeze-dried and kept at -20°C for the next step. Percent yield of freeze-dried Thai berries extracts was also recorded and calculated.

Equation 1 Yield (%) of freeze-dried Thai berries extracts

$$\text{Yield (\%)} = \left[\frac{\text{Amount (g) of freeze-dried Thai berry extract obtained}}{\text{Amount (g) of berry pulps}} \right] \times 100$$

1.2.3 Anthocyanins purification

Anthocyanins purification, using solid-phase extraction (SPE), was performed by following the protocol by McDougall, et al., 2011, with some minor modifications. The purification need very clear solution of aqueous freeze-dried Thai berries extracts for being loaded in a SPE cartridge (Stratar C18-E, 6 g capacity; Phenomenex Ltd., Macclesfield, UK). To prepare the clear solution for SPE, freeze-dried extracts of *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng.* and *Syzygium cumini* (L.) *Skeels.* were dissolved in a minimum volume of distilled water and then centrifuged at 4000 rpm for 5 min at 4 °C. In another way, freeze-dried extract of *Muntingia calabura* L. had to be dissolved by absolute methanol first and dried by an evaporator to get rid of its mucilage that actively obstructed the SPE cartridge flow. After clearing out the mucilage, freeze-dried *Muntingia calabura* L. extract was dissolved by distilled water and centrifuged at 4000 rpm for 5 min at 4°C.

The C18 SPE cartridges, which have a high affinity for the glycosylated anthocyanins, were preconditioned with 0.2% formic acid in acetronitrile and then equilibrated with acidified water (0.2% formic acid). The cartridges were washed with the acidified water and then distilled water after having been loaded by the aqueous samples. 80% acetonitrile was used to elute the SPE cartridges. The purified aqueous extracts were removed from the eluent (80% acetonitrile) through a rotary evaporation. All purified Thai-berries extracts, called as antocyanins-rich Thai berries extracts, were stored at -20°C for further investigations. They were also weighed, recorded and calculated the percent yield, using the equation below.

Equation 2 Yield (%) of anthocyanins-rich Thai berries extracts

$$\text{Yield (\%)} = \left[\frac{\text{Amount (g) of purified Thai berry extract obtained}}{\text{Amount (g) of freeze-dried extract}} \right] \times 100$$

1.3 Phytochemicals determinations

All anthocyanins-rich Thai berries extracts and chemical substances were weighted and prepared before the investigations

1.3.1 Chemical substances

1.3.1.1 Gallic acid

1.3.1.2 Folin-Ciocalteu's phenol reagent: Sigma-Aldrich, USA

1.3.1.3 Sodium carbonate (NaNO3): Ajax Finechem, Australia

1.3.1.4 Sodium nitrite (NaNO2): Sigma-Aldrich, USA

1.3.1.5 Catechin: Sigma-Aldrich, USA

1.3.1.6 AlCl3: Univar, Australia

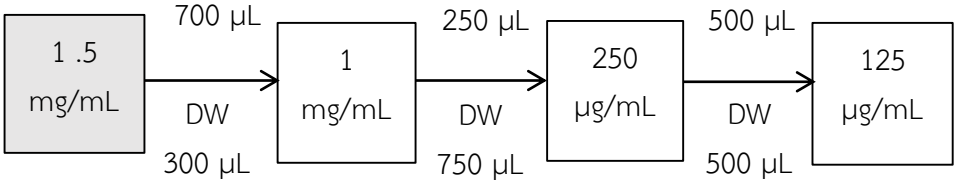
1.3.1.7 Sodium acetate (CH_3COONa)

1.3.1.8 Potassium chloride (KCl)

1.3.2 Preparation of aqueous anthocyanins-rich Thai berries extracts

(0.125 - 0.25 mg/mL) for phytochemical determination

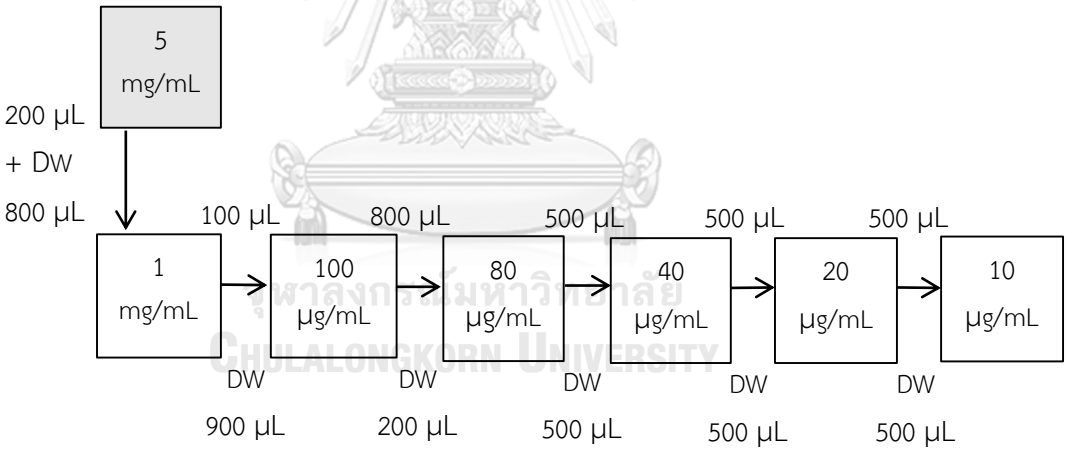
Stocks of aqueous anthocyanins-rich Thai berries extracts were freshly prepared by completely dissolving 1.5 mg of anthocyanins-rich Thai berries extracts with 1 mL distilled water (DW) in 1.5 mL tube. The stocked solution of anthocyanins-rich Thai berries extracts (1.5 mg/mL) was diluted by distilled water as described below



1.3.3 Determination of total phenolic (TP)

1.3.3.1 Preparation of Gallic acid (GA), (0 – 80 µg/mL), for total phenolic determination

Stock of Gallic acid solution was freshly prepared by completely dissolving Gallic acid powder (5 mg) with 1 mL distilled water (DW) in 1.5 mL tube. The stocked aqueous Gallic acid (5 mg/mL) was then diluted by distilled water for the serial dilution as following to



1.3.3.2 Preparation of Folin-Ciocalteu reagent (FCR) for total phenolic determination

This reagent was prepared before 5 min being used. 0.5 mL of Folin-Ciocalteu reagent was mixed well to DW (5 mL), and the mixture should be used with in 3 h due to its less sensitivity with light (Lowry et al., 1951).

1.3.3.3 Preparation of 10% (w/v) sodium carbonate ($NaCO_3$) for total phenolic determination

The stock solution (250 mL) of $NaCO_3$ was prepared by completely dissolve 25 g of $NaCO_3$ in DW (250 mL). The $NaCO_3$ solution was kept in 4°C for the investigation.

The determination of total phenolic was carried out by using the Folin-Ciocalteu method with some minor modifications (Mäkynen et al., 2013). An aliquot (50 µL) of aqueous anthocyanins-rich Thai berries extracts was mixed with 50 µL of Folin-Ciocalteu (FCR) reagent (FC:DW = 1:10). After 5 min incubation at room temperature, 50 µL of 10% (w/v) sodium carbonate ($NaCO_3$) was added. The reaction was then incubated further for 20 min at room temperature. 0.125 - 0.25 mg/mL of aqueous anthocyanins-rich Thai berries extracts were selected to measure their absorbance. The absorbance was measured at 760 nm by a microplate spectrophotometer (BioTek instruments, USA). Total phenolic was calculated to compare with a calibration curve of Gallic acid (GA), (0 – 80 µg/mL, as shown in Figure 14, and the results were expressed in milligram of Gallic acid equivalent per 1 gram extract (mg GAE/g extract).

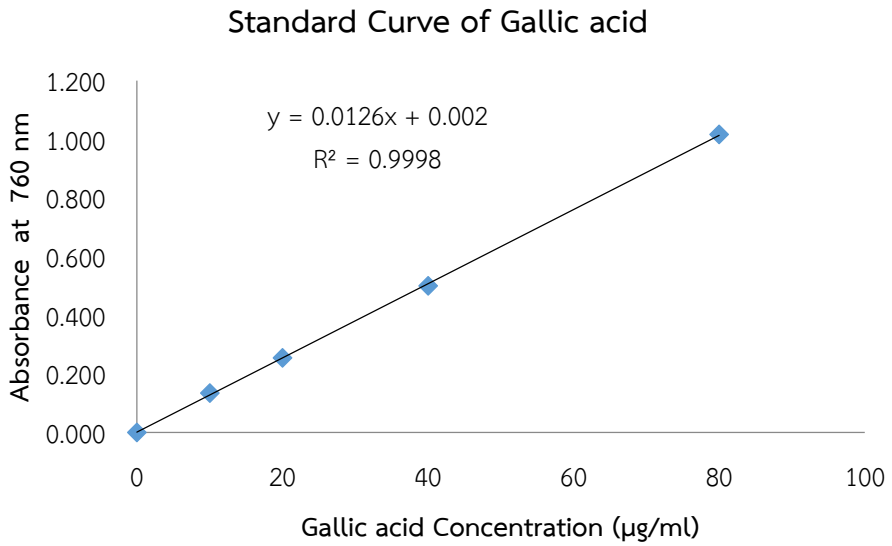


Figure 14 An example of calibration curve of Gallic acid (0 – 80 µg/mL)

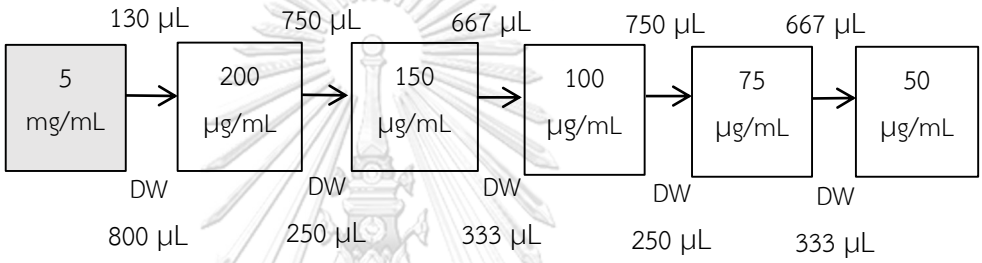
Table 3 Investigation of total phenolic determination

Investigation	Treatment	Standard	Reagent blank	Sample blank
Performed in the low light zone				
Extract (µL)	50	-	-	50
GA (µL)	-	50	-	-
DW (µL)	-	-	50	50
FCR (µL)	50	50	50	-
Incubated in the room temperature for 5 min				
$NaCO_3$ (µL)	50	50	50	50
Total (µL)	*150*	*150*	*150*	*150*
Incubated in the room temperature for 20 min				
Read the Abs at 760 nm through a microplate spectrophotometer				
Each conc. was done on triplicated reaction				

1.3.4 Determination of total flavonoids (TF)

1.3.4.1 Preparation of Catechin (CE), (0 – 200 µg/mL), for total flavonoids determination

Stock of Catechin acid solution was freshly prepared by completely dissolving Catechin powder (5 mg) with 1 mL distilled water (DW) in 1.5 mL tube. The stocked aqueous (5 mg/mL) was diluted by distilled water for the serial dilution as following to



1.3.4.2 Preparation of 5% (w/v) $NaNO_2$ for total flavonoids determination

The stock solution (250 mL) of $NaNO_2$ was prepared by completely dissolve 12.5 g of $NaNO_2$ powder in DW (250 mL). The $NaNO_2$ solution was then kept in 4°C for the investigations.

1.3.4.3 Preparation of 2% (w/v) aluminium tri-chloride ($AlCl_3$) for total flavonoids determination

The stock solution (250 mL) of $AlCl_3$ was prepared by completely dissolving 5 g of $AlCl_3$ powder in DW (250 mL). The $AlCl_3$ solution was then kept in 4°C for the investigation.

1.3.3.4 Preparation of 1 M NaOH for total flavonoids determination

The stock solution (250 mL) of NaOH was prepared by completely dissolving 10 g of NaOH powder in DW (250 mL). The NaOH solution was then kept in the room temperature for the investigations.

The determination of total flavonoids was performed by using a colorimetric assay, described by Sariburun et al. with some minor modifications (Sariburun et al., 2010). Aqueous anthocyanins-rich Thai berries extracts (50 µL) were mixed with 50 µL of 5% (w/v) $NaNO_2$. The reaction was incubated for 5 min at room temperature before being added 30 µL of 2% (w/v) aluminium tri-chloride ($AlCl_3$). The incubation was further incubated for 15 min at room temperature and 20 µL of 1 M sodium hydroxyl (NaOH) was added. The absorbance was determined at 510 nm after 5 min of incubation with NaOH, using a micro-plate spectrophotometer (BioTek instruments, USA). The equation was obtained from the calibration curve of Catechin (Figure 15). The results were expressed in milligram of Catechin equivalent per 1 extract gram extract (mg CE/g extract).

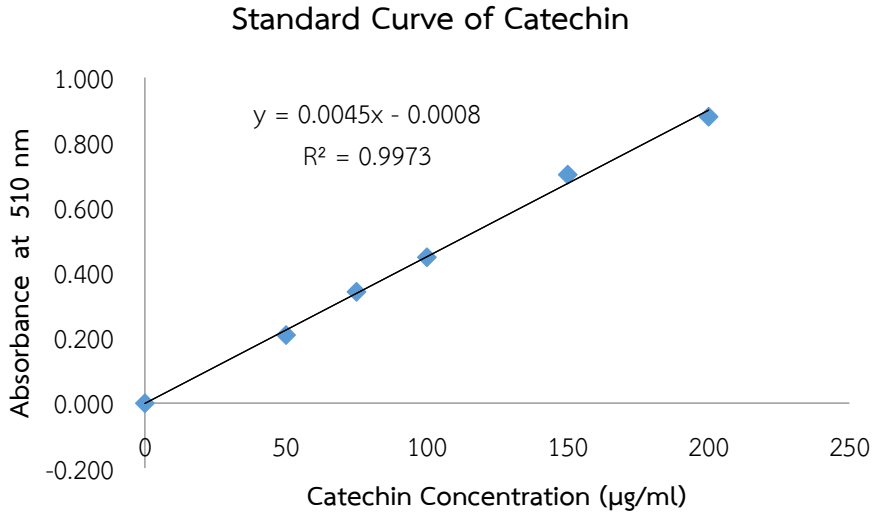


Figure 15 An example of a calibration curve of Catechin (0 – 200 µg/mL)

Table 4 Investigation of total flavonoids determination

Investigation	Treatment	Standard	Reagent blank	Sample blank
Extract (µL)	50	-	-	50
CE (µL)	-	50	-	-
DW (µL)	-	-	50	50
$NaNO_2$ (µL)	50	50	50	50
Incubated for 5 min at room temperature				
$AlCl_3$ (µL)	30	30	30	-
Incubated for 15 min at room temperature				
NaOH (µL)	20	20	20	20
Total (µL)	*150*	*150*	*150*	*150*
Incubated for 5 min at room temperature				
Read the Abs at 510 nm through a microplate spectrophotometer				
Each conc. was done on triplicated reaction				

1.3.5 Determination of total anthocyanin (TA)

1.3.5.1 Preparation of 0.4 M sodium acetate (CH_3COONa) buffer, pH 4.5 for total anthocyanins determination

The solution (250 mL) of 0.4 M CH_3COONa was prepared by completely dissolving 13.6 g of CH_3COONa powder in DW (250 mL). After well mixing, the aqueous CH_3COONa was then measured its pH value through a pH meter. To set the pH 4.5, the aqueous CH_3COONa was adjusted by adding 6 M HCl (~ 1-3 mL), and then added more distilled water to level at 250 mL in a volumetric flask. The CH_3COONa buffer was kept at 4°C for the investigation within a week.

1.3.5.2 Preparation of 0.025 M potassium chloride (KCl) buffer, pH 1 for total anthocyanins determination

The stock solution (250 mL) of 0.025 M KCl was prepared by completely dissolving 0.466 g of KCl powder in DW (250 mL). After well mixing, the aqueous KCl was then measured its pH value through a pH meter. To set the pH 1, the solution was adjusted by adding 6 M HCl (~ 1-10 mL), and added more distilled water to level at 250 mL in a volumetric flask. The KCl buffer was kept in 4°C for the investigation within a week.

The total anthocyanins contents were determined by pH differential method using a spectrophotometer (Unicam UV/VIS) (Sariburun et al., 2010). The absorbance of aqueous anthocyanins-rich Thai berries extracts was measured at 510 and 700 nm in pH 1.0 of 0.025 M potassium chloride (KCl) buffer and in pH 4.5 of 0.4 M sodium acetate (CH_3COONa) buffer, respectively. 500 µL of the aqueous extracts were mixed with 500 µL of each buffer, and left to equilibrate for 20 min at room temperature, in low light area. The absorbance (Abs) 510 and 700 nm was recorded and calculated. The results were expressed in milligrams of cyanidin-3-glucoside equivalent per 1 gram extract (mg of cy-3-glu/g extract), based on the equation below.

Equation 3 Anthocyanins pigment

$$\text{Anthocyanins pigment (Cyanidin-3-Glucoside equivalents, mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

When $A = (Abs_{510} - Abs_{700})_{pH\ 1.0} - (Abs_{510} - Abs_{700})_{pH\ 4.5}$

MW = Molecular weight of cyanidin-3-glucoside
= 449.2 g/mol
DF = Dilution factor = 2
l = Path length in cm = 1
ε = 26,900 molar extinction coefficient, L/mol/cm for cyanidin-3-glucoside
10^3 = Factor for conversion from g to mg

Table 5 1) Investigation of total anthocyanins determination at pH 1

Investigation	**Treatment**	**Reagent blank**
Extract (µL)	500	-
KCl buffer (µL)	500	-
DW (µL)	-	1000
Total (µL)	*1000*	*1000*
Left to equilibrate for 20 min at room temperature		
Read Abs at the wavelength of 510 and 700 nm		
Each conc. was done on fifth-plicate reaction		

Table 5 2) Investigation of total anthocyanins determination at pH 4.5

Investigation	**Treatment**	**Reagent blank**
Extract (µL)	500	-
CH_3COONa buffer (µL)	500	-
DW (µL)	-	1000
Total (µL)	*1000*	*1000*
Left to equilibrate for 20 min at room temperature		
Read Abs at the wavelength of 510 and 700 nm		
Each conc. was done on fifth-plicate reaction		

1.4 Statistical analysis of phytochemical determinations

Values are presented as Mean ± SEM (n = 3). Statistical analyses were performed by using one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test. P-value < 0.05 was considered to be statistically significant by using SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA).

2. Experiment 2

Investigations of aqueous anthocyanins-rich Thai berries extracts from *Muntingia calabura* L. (Takob), *Prunus domestica* L. (Thai plum), *Syzygium nervosum* A. Cunn. Ex DC. (Makiang), *Antidesma bunius* (L.) *Spreng.* (Mamao), *Syzygium cumini* (L.) *Skeels.* (Lukwha) on lipid lowering activities

2.1 Aims of experiment 2:

To determine lipid lowering activities of aqueous anthocyanins-rich Thai berries extracts via bile acids binding, pancreatic lipase and cholesterol esterase activities, and cholesterol micellization.

2.2 Materials and methods of experiment 2:

2.2.1 Plant material

Thai berries extracts were weighed and completely dissolved in distilled water and chemical substances were prepared.

2.2.2 Chemical substances

2.2.2.1 Oleic acid

2.2.2.2 P-nitrophenylbutylrate (p-NPB)

2.2.2.3 Lipase from porcine pancreas type II: Sigma-Aldrich, USA

2.2.2.4 Phosphatidylcholine

2.2.2.5 Taurocholic acid: Sigma-Aldrich, USA

2.2.2.6 Glycodeoxycholic acid

2.2.2.7 Taurodeoxycholic acid

2.2.2.8 Porcine cholesterol esterase: Sigma-Aldrich, USA

2.2.2.9 Cholesterol test kits: Genway

2.2.2.10 Total bile acid test kit: Genway

2.2.2.11 4-methylumbelliferyl oleate (4-MUO)

2.2.2.12 Dimethyl sulfoxide (DMSO): Sigma-Aldrich, USA

2.2.2.13 Hydrocholoric acid (HCl): Merck, Germany

2.2.2.14 Magnesium sulfate: Fisher Scientific, USA

2.2.2.15 Potassium cholride: Fisher Scientific, USA

2.2.2.16 Sodium chloride: Ajax Finechem, Australia

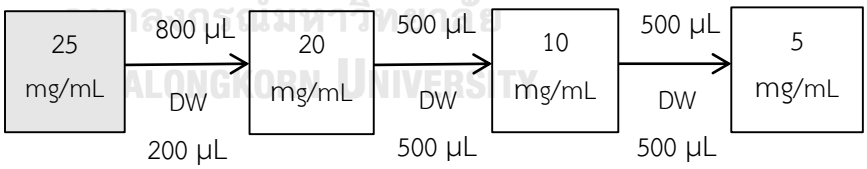
2.2.2.17 Methanol (analysis grade): Merck (Darmstadt, Germany)

2.2.2.18 Ethanol (analysis grade): Merck (Darmstadt, Germany)

2.2.3 Determination of bile acid binding

2.2.3.1 Preparation of aqueous anthocyanins-rich Thai berries for bile acid binding assay

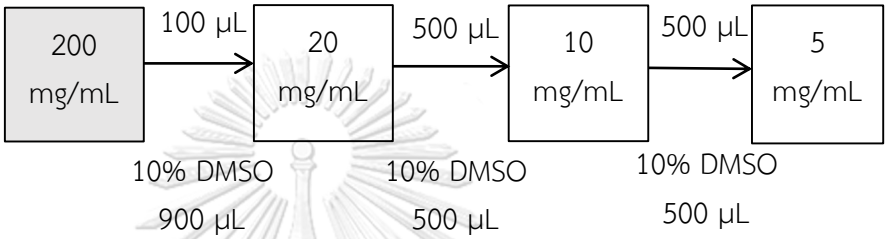
Stocks of aqueous anthocyanins-rich Thai berries extracts were freshly prepared by completely dissolving 25 mg of anthocyanins-rich Thai berries extract with 1 mL distilled water (DW) in 1.5 mL tube, and then this solution was diluted by distilled water as following;



2.2.3.2 Preparation of cholestyramine in 10% DMSO for bile acid binding assay

Stocks of cholestyramine were prepared by completely dissolving 4 g of resincolestiramina with 20 mL 10% DMSO in 50 mL tube. Because of toxicity (Violante et al., 2002), the final concentration of DMSO, which was used as a solvent of drugs, in this investigation had to be not greater than 1%. After well vortex,

the stocked cholestyramine solution (200 mg/mL) was centrifuged at 4000 rpm for 5 min at 4 ℃ to keep the supernatant for being centrifuged twice. In the last step, the clear supernatant of stock cholestyramine solution was diluted by 10% DMSO as following the diagram below, and the serial dilutions were stocked at 4 ℃ for the experiments.



2.2.3.3 Preparation of phosphate-buffered saline (PBS: 0.1 M, pH 7) for bile acid binding assay

The stock solution (1 L) of 0.1 M PBS was prepared by completely dissolved chemical substances as following to the table below in 800 mL of distilled water. After well mixing, the PBS solution was then measured its pH value through a pH meter. To set the pH 7, the PBS solution was adjusted by adding 1 M NaOH (~ 1-3 mL) before adding more distilled water to level at 1 L in a volumetric flask. The PBS buffer was kept in 4℃ for the investigations.

Table 6 Chemical substances of phosphate-buffered saline (PBS 0.1 M, pH 7) for bile acid binding assay

Substance	Molecular weight (g/L)	In a 1 L bigger		
		Amount (g)	+	DW (mL)
50 mM NaH_2PO_4	140	7.0	+	800
50 mM Na_2HPO_4	141.0	7.1	+	
100 mM NaCl	58.4	5.8	+	
Adjust to pH 7 by adding 1 M NaOH				
Fill distilled water to reach the total volume of 1000 mL				

2.2.3.4 Preparation of bile acids solution for bile acid binding assay

Taurocolic acid, taurodeoxycholic acid, and glycodeoxycholic acid were freshly prepared by dissolving in the PBS (0.1 M, pH 7). Bile acid solution (n) was prepared as following table below.

Table 7 Prepared concentration of bile acid solution for bile acid binding assay

Bile acids	Molecular weight (g/L)	In 15 mL tube		
		Bile acids (g)	+	PBS (mL)
2 mM Taurocholic acid	537.7	5.4	+	5
2 mM Taurodeoxycholic acid	521.7	5.2	+	5
2 mM Glycodeoxycholic acid	471.6	4.8	+	5

The role bile acids in the intestinal absorption of lipid at the microvillus membrane are to solubilize dietary fat and the other products of lipolysis, as well as to overcome the resistance of the unstirred water layer adjacent to the brush border. Therefore, any substance with capability for directly binding capability to

bile acid may cause a decrease in the intestinal bile acid activities, leading to lower lipid digestion and absorption in the small intestine.

Bile acid binding assay was performed according to the described method with minor modifications (Makynen et al., 2013). Taurocholic acid (TCA), glycodeoxycholic acid (GDA) and taurodeoxycholic acid (TDA) were used as bile acids in this assay. 20 µL of aqueous anthocyanins-acid Thai berries extracts (0.5 - 2 mg/mL) were incubated with 2 mM of each bile acid contained in phosphate-buffered saline (PBS: 0.1 M, pH 7) at 37 °C for 90 min. After the incubation, the mixtures (200 µL) were centrifuged at 5000 rpm for 5 min to maintain free bile acids, which have not been bound by the extracts, for analysis with a bile acid tested kit. Cholestyramine (CTR) in 1% DMSO (0.5 – 2 mg/mL) was used as a positive control, while distilled water and 1% DMSO were used as negative control. The results were calculated by absorbance (Abs) at 540 nm, 37 °C through a 96-well plate reader using the equation below.

Equation 4 Bile acid binding (%)

$$\text{Bile acid binding (\%)} = \left[\frac{\text{Abs of negative control} - \text{Abs of an inhibitor}}{\text{Abs of negative control} - \text{Abs of an inhibitor}} \right] \times 100$$

Table 8 Investigation of bile acid binding assay

Investigation	Treatment			Positive			Negative					
Extract (µL) (0.5, 1.0, 2.0 mg/mL)	20	20	20	-	-	-	-	-	-	-	-	-
CTR (µL) (0.5, 1.0, 2.0 mg/mL)	-	-	-	20	20	20	-	-	-	-	-	-
DW (µL)	-	-	-	-	-	-	20	20	20	-	-	-
1% DMSO (µL)	-	-	-	-	-	-	-	-	-	20	20	20
TCA	180	-	-	180	-	-	180	-	-	180	-	-
TDA	-	180	-	-	180	-	-	180	-	-	180	-
GDA	-	-	180	-	-	180	-	-	180	-	-	180
Total (µL)	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*
Incubated at 37 ℃ for 90 min and centrifuged at 5000 rpm for 5 min												
Determined amount of free bile acids BQ kits; Total bile acid colorimetric assay (BQ 092A-EALD), Std conc. = 34.8 µmol/L												

Table 9 Final concentration of substances in the bile acid binding assay

Investigation	Prepared conc. (mg/mL)	Final conc. (mg/mL)
Aqueous extracts	5	0.5
	10	1
	20	2
Cholestyramine	5	0.5
	10	1
	20	2
Negative	DW	0
	10% DMSO	1% DMSO
Bile acids solution	2 mM	1.8 mM

2.2.3.5 Analysis of fee bile acids

Free bile acid was evaluated by total bile acids assay kit (Colorimetric), Catalog Number: BQ 092A-EALD, (Appendix).

Assay principle: Substances that converts bile acids to 3-keto steroids and NADH include NAD, the enzyme 3-hydroxysteroidehydrogenase (3-HSD) produce the final molecule, NADH, to react with nitrotetrazolium blue (NBT) to form a formazan dye in the presence of diaphorase enzyme. The dye formation is used to measure the absorbance at 540 nm.

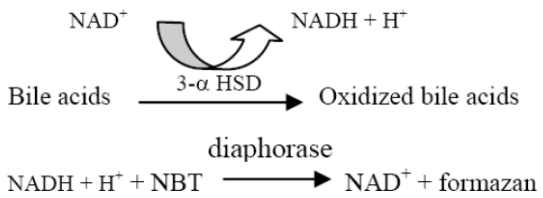
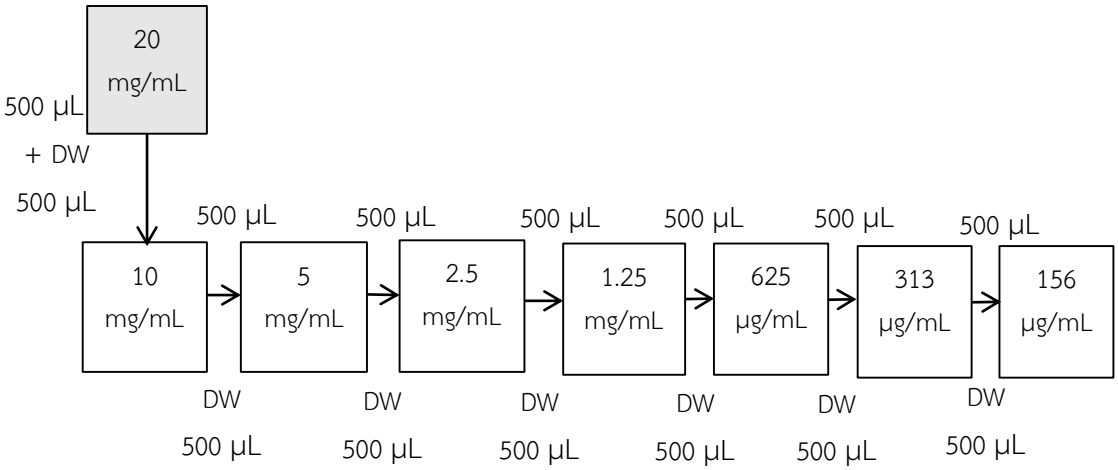


Figure 16 Reaction of free bile acid determination

2.2.4 Determination of pancreatic lipase inhibition (Lipase)

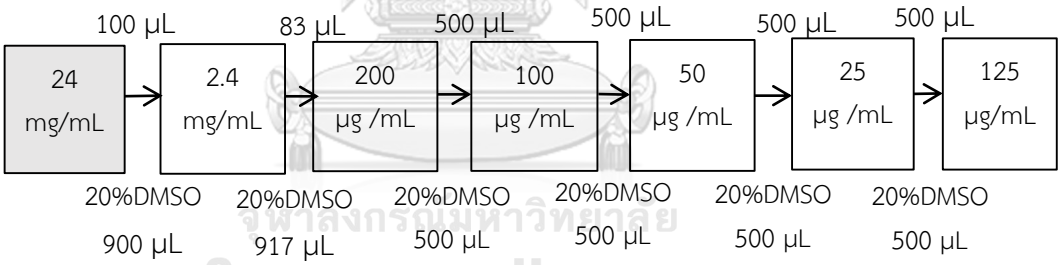
2.2.4.1 Preparation of aqueous anthocyanins-rich Thai berries extracts for pancreatic lipase inhibition assay

Stocks of aqueous anthocyanins-rich Thai berries extracts were freshly prepared by completely dissolving 20 mg of anthocyanins-rich Thai berries extract with 1 mL distilled water (DW) in 1.5 mL tube. The stocked aqueous extracts were diluted by distilled water as following diagram.



2.2.4.2 Preparation of orlistat in 20% DMSO (OST) for pancreatic lipase inhibition assay

Stock of orlistat solution, a positive control, was prepared by completely dissolving of 120 mg orlistat, (Xenical) with 1 mL absolute DMSO in 1.5 mL tube. Because of toxicity, the final concentration of



DMSO, which was used as a solvent of drugs, in this investigation had to be not greater than 1%. After well vortex, the stocked orlistat solution was centrifuged twice at 4000 rpm for 5 min at 4 °C to collect the supernatant. In the last step, the clear supernatant of 120 mg/mL orlistat (0.2 mL) was mixed to 0.8 mL of distilled water. The stocked orlistat solution in 20% DMSO (24 mg/mL) was then diluted by 20% DMSO, as shown below. The serial dilutions were stocked at -20 °C for the experiment.

2.2.4.3 Preparation of phosphate-buffered saline (PBS: 0.1 M, pH 7) for pancreatic lipase inhibition assay

The solution (1 L) of 0.1 M PBS was prepared by completely dissolving the chemical substances as following to the below table in 800 mL of distilled water. After well mixing, the PBS solution was then measured its pH value through a pH meter. To set the pH 7, the PBS solution was adjusted by adding 1 M NaOH (~ 1-3 mL) before being added more distilled water to level at 1 L in a volumetric flask. The PBS buffer was kept in 4°C for the investigations.

Table 10 Prepared concentration of chemical substances in phosphate-buffered saline (PBS: 0.1 M, pH 7) for pancreatic lipase inhibition assay

Substance	Molecular weight (g/L)	In a 1 L bigger		
		Amount (g)	+	DW (mL)
50 mM NaH_2PO_4	140.0	7.0	+	
50 mM Na_2HPO_4	141.0	7.1	+	800
100 mM NaCl	58.4	5.8	+	
Adjust to pH 7 by adding 1 M NaOH				
Fill distilled water to reach the total volume of 1000 mL				

2.2.5.4 Preparation of 4-Nitrophenyl butyrate (4-MUO) for pancreatic lipase inhibition assay

4-methylumbelliferyl oleate powder (0.48 mg) was prepared freshly by completely dissolving in 12 mL of 100 mM PBS (pH 7), containing 100 mM NaCl. This step had to be performed in low light

zone. The solution was then immediately stored in -20°C. This solution needed to be warm in the room temperature 5 min before testing.

2.2.5.5 Preparation of pancreatic lipase for pancreatic lipase assay

Lipase powder from porcine pancreas (3.34 mg) was prepared freshly by dissolving in 12 mL of mM PBS (pH 7), containing 100 mM NaCl. The solution was then immediately stored in -20°C. This solution needed to be warm in the room temperature 5 min before testing.

Inhibition of pancreatic lipase activity was slightly modified due to a previously described method by Makynen K, et al. with some minor modifications (Makynen et al., 2013). The porcine pancreatic lipase activity was measured by using 4-methylumbelliferyl oleate (4-MUO) as a substrate. The lipase stocked-solution (0.278 mg/mL) was freshly prepared in 0.1 M phosphate-buffered saline (PBS, 100 mM NaCl, pH 7) and shortly kept at -20 °C. To determine the lipase activity, aqueous anthocyanins-rich Thai berries extracts (0.4 – 500 µg/mL) and a positive control, orlistat (0.0625 - 10 µg /mL) in 1 % DMSO, were mixed with 50 µL of 0.05 mM 4-MUO that had been dissolved in the PBS. The reaction began when 45 µL of the lipase solution was added. This reaction required a low light environment. The reaction, having total volume of 100 µL was then immediately incubated at 37 °C for 20 min before adding 100 µL of sodium citrate (0.1 M, pH 4.2) to end the activity. Fractions of 4-MUO substrate, which had not been digested by the lipase, were measured with a fluorescence micro-plate reader at the excitation wavelength of 355 nm and the emission wavelength of 460 nm. Distilled water and 1% DMSO (negative controls) were also examined. The results can be expressed in IC_{50}, an inhibitory concentration that represents the effectiveness of a substance in inhibiting a specific biological or biochemical function at a single, specified

concentration by 50%. The percentage of inhibition was calculated by using the formula below

Equation 5 Inhibition (%) of pancreatic lipase activity

Inhibition (%) of pancreatic lipase activity

$$= \left[\frac{\text{Fi of negative control} - \text{Fi of an inhibitor)}}{\text{Fi of negative control}} \right] \times 100$$

When **Fi** = Fluorescence intensity value of each reaction

Table 11 Investigation of pancreatic lipase inhibition assay

Investigation	**Treatment**	**Positive**	**Test blank**		**Negative**		**Negative blank**	
Performed the test in very low light area								
Extract (µL)	5	-	5	-	-	-	-	-
OST (µL)	-	5	-	5	-	-	-	-
DW (µL)	-	-	-	-	5	-	5	-
1% DMSO (µL)	-	-	-	-	-	5	-	5
4-MUO (µL)	50	50	50	50	50	50	50	50
Lipase (µL)	45	45	-	-	45	45	-	-
Incubated at 37 °C for 20 min								
NaCi (µL)	100	100	100	100	100	100	100	100
PBS (µL)	-	-	45	45	-	-	45	45
Total (µL)	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*
Read abs at wavelength of 355 nm/460 nm								
Each conc. was done on triplicated reaction								

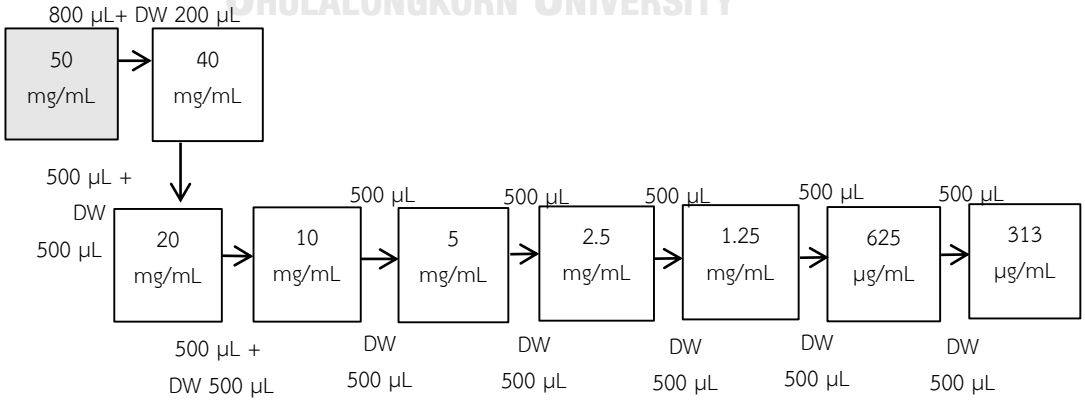
Table 12 Final concentration of substance in the pancreatic lipase inhibition assay

Substance	Prepared conc. (mg/mL)	Final conc. (µg/mL)
Sample or control	0 – 0.5	0 - 500
4-MUO	0.1 mM (0.04)	0.05 mM (0.02)
Lipase	0.278	0.125
Negative control	DW	0
	20% DMSO	1% DMSO

2.2.5 Determination of cholesterol esterase inhibition (CEsae)

2.2.5.1 Preparation of aqueous anthocyanins-rich Thai berries for cholesterol esterase inhibition assay

Stocks of aqueous anthocyanins-rich Thai berries extracts were freshly prepared by completely dissolving 50 mg of anthocyanins-rich extract with 1 mL distilled water (DW) in 1.5 mL tube. The stocked aqueous extract was diluted by distilled water as following to



2.2.5.2 Preparation of simvastatins in 40% DMSO (Sim) for cholesterol esterase inhibition assay

Stocks of simvastatin were prepared by completely dissolving of 40 mg simvastatin (ZOCOR) with 1 mL of 40% DMSO in 1.5 mL tube. Because of toxicity, the final concentration of DMSO, which was used as a solvent of drugs, in this investigation had to be not greater than 1%. After well vortex, the stocked simvastatin solution was centrifuged at 4000 rpm for 5 min at 4 ℃ to keep the supernatant for being centrifuged twice. The clear supernatant of simvastatin in 40% DMSO (40 mg/mL) was kept in -20 ℃ for the experiment.

2.2.5.3 Preparation of phosphate-buffered saline (PBS: 0.1 M, pH 7, TCA 5.16 mM) for cholesterol esterase inhibition assay

The stock solution (1 L) of 0.1 M PBS was prepared by completely dissolving chemical substances as following to the table below in 800 mL distilled water. After well mixing, the PBS solution was then measured its pH value through a pH meter. To set the pH 7, the PBS solution was adjusted by adding 1 M NaOH (~ 1-5 mL) before being added more distilled water to level at 1 L in a volumetric flask. The PBS buffer was kept in 4℃ for the investigations.

Table 13 Prepared concentration of chemical substances in phosphate-buffered saline (PBS: 0.1 M, pH 7, TCA 5.16 mM) for cholesterol esterase inhibition assay

Substance	Molecular weight(g/L)	In a 1 L bigger		
		Amount (g)	+	DW (mL)
50 mM NaH_2PO_4	139.99	7.0	+	800
50 mM Na_2HPO_4	141.96	7.1	+	
100 mM NaCl	58.44	5.8	+	
5.16 mM Taurocholic acid	537.68	2.8	+	
Adjust to pH 7 by adding 1 M NaOH				
Fill distilled water to reach the total volume of 1000 mL				

2.2.5.4 Preparation of 4-Nitrophenyl butyrate (p-NBP) for cholesterol esterase inhibition assay

4-Nitrophenyl butyrate powder (0.6 mg) was prepared freshly by dissolving in 10 mL of 100 mM PBS (pH 7), containing 5.16 mM taurocholic acid. The solution was then stored in 4°C. This solution needed to be warm in the room temperature 5 min before testing.

2.2.5.5 Preparation of cholesterol esterase for cholesterol esterase inhibition assay

Cholesterol esterase powder (0.04 mg) was prepared freshly by dissolving in 10 mL of 100 mM PBS (pH 7), containing 5.16 mM taurocholic acid. The solution was then stored in -20°C. This solution needed to be warm in the room temperature 5 min before testing.

Cholesterol esterase has been proposed in three functions in the small intestine: first, to control absorption of cholesterol from dietary cholesterol esters: second, to contribute to the forming of cholesterol into mixed micelles and: third, to help in the transportation of free cholesterol to the enterocyte. Thus, it is predicted that the inhibition of cholesterol esterase can be used to limit the intestinal cholesterol absorption.

Cholesterol esterase inhibition was performed by using the described protocol with some minor modifications (Makynen et al., 2013). Aqueous anthocyanins-rich Thai berries extracts (7-1000 µg/mL) were mixed with 75 µL of 100 mM phosphate-buffered saline, containing 5.16 mM taurocholic acid (TCA) and 100 mM NaCl, pH 7.0, and 75 µL 0.2 mM p-NPB and that had been already dissolved in the PBS. The reaction was started by adding 45 µL cholesterol esterase (0.556 µg/mL). After the incubation at room temperature for 20 min, the mixture (200 µL) was immediately measured at the 405 nm absorbance (Abs) through a spectrophotometry 96-well micro-plate reader. Simvastatin (ZOCOR) in 1% DMSO was used as a positive control while distilled water and 1% DMSO were played as negative controls in this assay. The results were expressed in IC_{50} and the percentage of inhibition was calculated using the formula below.

Equation 6 Inhibition (%) of cholesterol esterase activity

Inhibition (%) of cholesterol esterase activity

$$= \left[\frac{(\text{Abs of negative control} - \text{Abs of an inhibitor})}{\text{Abs of negative control}} \right] \times 100$$

Table 14 Investigation of cholesterol esterase inhibition assay

Investigation	**Treatment**	**Positive**	**Test blank**		**Negative**		**Negative blank**	
Extract (µL)	5	-	5	-	-	-	-	-
Sim (µL)	-	5	-	5	-	-	-	-
DW (µL)	-	-	-	-	5	-	5	-
1% DMSO (µL)	-	-	-	-	-	5	-	5
PBS (µL)	75	75	120	120	75	75	120	120
p-NBP (µL)	75	75	75	75	75	75	75	75
CEase (µL)	45	45	-	-	45	45	-	-
Total (µL)	*200*	*200*	*200*	*200*	*200*	*200*	*200*	*200*
Incubated at room temperature for 20 min, and the mixture (200 µL) was then immediately measured Abs at the 405 nm								
Each conc. was done on triplicated reaction								

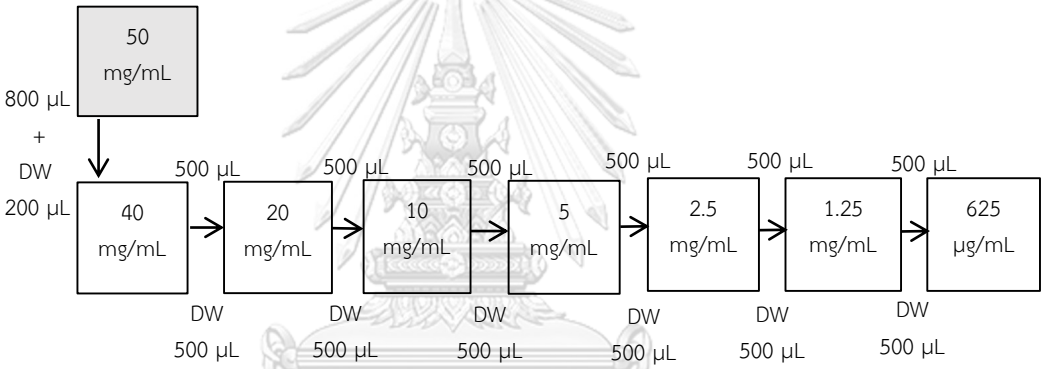
Table 15 Final concentration of chemical substances for cholesterol esterase inhibition assay

Substance	**Prepared conc. (mg/mL)**	**Final conc. (µg/mL)**
Sample or control	0 – 40	0 - 1000
p-NBP	0.3 mM (0.06)	0.2 mM (0.04)
CEase solution	0.556	0.125
Negative control	DW	0
	40% DMSO	1% DMSO

2.2.6 Determination of cholesterol micellization (Micelle)

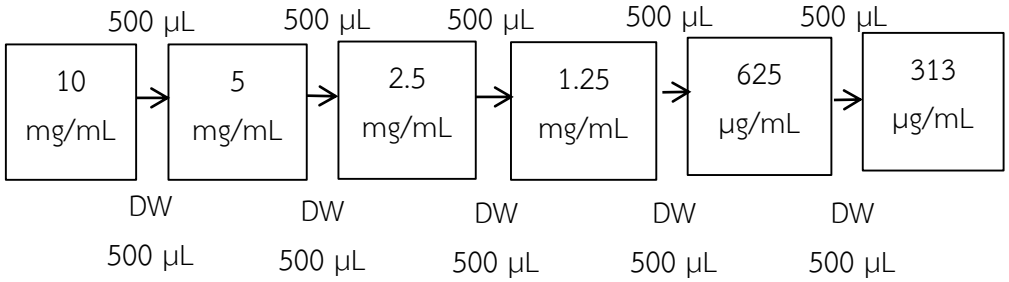
2.2.6.1 Preparation of aqueous anthocyanins-rich Thai berries extract for cholesterol micellization assay

Stocks of aqueous anthocyanins-rich Thai berries extracts were freshly prepared by completely dissolving 50 mg of anthocyanins-rich Thai berries extract with 1 mL distilled water (DW) in 1.5 mL tube. The stocked aqueous extract (50 mg/mL) was diluted by distilled water as following to



2.2.6.2 Preparation of aqueous Gallic acid for cholesterol micellization assay

Stock of aqueous Gallic acid was freshly prepared by completely dissolving Gallic acid powder of 5 mg with 0.5 mL distilled water (DW) in 1.5 mL tube. The stocked aqueous Gallic acid (10 mg/mL) was diluted by distilled water for the serial dilution as following to



2.2.6.3 Preparation of phosphate-buffered saline (PBS: 15 mM, taurocholate 6.6 mM, pH 7) for cholesterol micellization assay

The stock solution (1 L) of PBS was prepared by completely dissolving chemical substances following to the table below in 800 mL distilled water. After well mixing, the PBS solution was then measured its pH value through a pH meter. To set the pH 7, the PBS solution was adjusted by adding 1 M NaOH (~ 1-5 mL) before being added more distilled water to level at 1 L in a volumetric flask. The PBS buffer was kept in 4°C for the investigations.

Table 16 Prepared concentration of phosphate-buffered saline (PBS: 15 mM, TCA 6.6 mM, pH 7) for cholesterol micellization assay

Substance	Molecular weight (g/L)	In a 1 L bigger		
		Amount (g)	+	DW (mL)
7.5 mM NaH_2PO_4	140.0	1.1	+	800
7.5 mM Na_2HPO_4	142.0	1.1	+	
15 mM NaCl	58.4	0.9	+	
6.6 mM Taurocholic acid	537.7	3.6	+	
Adjust to pH 7 by adding 1 M NaOH				
Fill distilled water to reach the total volume of 1000 mL				

A typical mixed micelle in aqueous aggregates when the hydrophilic head regions are surrounded by solvent and sequesters with hydrophobic single-tail regions (cholesterol, free fatty acids, or phospholipids) at the micelle center. The solubility of cholesterol in mixed micelle may alter the uptake of cholesterol by the brush border membrane and may decrease cholesterol binding capability to cholesterol receptors since mixed micelles generally plays a role in the transportation of lipid-particles (Iqbal & Hussain, 2009). Therefore, decreases in cholesterol solubility in mixed micelle may potentially affect the dietary cholesterol absorption in the small intestine as well as others lipid-particles.

Artificial micelle was prepared according to the described method with some minor modifications (Makynen et al., 2013). Micelle solution (2 mM cholesterol, 1 mM oleic acid, and 2.4 mM phosphatidylcholine) was completely dissolved in methanol and then dried under nitrogen. After being re-dissolved by 15 mM phosphate-buffered saline (PBS), containing 6.6 mM taurocholate salt, pH 7, the suspension was sonicated twice for 20 min via a sonicator. The micelle solution was then incubated overnight at 37 °C. In the investigation, 20 µL of aqueous anthocyanins-rich Thai berries extracts (7-500 µg/mL) were incubated with the micelle solution at 37 °C for 2 h. The mixture (200 µL) was then centrifuged at 10,000 rpm for 25 min. The supernatant was collected in order to investigate free cholesterol, using total cholesterol test kits. Gallic acid (15 - 250 µg/mL) was used as a positive control, whereas distilled water was used as negative control in the assay. The absorbance (Abs) was measured via a 96-well plate reader at 500 nm. The results were expressed in IC_{50}, and the inhibitory percentage was calculated using the following formula:

Equation 7 Inhibition (%) of cholesterol micellization

Inhibition (%) of cholesterol micellization

$$= \left[\frac{\text{Abs of negative control} - \text{Abs of an inhibitor}}{\text{Abs of negative control}} \right] \times 100$$

Table 17 Investigation of cholesterol micellization assay

Investigation	**Treatment**	**Positive**	**Test blank**	**Negative**	**Negative blank**	
Extract (µL)	20	-	5	-	-	-
GA (µL)	-	20	-	5	-	-
DW (µL)	-	-	-	-	5	5
Micellar sol (µL)	180	180	-	-	180	180
PBS (µL)	-	-	180	180	-	180
Total (µL)	*200*	*200*	*200*	*200*	*200*	*200*
Incubated at 37 °C for 2 h, and then centrifuged at 10,000 rpm for 25 min						
Measured Abs via a 96-well plate reader at 500 nm						
Amount of free cholesterol Human cholesterol test kits; Cholesterol liquicolor Std. conc. = 200 mg/dl (5.17 mmol/l)						
Each conc. was done on triplicated reaction						

Table 18 Final concentration of chemical substances for cholesterol micellization assay

Substance	Prepared conc.(mg/mL)	Final conc. (µg/mL)
Sample/control	0 – 5	0 - 500
Micelle solution (Mic Sol)		
Cholesterol	0.773	0.696
Oleic acid	0.282	0.254
Phosphatidylcholine	1.843	1.659
Negative control	DW	DW

2.3 Statistical analysis

Values are presented as Mean ± SEM (n = 3). Statistical analyses were performed by using one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test. P-value < 0.05 was considered to be statistically significant by SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA). The IC_{50} (half maximal inhibitory concentration) was calculated by plotting a curve of log concentration between concentration of samples and percentages of inhibition. Correlation analysis was obtained by using bivariate correlations and expressed with Pearson's correlation coefficient (*r*).

3. Experiment 3

Determination of an anthocyanins-rich Thai berry extract on cholesterol uptake into Caco-2 cells

3.1 Aims of experiment 3:

To investigate the inhibitory effect of an anthocyanins-rich Thai berry extract on cholesterol uptake into Caco-2 cells, chemical substances were prepared. Thai berry extract was selected to perform based on the following criteria:

3.1.1 Highest amount of anthocyanins content

3.1.2 Highest binding (%) to bile acids (at least 2 types of bile acids)

3.1.3 Lowest IC_{50} values of pancreatic lipase and cholesterol esterase activities

3.1.4 Highest inhibition (%) of cholesterol micellization

3.2 Materials and methods of experiment 2:

3.2.1 Plant material

Thai berry extract and ezetimibe were weighed and dissolved by 100% DMSO. Chemical substances were weighed and prepared before the investigations

3.2.2 Chemical substances

3.2.2.1 The Caco-2 cells, reported to morphologically and functionally express a combination of colonocytes and enterocytes under specific conditions were obtained from ATCC® Number: HTB-37™.

3.2.2.2 High and low-glucose Dulbecco's modified Eagle medium (DMEM)

3.2.2.3 Non-essential amino acids

3.2.2.4 Fetal bovine serum (FBS): Gibco, USA

3.2.2.5 Penicillin/streptomycin (10,000 U/mL penicillin, 10,000 µg/mL streptomycin): Gibco, USA

3.2.2.6 Ezetimibe (EZETROL™)

3.2.2.7 22-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-23, 24-bisnor-5-cholen-3-ol (NBD-cholesterol)

3.2.2.8 HEPES sodium salt: Sigma-Aldrich, USA

3.2.2.9 Thiazolyl blue tetrazolium bromide (MTT): Sigma-Aldrich, USA

3.2.2.10 Tris (hydrodroxymethylo) amonomethane: Bio-Rad, USA

3.2.3 Caco-2 cell culture

Colon adenocarcinoma cells (Caco-2 cell) from human colon adenocarcinoma were routinely maintained in Dulbecco's Modified Eagle's medium (DMEM) high glucose formula containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% nonessential amino acids (Sambuy et al., 2005; Yao et al., 2013). Caco-2 cells with passage-numbers 30 to 45 were used in this study: they were cultured at 37 ºC in a 90% humidified incubator with 5% CO_2. The fresh medium was changed every other day. At 80% confluence, the cells were ready to be seeded for the investigations.

Table 19 Preparation of fresh medium (DMEM) high glucose formula (1 L) for Caco-2 cells culture

Substance	**Prepared volume in 1 L Duran bottle (mL)**
Sterile DMEM mixture	900
10% fetal bovine serum	100
1% penicillin/streptomycin	10
1% nonessential amino acids	10

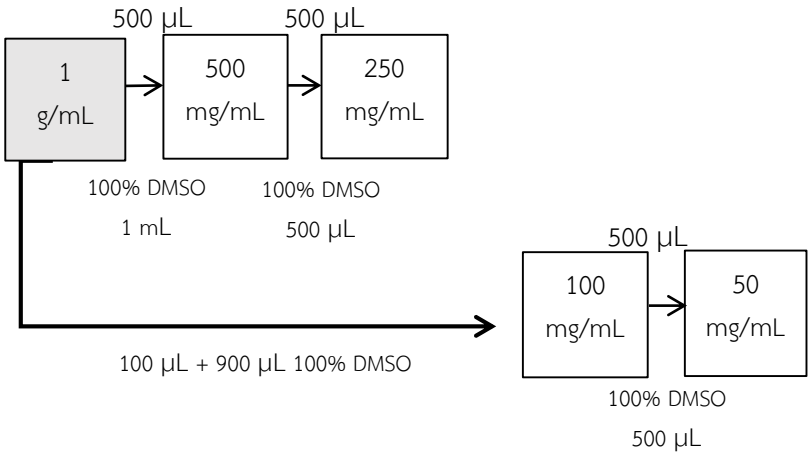
3.2.4 Cytotoxicity evaluation

3.2.4.1 Preparation of 0.5 MTT solution for cytotoxicity evaluation

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) stock solution (5 M) was already prepared and kept at -20 ℃. 0.5 M MTT solution (10 mL) was mixed well between 1 mL of MTT stocked solution was mixed well with 9 mL of free serum medium (Dulbecco's Modified Eagle's medium (DMEM) high glucose formula containing 1% penicillin/streptomycin, and 1% nonessential amino acids).

3.2.4.2 Preparation of Thai berry solution for cytotoxicity evaluation

Stock solution of Thai berry extract was prepared as a tock by completely dissolving 500 mg of purified extract with 500 µL absolute DMSO in 1.5 mL tube, and then filtered with syringe filter, using nylon filter membranes, pore size 0.45 µm. Because of toxicity to the cells, the final concentration of DMSO, which was used as a solvent, in this investigation had to be not greater than 0.1%. The stock solution (1000 mg/mL) was diluted by 0.1 % DMSO as following to



3.2.4.3 Preparation of Hanks' Balanced Salt Solution (HBSS: pH 7) (Wu et al., 2014) for cytotoxicity evaluation

The stock solution (2 L) of HBSS was prepared by completely dissolve chemical substances as following to the table below in 1.5 L of distilled water. After well mixing, the solution was then measured its pH value through a pH meter. To set the pH 7, the solution was adjusted by adding 1 M NaOH (~ 1-5 mL) before adding more distilled water to level at 2 L, using 1 L cylinder. The buffer was then filtered to be sterile and kept in 4°C for the investigations.

Table 20 Preparation of chemical substances in Hanks' Balanced Salt Solution (HBSS: pH 7) for cytotoxicity evaluation

Substance	Molecular weight (g/L)	In a 2 L Duran		
		Amount (g)	+	DW (L)
140 mM NaCl	58.44	16.4	+	
5 mM KCl	74.56	0.75	+	
1.2 mM Na_2HPO_4	141.96	0.34	+	
2 mM $CaCl_2$	147.02	0.59	+	1.5
1.2 mM $MgSO_4$	246.47	0.6	+	
20 mM HEPES	238.3	9.53	+	
0.2% w/v bovine serum albumin	-	0.4	+	
Adjust to pH 7 by adding 1 M NaOH				
Fill distilled water to reach the total volume of 2000 mL				

Cytotoxicity of formulation was assessed by using MTT assay or 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay to determine the cell

viability according to reported methods with minor modifications (Chinthala & Chinde, 2014; Peng et al., 2007). Principle of this method is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells into purple formazan crystals, which was dissolved in dimethyl sulfoxide (DMSO). 5×10^4 cells/mL of Caco-2 cells was seeded into a 96-well plate by counting trough Trypan blue exclusion dye method. They were allowed to grow until 50-60% confluence before being treated with various concentration of an aqueous anthocyanins-rich Thai berry extract (50, 100, 250, 500 and 1000 µg/mL). The culture medium containing 0.1% DMSO and hanks' balanced salt solution (HBSS; containing 140 mM NaCl, 5 mM KCl, 1.2 mM Na_2HPO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, 0.2% bovine serum albumin, pH 7.4), represented as controls, were also performed. The mixture (200 µL) was incubated for 2 h in the 37°C incubator with 90% humidified atmosphere and 5% CO_2. After the incubation, the mixture was replaced with 100 µL of fresh serum free medium containing 0.5 M of MTT solution and incubated further for 2 ½ h in a 37°C incubator. Then the solution was substituted by absolute DMSO in order to let purple crystals dissolve. The absorbance (Abs) was read at 570 nm via a 96-well microplate spectrophotometry. The result was expressed in % cell viability based on the below calculation.

Equation 8 Cell viability (%)

$$\text{Cell viability (\%)} = \left[\frac{(\text{Abs of negative control} - \text{Abs of extract})}{\text{Abs of negative control}} \right] \times 100$$

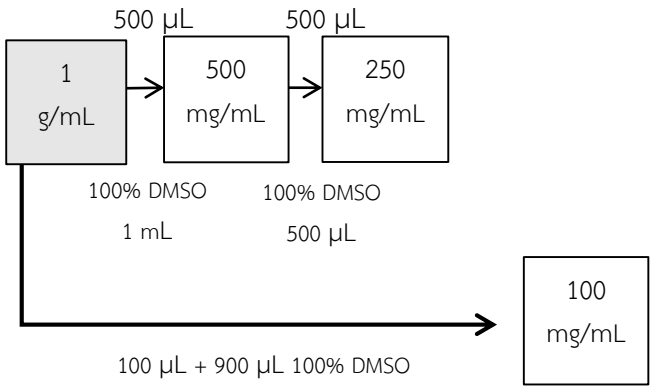
Table 21 Investigation of cytotoxicity evaluation

Investigation with 5×10^4 cells/mL	**Treatment**	**Negative**	**Reagent blank**
Extract (µL)	200	-	-
0.1 %DMSO (µL)	-	200	-
HBSS (µL)	-	200	-
Incubated for 2 h in the 37°C incubator with 90% humidified atmosphere and 5% CO_2			
MTT (µL)	100	100	100
Incubated further for 2 ½ h in a 37°C incubator			
DMSO (µL)	100	100	100
Read the Abs at 570 nm			
Each conc. was done on six-replicated reaction			

3.2.5 Cholesterol uptake by Caco-2 cells assay

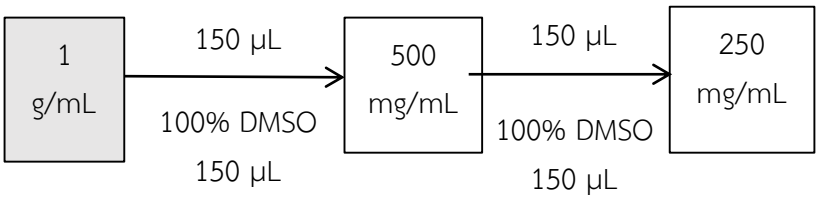
3.2.5.1 Preparation of Thai berry solution for cholesterol uptake into Caco-2 cells assay

Stocks solution of Thai berry extract were prepared as a tock by completely dissolving 500 mg of purified extract with 500 µL absolute DMSO in 1.5 mL tube, and then filtered with syringe filter, using nylon filter membranes, pore size 0.45 µm. Because of toxicity to the cells, the final concentration of DMSO, which was used as a solvent, in this investigation had to be not greater than 0.1%.The stock solution (1000 mg/mL) was diluted by 0.1 % DMSO, as shown below, and it was kept at -20 °C.



3.2.5.2 Preparation of ezetimibe (EZT) for cholesterol uptake into Caco-2 cells assay

Stock of ezetimibe was prepared by completely dissolving 100 mg of ezetimibe with 100 µL absolute DMSO in 1.5 mL tube. Because of toxicity, the final concentration of DMSO, which was used as a solvent, in this investigation had to be not greater than 0.1%. After well vortex, the stock solution (1 g/mL) was centrifuged at 4000 rpm for 5 min at 4 °C and kept the supernatant for being centrifuged twice. In the last step, the clear supernatant of ezetimibe stock solution was diluted by 0.1% DMSO, as shown below. The serial dilutions were stocked at -20 °C for the experiment.



3.2.5.3 Preparation of lysis buffer (pH 7) for cholesterol uptake into Caco-2 cells assay

The stock solution (1 L) of lysis buffer was prepared by completely dissolve chemical substances as following to the table

below in 800 mL of distilled water. After well mixing, the solution was then measured its pH value through a pH meter. To set the pH 7, the solution was adjusted by adding 1 M NaOH (~ 1-5 mL) before adding more distilled water to level at 1 L, using 1 L volumetric flask. The buffer was kept in 4°C for the investigations.

Table 22 Prepared concentration of chemical substances in lysis buffer (pH 7) for cholesterol uptake into Caco-2 cells assay

Substance	Molecular weight (g/L)	In a 1 L bigger		
		Amount (g)	+	DW (L)
10 mM Tris-HCl,	121.41	1.2	+	
1% Triton-x-100	-	10 mL	+	
1 mM EDTA	292.24	0.29	+	1
0.1% SDS	-	1	+	
150 mM NaCl	58.44	8.7	+	

3.2.5.4 Preparation of 0.5 mM taurocholic acid containing in HBSS, pH 7 for cholesterol uptake into Caco-2 cells assay

The taurocholic acid stocked solution (10 mM) was already prepared by dissolving taurocholic acid powder and the stocked solution was kept at 4 °C. 0.5 mM taurocholic acid (50 mL) was mixed well between 2.5 mL of 10 μM of taurocholic acid stocked solution and 47.5 mL of HBSS. The 0.5 mM taurocholic acid stocked solution was kept at 4 °C.

3.2.5.5 Preparation of 0.25 mM NBD-cholesterol for cholesterol uptake into Caco-2 cells assay

NBD-cholesterol stock solution (100 μM) was already prepared and kept at -20 °C. 0.025 μM NBD-cholesterol (3 mL) was mixed well between 15 μL of NBD-cholesterol stocked solution was mixed well with 2, mL of HBSS containing 0.5 mM taurocholic acid. The 0.025 μM NBD-cholesterol solution had to be freshly performed before being used in the very low light area.

A study previously reported that anthocyanins from blueberries can be transported through Caco-2cell with an efficiency averaged 3-4% (P. Wu et al., 2014; Yao et al., 2013). *In vitro* study, the result was reported cyanidin-3-glucoside and peonidin-3-lucoside (40 μg/mL) found in black rice anthocyanin exhibited a significant reduction in cholesterol uptake by Caco-2 cells (Yao et al., 2013). Thus, the anthocyanins containing in the selected Thai-berry extract may show a decrease in cholesterol uptake into Caco-2 cells. Caco-2 cells with density of 50,000 cells/mL were seeded into a 24-well plate and cultured for 6-7 days to let them differentiate. During this period, the cells were fed with fresh medium every other day. After culturing, the cells were starved 24 h with low glucose medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% nonessential amino acids. They were then starved further with HBSS for 1 h. Various concentrations of an aqueous anthocyanins-rich Thai berry extract, a positive control (Ezetimibe), and 0.1% DMSO in HBSS were added to treat the cells. The reaction was started by adding 0.025 mM NBD-cholesterol in HBSS containing 0.5 mM taurocholic acid (TCA). After 37°C incubation for 1 h, the reaction was stopped by the removal of the solution and cold HBSS with free taurocholic acid was added

to wash the cells by 5 times. The fluorescent intensity was determined at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. To determine protein concentration of the treated cells, cold lysis buffer (10 mM Tris-HCl, 150 mM, 1% Triton-x-100, 1 mM EDTA, 0.1% SDS, pH 7.4) was added to break the cells. Cell suspension was also stored in freezer (-20°C) switching to be thawed at room temperature for 3 times. The amount of protein was evaluated by BCA colorimetric assay, using bovine serum albumin (BSA) as a standard. The result was expressed in inhibitory percentage, calculated by the formula below.

Equation 9 Inhibition (%) of choesterol uptake

$$\text{Inhibition (\%) of cholesterol uptake} = \left[\frac{B - A}{B}\right] \times 100$$

When **A** =

$$\left[\frac{\text{Fluorescent intensity of treated cells with sample}}{\text{Abs of protein contents from treated cells with sample}}\right]$$

When **B** =

$$\left[\frac{\text{Fluorescent intensity of untreated cells}}{\text{Abs of protein contents from untreated cells}}\right]$$

Table 23 Investigation of cholesterol uptake into Caco-2 cells

Investigation with 5×10^4 cells/mL	**Treatment**	**Negative**	**Test blank**		**Negative blank**	
Extract (µL)	200	-	200	-	-	-
EZT (µL)	-	200	-	200	-	-
0.1 %DMSO (µL)	-	-	-	-	200	-
HBSS (µL)	-	-	200	200	-	400
NBD cholesterol (µL)	200	200	-	-	-	-
Total (µL)	*400*	*400*	*400*	*400*	*400*	*400*
Incubated for 1 h in the 37°C incubator with 90% humidified atmosphere and 5% CO2						
Replaced the solution with cold HBSS with free TCA + wash the cells by 5 times						
Read the fluorescent intensity at 485 nm/535 nm						
Breaking the cell with cold lysis buffer together with freezing + thawing them						
Determination amount of protein was evaluated by BCA colorimetric assay						
Each conc. was done on duplicated reaction						

3.2.5.6 Determination of protein by using BCA colorimetric assay

The measurement of protein in this assay was performed after the cells had already broken in the lysis buffer. The solution was also centrifuged at 5000 rpm for 5 min to collect the supernatant for this determination. The BCA (bicinchoninic acid) protein assay kit was set to be measure the absorbance (Abs) at 562 nm as followed to the description in Appendix. The result was expressed by compared with a standard curve (BSA: Bovine Serum Albumin, 0 – 1 mg/mL) (Figure 17).

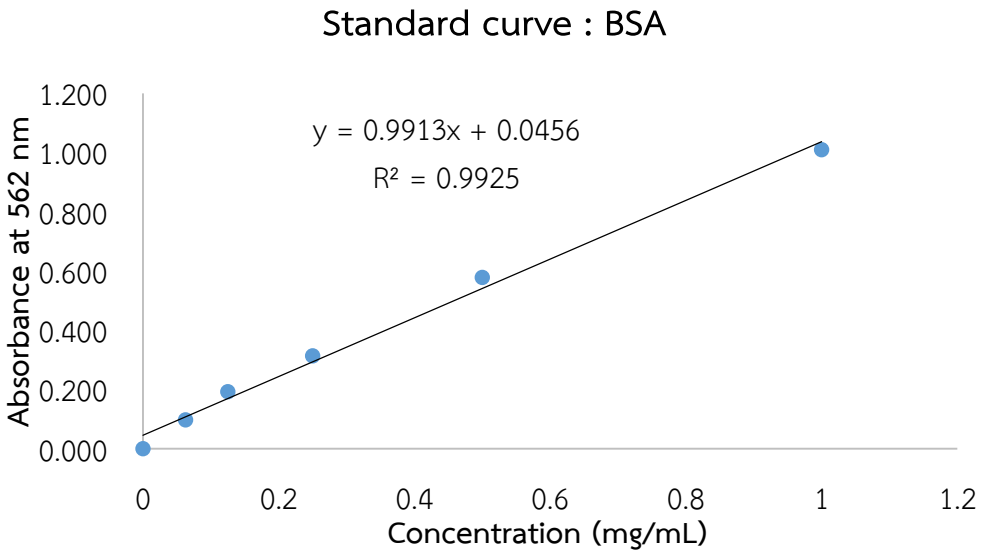


Figure 17 An example of calibration curve of Bovine Serum Albumin (0 – 1 mg/mL)

3.3 Statistical analysis

Statistical analyses were performed by using one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test. P-value < 0.05 was considered to be statistically significant by SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER IV

RESULTS

1. Percent yield of freeze-dried Thai berries extracts (Non-purified Thai berries extracts)

In this study, *Muntingia calabura* L.*, Prunus domestica* L., *Skeels*, *Syzygium nervosum* A. Cunn. Ex DC.*, Antidesma bunius* (L.) *Spren g*., and *Syzygium cu*mini (L.) *Skeels*. were overnight thawed for taking their pulps to be mashed, squeezed and extracted with water. Their aqueous crude extracts were then freeze-dried. The percent yield of freeze dried Thai berries extracts were reported as shown in Table 24. *Muntingia calabura* L. exhibited the highest percent yield, followed by *Antidesma bunius (*L.*) Spreng*., *Prunus domestica* L., *Syzygium cumini (*L.) *Skeels*., and *Syzygium nervosum* A. Cunn. Ex DC.

Table 24 Percent yield of freeze-dried Thai berries extracts (Non-purified Thai berries extracts)

Sample		Yield (%) (Freeze-dried wt./Fresh pulp wt.)
Takob	*Muntingia calabura* L.	9.7 ± 0.0
Thai plum	*Prunus domestica* L.	6.8 ± 0.0
Makiang	*Syzygium nervosum* A. Cunn. Ex DC	1.3 ± 0.4
Mamao	*Antidesma bunius (*L.*) Spreng*	8.2 ± 0.1
Lukwha	*Syzygium cumini (*L.) *Skeels*	6.0 ± 0.0

2. Percent yield of anthocyanins-rich Thai berries extracts (Purified Thai berries extracts)

Percent yield of purified freeze dried extracts from *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC*, Antidesma bunius* (L.) *Spreng*., and *Syzygium cu*mini (L.) *Skeels* were shown in Table 25. It was found that *Muntingia calabura* L. presented the highest percent yield of dried weight. On the other hand, *Antidesma bunius (*L.*) Spreng* was found the lowest percent yield.

Table 25 Percent yield of anthocyanins-rich Thai berries extracts (Purified Thai berries extracts)

Sample		Yield (%) (Purified wt./Freeze-dried wt.)
Takob	*Muntingia calabura* L.	3.5 ± 0.5
Thai plum	*Prunus domestica* L.	3.1 ± 0.0
Makiang	*Syzygium nervosum* A. Cunn. Ex DC	1.2 ± 0.6
Mamao	*Antidesma bunius (*L.*) Spreng*	0.6 ± 0.6
Lukwha	*Syzygium cumini (*L.) *Skeels*	2.6 ± 0.0

Before purification, freeze-dried extracts of *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*., and *Syzygium cumini* (L.) *Skeels*. were completely dissolved by distilled water, whereas *Muntingia calabura* L. was firstly dissolved in methanol, dried under nitrogen gas, and then re-dissolved by distilled water. Purified wt. was weight of anthocyanins-rich Thai berries extracts, and freeze-dried wt. was weight of freeze-dried Thai berries extracts.

3. Phytochemical contents of aqueous freeze-dried Thai berries extracts (Non-purified Thai berries extracts)

Freeze-dried Thai berries extracts were also quantified total phenolic, flavonoids, and anthocyanins contents by spectrophotometric method and reported as shown in Table 26. *Prunus domestica* L. presented the highest amount of total phenolic and flavonoids contents and followed by *Antidesma bunius* (L.) *Spreng., Muntingia calabura* L., *Syzygium nervosum* A. Cunn. Ex DC., and *Syzygium cumini* (L.) *Skeels*., respectively. Total anthocyanins content was found highest in *Syzygium cumini* (L.) *Skeels*., followed by *Antidesma bunius* (L.) *Spreng.*, *Muntingia calabura* L., and *Prunus domestica* L. However, anthocyanins content was not detected in *Muntingia calabura* L. in this observation.

Table 26 Total phenolic, flavonoids and anthocyanins contents of aqueous freeze-dried Thai berries extracts (Non-purified Thai berries extracts)

Freeze-fried extracts	Total Phenolic (mg GAE/g extract)	Total Flavonoids (mg CE/g extract)	Total Anthocyanins (mg Cy-3-glu/g extract)
Takob	18.5 ± 0.0[a]	11.2 ± 0.4[a]	ND
Thai plum	25.8 ± 0.4[b]	16.2 ± 0.4[b]	2.3 ± 0.0[a]
Makiang	17.7 ± 0.3[ac]	10.8 ± 0.4[c]	2.4 ± 0.0[a]
Mamao	23.7 ± 0.5[d]	12.4 ± 0.1[a]	4.6 ± 0.0[b]
Lukwha	16.6 ± 0.3[c]	10.0 ± 0.7[d]	5.1 ± 0.0[c]

Values are expressed as Mean ± SEM (n = 3). ND = data was not detected. Gallic Acid Equivalent: GAE; Cathechin Equivalent: CE; Cyanidins-3-glucosides: Cy-3-glu. Takob, Thai plum, Makiang, Mamao, and Lukwha are represented to *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*, and *Syzygium cumini* (L.) *Skeels*, respectively. The different superscripted letters are statistically significant difference (P-value < 0.05).

4. Phytochemicals contents of aqueous anthocyanin-rich Thai berries extracts

Total phenolic (TP), total flavonoids (TF), and total anthocyanins (TA) contents were quantified by spectrophotometric method. The results in Table 27 demonstrated that total phenolic and flavonoids contents were found highest in *Muntingia calabura* L. (579.5 ± 4.5 mg GAE/g extract) and *Prunus domestica* L. (184.3 ± 0.7 mg CE/g extract), respectively. The highest anthocyanins content was found in both *Prunus domestica* L. and *Antidesma bunius* (L.) *Spreng*. with value of 49.5 ± 0.2 and 48.8 ± 0.2 mg Cy-3-glu/g extract, respectively. In contrast, *Syzygium nervosum* A. Cunn. Ex DC. had the lowest amount of total phenolic, flavonoids, and anthocyanins contents with values of 222.7 ± 1.4 mg GAE/g extract, 91.2 ± 2.4 mg CE/g extract and 37.9 ± 0.2 mg Cy-3-glu/g extract, respectively. However, total anthocyanins content was not detected in *Muntingia calabura* L. in this study.

Table 27 Total phenolic, flavonoids and anthocyanins contents of aqueous anthocyanins-rich Thai berries extracts

Sample	Total Phenolic	Total Flavonoids	Total Anthocyanins
	(mg GAE/g extract)	(mg CE/g extract)	(mg Cy-3-glu/g extract)
TKE (Takob)	579.5 ± 4.5^{a}	92.2 ± 1.3^{a}	ND
TPE (Thai plum)	283.4 ± 0.4^{b}	184.3 ± 0.7^{b}	48.8 ± 0.6^{a}
MKE (Makiang)	222.7 ± 1.4^{c}	91.2 ± 2.4^{a}	37.9 ± 0.2^{b}
MME (Mamao)	276.6 ± 0.6^{d}	135.6 ± 1.4^{c}	49.5 ± 0.2^{a}
LWE (Lukwha)	268.2 ± 0.6^{e}	106.7 ± 2.4^{d}	44.0 ± 0.4^{c}

Values are expressed as Mean ± SEM (n = 3). ND = data was not detected. Gallic Acid Equivalent: GAE; Cathechin Equivalent: CE; Cyanidins-3-glucosides: Cy-3-glu. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC.,

Antidesma bunius (L.) *Spreng*., and *Syzygium cumini* (L.) *Skeels*., respectively. The different superscripted letters are statistically significant difference (P-value < 0.05).

5. Effect of aqueous anthocyanins-rich Thai berries extracts on bile acid binding

The potential of five aqueous anthocyanins-rich Thai berries extracts on bile acids binding were presented in Figure 18. It was found that all the extracts bound to both primary (taurocholic acid) and secondary bile acids (taurodeoxycolic acid and glycodeoxycholic acid) with concentration-dependent manner (0.5, 1.0, and 2.0 mg/mL), as shown in Figure 18a)-c).

a)

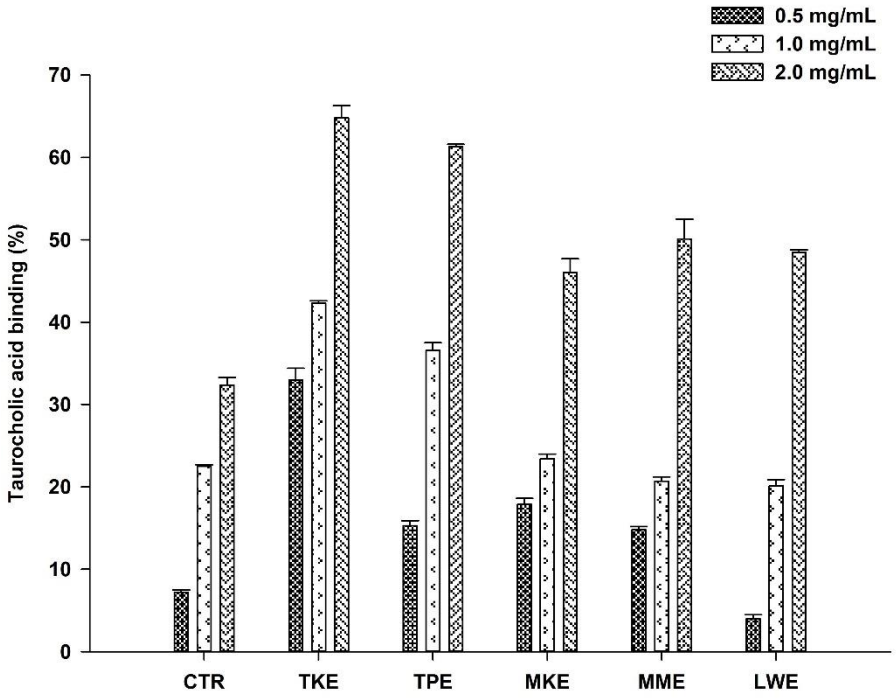
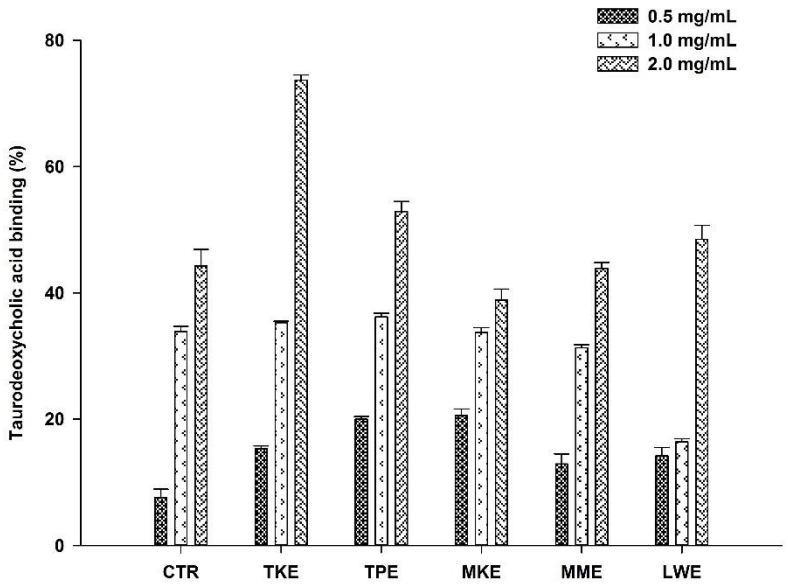


Figure 18 Effect of TKE, TPE, MKE, MME, and LWE (0.5, 1.0, and 2.0 mg/mL) on a) taurocholic acid binding. Values are expressed as Mean ± SEM (n = 3). Cholestyramine (CTR) was used as positive control. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*, and *Syzygium cumini* (L.) *Skeels*, respectively.

b)



c)

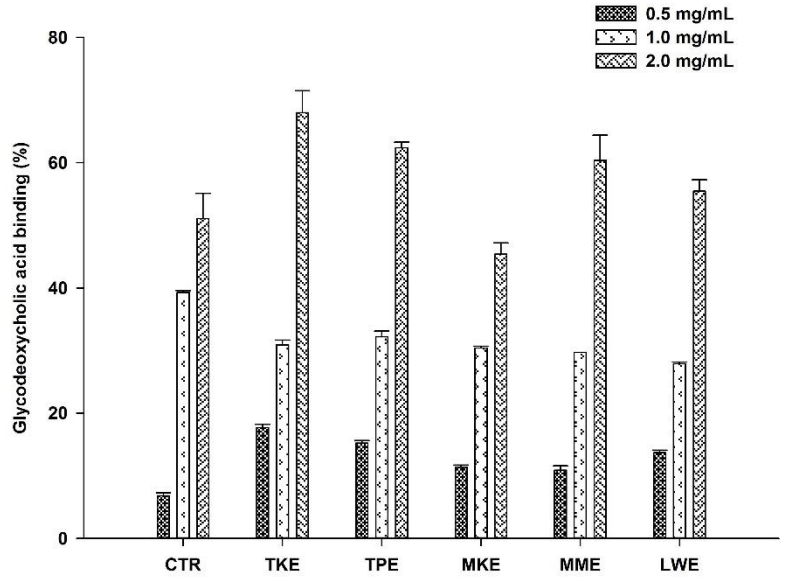


Figure 18 Effect of TKE, TPE, MKE, MME and LWE (0.5, 1.0, and 2.0 mg/mL) on b) taurodeoxycholic acid binding and c) glycodeoxycholic acid binding. Values are expressed as Mean ± SEM (n = 3). Cholestyramine (CTR) was used as positive control. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*, and *Syzygium cumini* (L.) *Skeels*, respectively.

At concentration of 1 mg/mL anthocyanins-rich Thai berries extratcs had ability to bind to taurocholic acid (primary bile acid) and taurodeoxycolic acid and glycodeoxycholic acid (secondary bile acids) with the binding values ranged from 20.1 - 42.3%, 16.4 - 36.2%, and 27.9 - 30.9%, respectively (Figure 19 and Table 28). Additionally, the binding efficacy of aqueous anthocyanins-rich Thai berries extracts was also compared with cholestyramine, a bile acid sequestrant. *Muntingia calabura* L. and *Prunus domestica* L. had the significant-higher ability to bind to taurocholic acid (42.3 ± 0.3% and 36.6 ± 0.9%, respectively) when compared to cholestyramine (22.5 ± 0.2%) at the same concentration (1 mg/mL). Interestingly, *Prunus domestica* L. had the highest ability to bind with taurodeoxycholic acid and glycodeoxycolic acid (36.2 ± 0.6% and 32.2 ± 0.9%) when compared to other Thai berries extracts. However, *Prunus domestica* L. had less potent of binding with glycodeoxycholic acid than cholestyramine (39.3 ± 0.3%).

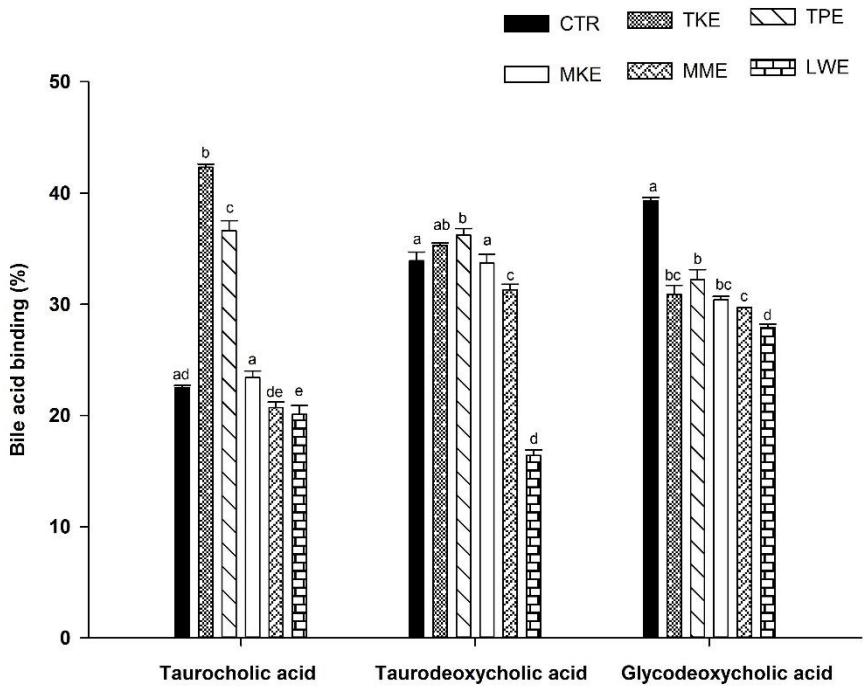


Figure 19 Effect of TKE, TPE, MKE, MME and LWE at concentration 1 mg/mL on taurocholic acid binding, taurodeoxycholic acid binding and glycodeoxycholic acid binding.

Values are expressed as Mean ± SEM (n = 3). Cholestyramine (CTR) was used as positive control. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*, and *Syzygium cumini* (L.) *Skeels*, respectively. The different superscripted letters of each bile acid are statistically significant difference (P-value < 0.05)

Table 28 Percent bile acid binding of TKE, TPE, MKE and LWE (1 mg/mL) on taurocholic acid, taurodeoxycholic acid and glycoldeoxycholic acid

Sample	Bile acid binding (%)		
	Taurocholic acid	Taurodeoxycholic acid	Glycodeoxycholic acid
TKE	42.3 ± 0.3^{b}	35.3 ± 0.2^{ab}	30.9 ± 0.8^{bc}
TPE	36.6 ± 0.9^{c}	36.2 ± 0.6^{b}	32.2 ± 0.9^{b}
MME	20.7 ± 0.5^{de}	31.3 ± 0.5^{c}	29.7 ± 0.0^{c}
MKE	23.4 ± 0.6^{a}	33.7 ± 0.8^{a}	30.4± 0.3^{bc}
LWE	20.1 ± 0.80^{e}	16.4 ± 0.5^{d}	27.9 ± 0.3^{d}
CTR	22.5 ± 0.2^{ad}	33.9 ± 0.8^{a}	39.3 ± 0.3^{a}

Values are expressed as Mean ± SEM (n = 3). Cholestyramine (CTR) was used as positive control. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*, and *Syzygium cumini* (L.) *Skeels*, respectively. The different superscripted letters of each bile acid are statistically significant difference (P-value < 0.05)

6. Inhibitory effects of aqueous anthocyanins-rich Thai berries extracts on pancreatic lipase and cholesterol esterase activities

To absorb triglycerides into enterocytes, pancreatic lipase hydrolyses triglycerides into diglycerides and subsequently into monoglycerides and free fatty acids. In the same way, cholesterol esterase is a key enzyme to digest dietary cholesterylester into free cholesterol and free fatty acids. Hence, inhibitions of pancreatic lipase and cholesterol esterase activities may eventually reduce hydrolysis of triglyceride and cholesterol, leading to reduce triglyceride and cholesterol absorption. The inhibitory concentration of aqueous anthocyanins-rich Thai berries extracts at 50% (IC_{50}) on pancreatic lipase and cholesterol esterase activities were shown in Table 29. It was found that all aqueous anthocyanins-rich Thai berries extracts inhibited activities of pancreatic lipase with IC_{50} values ranged from 90.6 - 336.9 µg/mL. *Prunus domestica* L. illustrated the greatest inhibitory activity on pancreatic lipase with IC_{50} value of 90.6 µg/mL followed by *Antidesma bunius* (L.) *Spreng*., *Syzygium nervosum* A. Cunn. Ex DC., *Syzygium cumini* (L.) *Skeels*., and *Muntingia calabura* L., respectively (Table 28). However, the inhibitory activity of all the extracts on such enzyme were less than orlistat (IC_{50} value = 1.8 µg/mL).

IC_{50} values of aqueous anthocyanins-rich Thai berries extracts on cholesterol esterase inhibition were also illustrated in Table 29. *Antidesma bunius* (L.) *Spreng*. demonstrated the highest inhibitory effect on cholesterol esterase activity with IC_{50} value of 288.7 µg/mL, whereas *Muntingia calabura* L. demonstrated the lowest inhibitory ability of that enzyme with IC_{50} value of 469.1 µg/mL (Table 29). Moreover, simvastatin (1 µg/mL) attenuated cholesterol esterase activity with the inhibition of 9.19%.

Table 29 The inhibitory effects of aqueous Thai berries extracts on pancreatic lipase and cholesterol esterase activities

Sample	IC_{50} values (μg/mL)	
	Pancreatic lipase activity	Cholesterol esterase activity
TKE (Takob)	336.9 ± 1.6[b]	469.1 ± 1.6[a]
TPE (Thai plum)	90.6 ± 0.4[c]	417.7 ± 2.4[b]
MKE (Makiang)	146.6 ± 2.1[d]	333.8 ± 4.5[c]
MME (Mamao)	118.7 ± 1.1[e]	288.7 ± 1.7[d]
LWE (Lukwha)	181.7 ± 0.8[f]	455.0 ± 2.2[e]
Orlistat	1.8 ± 0.0[a]	NA
Simvastatin	NA	≥ 1

Values are expressed as mean ± SEM (n = 3). NA = data was not analyzed. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*, and *Syzygium cumini* (L.) *Skeels*, respectively. Orlistat and simvastatin were used as positive control for pancreatic lipase inhibition and cholesterol esterase inhibition assays, respectively. Simvastatin (1 μg/ml) showed inhibitory percentage of 9.19 ± 0.12. P-value < 0.05 significant differences of each assay, represented with different superscripted letters.

7. Inhibitory effect of aqueous anthocyanins-rich Thai berries extracts on inhibition of cholesterol micellization

According to mixed micelles plays as a vehicle for digested lipid to be transported into the enterocyte, especially free cholesterol, an interference of cholesterol micellization has been purposed as an essential step to reduce cholesterol absorption. As shown in Figure 20, aqueous anthocyanins-rich Thai berries extracts were able to decrease in cholesterol solubility in mixed micelles with concentration-dependent manner (0.03, 0.13, and 0.25 mg/mL).

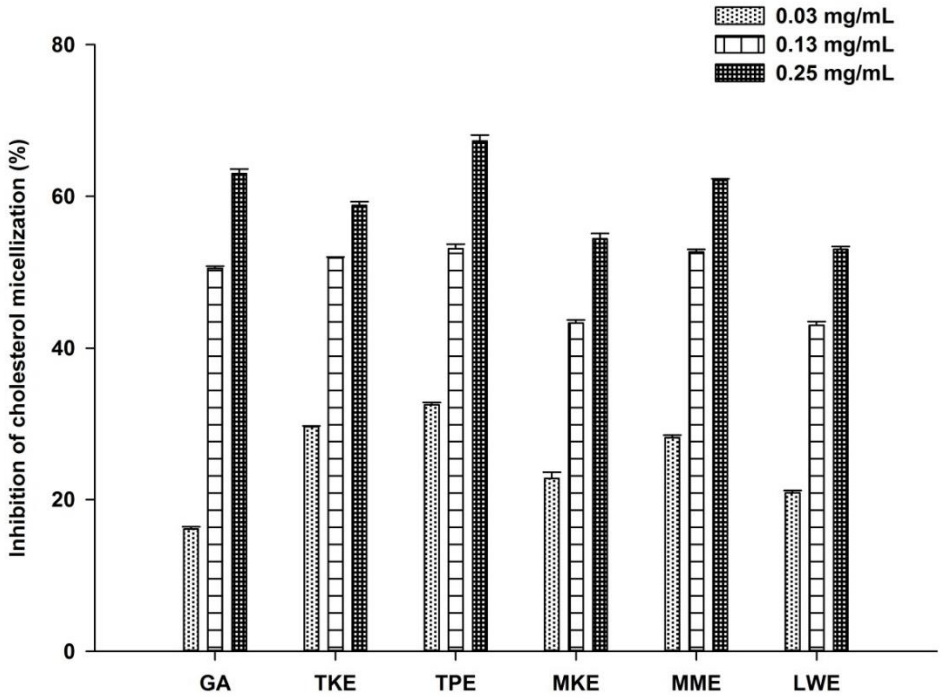


Figure 20 Effect of TKE, TPE, MKE, MME and LWE (0.03, 0.13, and 0.25 mg/mL) on inhibition of cholesterol micellization.

Values are expressed as Mean ± SEM (n = 3). Gallic acid (GA) was used as positive control. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*., and *Syzygium cumini* (L.) *Skeels*, respectively.

In addition, *Prunus domestica* L. (0.25 mg/mL) expressed the highest potential interfering cholesterol solubility in mixed micelle with the inhibition of 67.3 ± 0.8%, as shown in Figure 21. On the other hand, *Syzygium cumini* (L.) *Skeels* at the same concentration showed the lowest inhibitory activity of this event with inhibition of 53.0 ± 0.4%. Interestingly, *Prunus domestica* L. had more inhibitory ability on cholesterol micellization than gallic acid (%inhibition = 63.0 ± 0.6%).

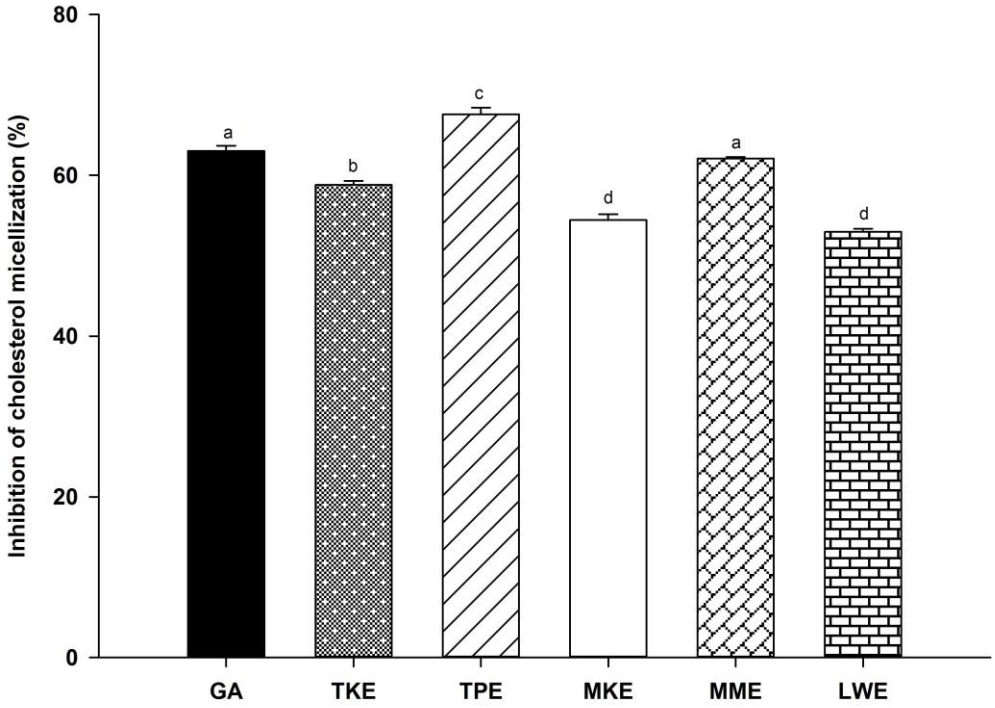


Figure 21 Effects of aqueous anthocyanins-rich Thai berries extracts (0.25 mg/mL) on inhibition of cholesterol micellization.

Values are expressed as Mean ± SEM (n = 3). Gallic acid (GA) was used as positive control. TKE, TPE, MKE, MME, and LWE are represented to anthocyanins-rich Thai berries extracts of *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng*., and *Syzygium cumini* (L.) *Skeels*., respectively. Significant differences were represented with different superscripted letters (P-value < 0.05).

8. Correlation between activities of lipid digestion and absorption (Bile acids: TCA, TDA, and GDA, Lipase, CEase, and Micelle) and TP, TF, and TA of aqueous anthocyanins-rich Thai berries extracts

Pearson's correlation coefficient (*r*) was resulted from bivariate correlation analysis and used to describe the correlation between activities of lipid digestion and absorption and phytochemical contents of five aqueous anthoyanins-rich Thai berries extracts (TP, TF, and TA) (Table 30). Three correlation levels were defined as strong (*r* = (+/–) 0.600 – 1.000), moderate (*r* = (+/–) 0.400 – 0.599), and weak (*r* = (+/–) 0.000 – 0.399) (Ngamdee, Wichai, & Jiamyangyuen, 2016). As shown in Table 30, the results were found that total phenolic was strongly positive correlated to total anthocyanins content, binding to taurocholic acid, and strongly negative correlated to pancreatic lipase with that coefficient (*r*) of 0.926, 0.777 and 0.899 (P-value < 0.05), respectively. Total flavonoids had the strong negative associations with inhibition of pancreatic lipase (*r* = -0.667) and the strong positive associations with cholesterol micellization (*r*=0.868) (P-value < 0.05). Moreover, total anthocyanins content was found powerfully related to taurocholic acid binding (*r* = 0.683) and pancreatic lipase (*r* = -0.959) (P-value < 0.05).

Table 30 Correlation between activities of lipid digestion and absorption (Bile acids: TCA, TDA, and GDA, Lipase, CEase, and Micelle) and TP, TF, and TA of aqueous anthocyanins-rich Thai berries extracts

	TF	TA	TCA	TDA	GDA	Lipase	CEase	Micelle
TP	.305	.926**	.777**	.300	.227	-.899**	-.574*	.075
TF	-	.581*	.159	.268	.423	-.667**	-.115	.868**
TA	-	-	.683**	.288	.167	-.959**	-.530*	.204
TCA	-	-	-	.621*	.663**	.528*	.575*	.478
TDA	-	-	-	-	.774**	.020	-.252	.626*
GDA	-	-	-	-	-	-.061	.015	.619*
Lipase	-	-	-	-	-	-	.599*	-.360
CEase								-.131

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

TP, TF and TA are represented as total phenolic, flavonoids and anthocyanins content found in all anthocyanins-rich Thai berries extracts. TCA, TDA and GDA are mentioned to percent binding of 1 mg/mL of all the extracts on taurocholic acid, taurodeoxycholic acid and glycodeoxycholic acid, respectively. Lipase and CEase are IC_{50} values of all the extracts on pancreatic lipase and cholesterol esterase, respectively. Micelle is referred to Inhibition of 0.25 mg/mL anthocyanins-rich Thai berries extracts on cholesterol micellization.

9. Effect of *Prunus domestica* L. (TPE) on Caco-2 cells viability

In this experiment, anthocyanins-rich *Prunus domestica* L. extract (TPE) contained higher both in total flavonoids and anthocyanins contents than other Thai berries extracts. Moreover, abilities of anthocyanins-rich *Prunus domestica* L. extract on lipid lowering mechanisms were superior ranking among five Thai berries extracts. Thus, *Prunus domestica* L. was chosen for determination its ability on inhibition of cholesterol uptake into the intestinal cells. To observe further cholesterol uptake into Caco-2 cells, the cytotoxicity of *Prunus domestica* L. extract (0.10, 0.25, 0.50 and 1.00 mg/mL) was tested. As shown in Figure 22, *Prunus domestica* L. extract at the concentration of 1 mg/mL illustrated a significant reduction of cell viability when compared to control (0.1% DMSO). Thus, the concentrations of *Prunus domestica* L. extract at 0.10 – 0.50 mg/mL were selected for cholesterol uptake assay.

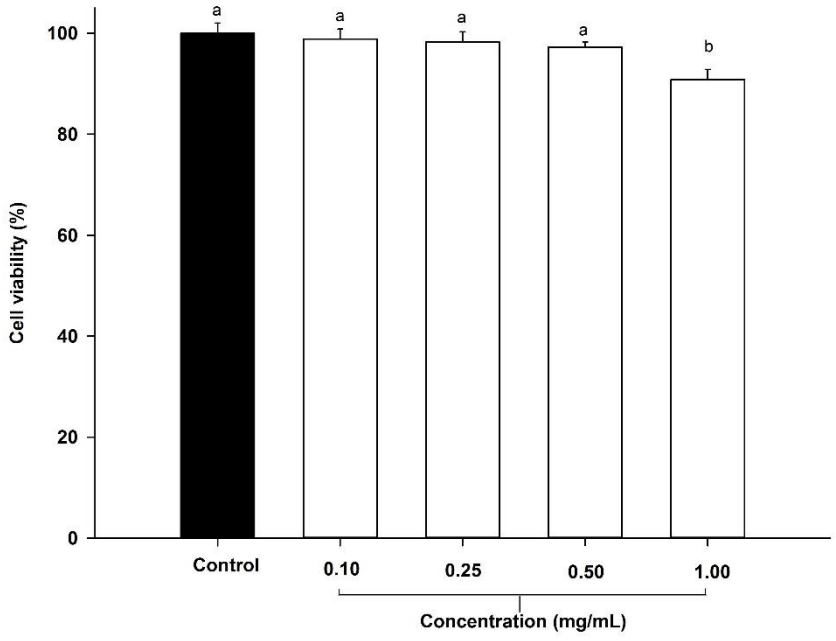


Figure 22 Cell viability (%) of Caco-2 cells after 2 h incubation with *Prunus domestica* L. (TPE) with (0.10, 0.25, 0.50, and 1.00 mg/mL) before adding 3-(4, 5-dimethlthaizol-2-yl)-2, 3-diphhenyl tetrazolium bromide (MTT) to evaluate the cell viability.

Values are expressed as Mean ± SEM (n = 3). 0.1 % DMSO was represented as control. Significant differences were represented with different superscripted letters (P-value < 0.05).

10. Effect of *Prunus domestica* L. extract (TPE) on cholesterol uptake into Caco-2 cells

Caco-2 cells were used as an *in vitro* model in this study. After culture the Caco-2 cell under the specific condition, they were differentiated and presented the characteristic of intestinal cells. Ezetimibe, a cholesterol lowering drug, was used as a positive control. The results were exhibited that anthocyanins-rich *Prunus domestica* L. extract (0.10, 0.25, and 0.50 mg/mL) significantly decreased cholesterol uptake into differentiated Caco-2 cells in a concentration-dependent manner. The inhibitory values were ranged from 14.9% - 34.1%, as shown in Figure 23.

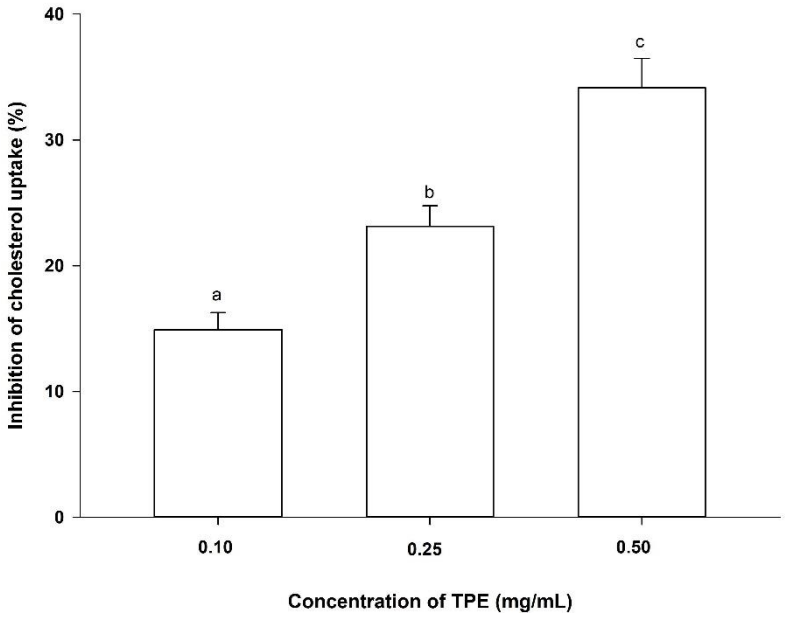


Figure 23 Effect of *Prunus domestica* L. (TPE) on cholesterol uptake into Caco-2 cells 0.1% DMSO was used as negative control. Values are expressed as Mean ± SEM (n = 3 to 5). Significant differences were represented with different superscripted letters (P-value < 0.05).

Prunus domestica L. extract at concentration of 0.10 mg/mL was also compared to ezetimibe (0.05 and 0.10 mg/mL), as shown in Figure 23. The inhibitory effect of 0.10 mg/mL *Prunus domestica* L. extract (% inhibition = 14.9 ± 1.3) showed lower ability than ezetimibe at the same concentration (% inhibition = 44.1 ± 3.0), as shown in Figure 24. In addition, the combined treatment between TPE (0.10 mg/mL) and ezetimibe (0.05 mg/mL) on inhibition of cholesterol uptake into Caco-2 cells was also observed. Interestingly, the additive inhibitory effect of cholesterol uptake into Caco-2 cells was found in the combined treatment with percent inhibition of 37.7 ± 2.7 which showed the similar inhibitory ability of ezetimibe at 0.10 mg/mL alone (% inhibition = 44.1 ± 3.0).

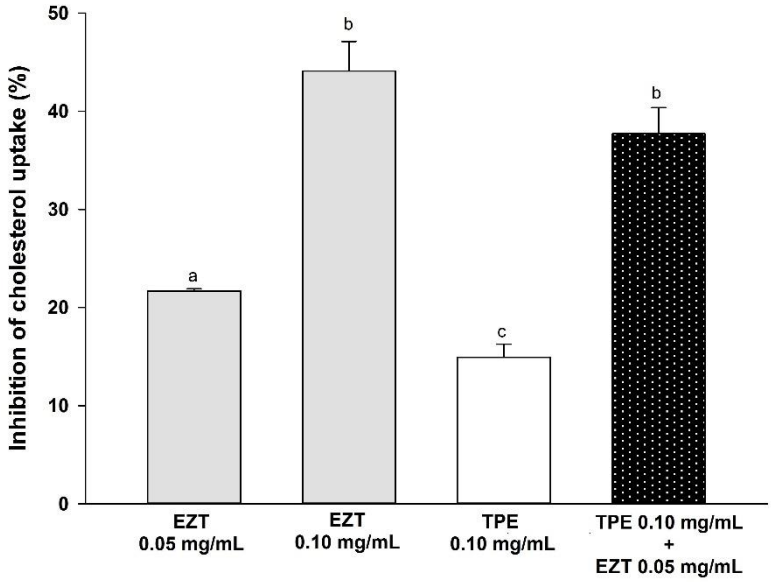


Figure 24 Effects of single and combined effect of *Prunus domestica* L. (TPE) and ezetimibe (positive control) on cholesterol uptake by Caco-2 cells.
Values are expressed as Mean ± SEM (n = 3 to 5). Significant differences were represented with different superscripted letters (P-value < 0.05).

CHAPTER V

DISCUSSION

1. Phytochemical contents of aqueous anthocyanins-rich Thai berries extracts

The amount of phytochemical contents containing in Thai berries extracts was different among the studies. This may be partly due to growing location or environment factors including soil, temperature, fertilizer, or even the period of their ripeness (Miletic et al,.2012). In the previous reports, *Muntingia calabura* L. extracted by mixture sovent was found total phenolics (121.1 mg GAE/ gextract), flavonoids (173.2 mg RE/g extract), and anthocyanins (82.4 mg CGE/g extract) were estimated through standard spectrophotometric method. Ripening *Prunus domestica* L. was found total phenolic within the range of 70 to 214 mg GAE/100 g fresh weight and total anthocyanins content that ranged from 5 to 57 mg/100 g (Miletic et al., 2012). The 95% ethanol freeze dry extract of *Cleistocalyx nervosum* var. *paniala* (Makiang) was reported their total phenolic compound = 111.28 ± 2.54 mg GAE/g total monomeric anthocyanin with 5.36 ± 0.12 mg/g. Fifteen cultivars of *Antidesma bunius* L. from Northeastern Thailand contained total phenolic contents with ranged values of 54.67- 949.73 mg/100 g DW (Jorjong et al., 2015). Another previous report of *Syzygium cumini* (L.) *Skeels*. was noted that the highest anthocyanin yield of 763.80 mg/100 mL could be obtained when 20% ethanol was used in combination with 1% acetic acid (Chaudhary & Mukhopadhyay, 2013). In this study, phytochemical compounds of five Thai berries were slightly-higher than the previous reports (Chagas et al., 2015; Islary et al., 2017; Jorjong et al., 2015; Usenik et al., 2008; Usenik et al., 2009), in particular the amount of anthocyanins containing in both *Prunus domestica* L. and *Antidesma bunius* (L.) *Spreng* because the difference of extraction method, solvent extraction, and a process of anthocyanins purification by using solid phase extraction (SPE C18). In correlation-

analysis, total anthocyanins of anthohcyanins-rich Thai berries extracts were showed a very strong correlation with total phenolic (r = 0.581, p-value<0.01), and had a moderate correlation with total flavonoids (r = 0.926, p-value<0.05). The correlation was concomitant with the previous reports (Sellappan et al., 2002) that showed the amount of total phenolic compound increased proportionally with the number of anthocyanin content (Ortega-Regules et al., 2006). However, antioxidant properties of phenolic compound, flavonoids, and anthocyanins have a complex structure of phenol and acyl groups that were very reactive towards reactive oxygen species (ROS) by acting as hydrogen atom donors owing to their resonance stabilization (Wang et al., 1999; Zhu et al., 2012). The biological activities of those phytochemical compounds were purposed in different level, so it should be deeply analyzed.

2. Effect of aqueous anthocyanins-rich Thai berries extracts on bile acid binding

The potential of protective effects of anthocyanins toward the onset of cardiovascular disease has been purposed in all clinical, *in vivo* and *in vitro* cell studies. Blood lipid profile, known as a common biomarker of cardiovascular health and to diagnose cardiovascular diseases, could be alternatively controlled into the normal level by biological properties of anthocyanins. The reduction of bile acids pool size has been proposed to be the first part to control lipid profile because bile acids are important emulsifier for the lipid digestion and absorption (Mukhopadhyay & Maitra, 2004). Previously, supplementation of blueberry anthocyanins (0.5 and 1.0 %) for 6 weeks decreased total cholesterol concentration by 6–12 % in a dose-dependent manner (Liang et al., 2013; Nile & Park, 2014). It was suggested that the decrease of cholesterol was accompanied by increasing the excretion of fecal neutral and acidic sterols by 22–29 % and 41–74 %, respectively (Kahlon & Smith, 2007). In this study, the results were clearly demonstrated that all Thai berries extracts directly bound to both primary and secondary bile acids. A great amount of bile acids binding may

increase their excretion which leads to reduce bile acid pool. This event eventually causes the use of cholesterol to produce the primary bile acid to maintain the bile acid pool size (Kahlon & Smith, 2007; Moundrasett et al., 1997). Because of the conjugation, taurine or glycine increase the ionized capacity of active bile acids in order to disperse the big-lipid molecules to be digested by enzyme. Another study was previously discussed that taurocholic acid was more highly bound than glycocholate as the longer taurine side chain may change the molecular configuration of the carboxyl group to be more easily bound to active groups on the resin (Nazir et al., 1972). Like cholestyramine, administration of cholestyramine (12 g/day) in four subjects with familial hyperchloesterolemia (Type II) for 12 to 15 days had showed the fallen plasma cholesterol values (24 to 28%) in all subjects (Levy et al., 1973). Based on the dramatic effect of cholestyramine treatment, the researchers also reported the fecal loss of bile acids and the fecal excretion of neutral steroids, as well. This study was further found *Muntingia calabura* L. and *Prunus domestica* L. were the most effective resulted in a competitive binding to taurocholic acid and taurodeoxycholic acid, respectively. The binding ability has positive effects on pancreatic lipase function, leading to decrease hydrolyzed triglycerides level and other absorbable lipid-molecules (Watanabe et al., 2004). Because of many functions of bile acids, bile acids could solubilize nonpolar lipids such as cholesterol and fat-soluble vitamins, increasing their water-solubility and promoting their diffusion across the unstirred water layer for delivery to the intestinal epithelium (Dietschy, 1968). It can be markedly noted that increase of binding to bile acids may alternative decrease both cholesterol and triglycerides levels through the bile acid excretion, the use of cholesterol for bile acid production, and the lack of small lipid particles for hydrolysis of triglycerides. Additionally, highly binding to secondary bile acids had some beneficial properties as anti-inflammation and anti-cancer. According to bile acids are absorbed primarily in the distal small intestine (ileum), passive absorption of protonated uncharged bile acid

species, unconjugated bile acids with only about 5% of intestinal bile acids escaping reabsorption to undergo fecal elimination (Dawson & Karpen, 2014). The accumulation of bile acids leads to tissue inflammation and injury that can be developed into colon cancer (Li & Apte, 2015). Our result indicated the very effective potent of *Prunus domestica* L. with the highest potent among those Thai berries extracts on secondary bile acids (taurodeoxycholic acid and glycodeoxycholic acid) may have ability protect against intestinal inflammation. The result was accordant to the previous report of plums (*Prunus domestica* L.) that its fresh and dried exhibited a strong capacity to bind bile acids (Kahlon & Smith, 2007). The author suggested that the effectiveness of bile acid binding may be related to antioxidant property of those fruits. In our correlation-analysis, binding to taurocholic acid was strongly influenced by total phenolic compound and anthocyanins. The binding between plant extracts and bile acids may be related to the number and position of phenolic hydroxyl groups in structure of phenolic compounds (Kuo et al., 2005; Wu et al., 2015). A study of green, black and dark tea polyphenols was recently revealed a significant positive correlation between tea phenolic including (-) epigallocatechingallate (EGCG), (-) gallocatechin gallate (GCG), and (-) epicatechingallate (ECG) contents and bile acids-binding capacity (Adisakwattana et al., 2012; Ikeda et al., 1992). Shortly, phenolic and anthocyanins of Thai berries extracts may influence the increase binding to bile acids due to the active groups and the similar structure as tea catechin.

3. Effects of aqueous anthocyanins-rich Thai berries extracts on pancreatic lipase and cholesterol esterase activities

Studies have been reported the supplementation on anthocyanin resulted in a reduction of total cholesterol and triglycerides (TG) (Alvarez-Suarez et al., 2014; Qin et al., 2009). Some publications revealed the inhibition of pancreatic lipase was functioned by several edible fruits, leading to delaying dietary fat digestion and absorption (Garza et al., 2011). Consumption of 1.5% and 3.0% of Haskap fruit effected

to decreased postprandial blood lipids and blood glucose in hyperlipidemia rats (Takahashi et al., 2014). At the concentration of 16.95 mg/mL Muscadine grape extract accessed to inhibit pancreatic lipase activity by 50% (You et al., 2012). In this current work, *Prunus domestica* L. presented the great function on the of pancreatic lipase inhibitiory activity. However, five Thai berries extracts had less effective than orlistat. The mode of orlistat action would act dependently to make covalent bond with the serine residue on the active site of the lipases, leading to inhibit pancreatic lipase function (Guerciolini, 1997). The inhibitory occurrence may be partly followed by several investigations on plant polyphenols including flavonoids, hydroxycinnamic acids, hydroxybenzoic acids, and lignans (McDougall et al., 2009; Nakai et al., 2005). The number and position of phenolic hydroxyl groups and the degree of polymerization were summarized to have high affinity to the active group of such an enzyme (Mcdougall & Stewart, 2005). There was a reported effect of EGCG inhibited lipase activity by forming a non-covalent bond with the lipase protein (Nakai et al., 2005). However, the study of Muscadine anthocyanins was explored the correlation, using Lineweaver-Burk plots, between enzymatic kinetic mode against lipase and the inhibitors including extracts, single anthocyanin and anthocyanidin. The result was fell into the competitive inhibition mode (You et al., 2012). Our analysis was also found a very strong correlations between pancreatic lipase inhibition and total anthocyanins (r=0.959, p<value 0.05), whereas total phenolic and flavonoids contents showed lower relative to the lipase inhibition, r=0.899 and r=0.677, p<value 0.05, respectively. In addition, our finding exhibited similarly with an *in vitro* study conducted by Fabroni and his colleagues. The result was found a strong relationship between total anthocyanin content containing in thirteen anthocyanins extracts and pancreatic lipase inhibition (Fabroni et al., 2016).

Not only pancreatic lipase, which is a key enzyme in dietary fat absorption, but also cholesterol esterase, which hydrolyses dietary cholesterol into free cholesterol

and free fatty acids prior being absorbed by enterocytes (Adisakwattana et al., 2012; Ngamukote et al., 2011). Extracts from edible part of plants were purposed to inhibit cholesterol esterase activity, as well. Previously, inhibition of cholesterol esterase reduced cholesterol absorption in hamster (Heidrich et al., 2004). Phenolic compound including gallic acid, catechin, epicatechin, or even anthocyanins had potentially ability as therapeutics for limiting cholesterol absorption trough the inhibition of cholesterol esterase according to gallic acid, catechin, and epicatechin were revealed to have a significant ability on cholesterol esterase inhibition with dose-dependent manner (Ngamukote et al., 2011). The researchers suggested that phenolic, flavonoids and anthocyanins possibly effected to covalent bond formation to catalyzing site of digestive enzyme, based on their antioxidant property (Deck & Vander Jagt, 2000; Kroll., 2003). The results in this present study were reported that five aqueous anthocyanins-rich Thai berries extract reduced activity of cholesterol esterase. Although our result had the limited finding IC_{50} value of simvastatin as it may have different action on such a substrate (Neuvonen et al., 2006), the inhibitory percentage could be identified. Our data was also found the moderate relationship between the inhibitory value of cholesterol esterase activity and total phenolic and anthocyanins content of Thai berries extracts with the coefficient (*r*) of 0.574 and 0.530 (p-value < 0.01), respectively. Hence, it can be markedly inferred that number and position of hydroxyl group and changing the structure of phenolic compounds are potentially restrain the pancreatic lipase and cholesterol esterase activities.

4. Inhibitory effect of aqueous anthocyanins-rich Thai berries extracts on cholesterol micellization

The effects of berries anthocyanins on a reduction of total cholesterol, especially LDL-cholesterol, were found in several subgroups of individuals including longer-term intervention and parallel study design (He & MacGregor, 2004). The reduced

cholesterol in mixed micelles is essential step for inhibition of cholesterol absorption. Sevreral phytochemical have been proved to decrease cholesterol micellization through reducing cholesterol solubility in mixed micelles. Since mixed micelles can be produced by combining natural phospholipids with specific surfactants including free fatty acid and free cholesterol, core of mixed micelles, the hydrophilic head forming of bile salts, which have a very good ability to solubilize polar lipids next to water (Oakenfull & Fisher, 1977), and phosphatidylcholine that served as a water insoluble (Almgren, 2000). The interference of forming mixed micelles have been elucidated by phytosterol, tea catechins, and saponins (Brown et al., 2016; Su et al., 2016), leading to increase particle size of micelles and lower their absorption ability. Our result was also indicated the potential of anthocyanins-rich Thai berries extracts inhibited cholesterol solubility in micellar solution with concentration dependent manner. It was also found that *Prunus domestica* L. had the highest protential to prevented cholesterol incorporation into the micelles than other extracts at the same concentration. It is possible that the presence of anthocyanins in the extract, especially cyanidins-3-glucoside and peonidin-3-glucoside, causes cholesterol precipitation (Yao et al., 2013). This phenomenon may prevent forming mixed micelles. In our result, inhibition on cholesterol micellization was slightly accorded with the analysis of black rice anthocyanins because of a strong association, found between total flavonoids (r = 0.868, p-value < 0.05) and the inhibition. The previous studies was additionally purposed that *Prunus domestica* L. contained the major flavonoids as flavonols (rutin, isorhamnetin3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, and kaempferol3-rutinoside), and the major anthocyanins as cyanidin-3-rutinoside, cyanidin-3-glucoside, peonidin-3-rutinoside, and peonidin-3-glucoside (Slimestad et al., 2009; Usenik et al., 2009). Thus, it may imply that other component of flavonoids group in Thai berries probably rule out cholesterol micellization in this investigation. Moreover, besides cholesterol, interference of forming mixed micelles may involve to

bile acids, another important component in micelle structure. The bound bile acids that had been already mentioned by the potential of Thai berries extracts may disrupt the micellization, as well as, decreasing in cholesterol solubility in micellar solution. Overall of this observation, it may be concluded that that component of anthocyanins may actively inhibit cholesterol micellization through directly binding to either bile acids or cholesterol, which caused to increase in size of micelles and the elimination of cholesterol from the mixed micelle. The further study is also suggested for reason of binding between cholesterol and bile acids.

5. Effect of *Prunus domestica* L. extract (TPE) on cholesterol uptake into Caco-2 cells

Cholesterol absorption represents in multi-step processes in which cholesterol is hydrolyzed, formed into mixed micelles, taken up by the enterocyte, assembled into lipoproteins, and transported to the lymph and the circulation (Phan & Tso, 2001). The blocking cholesterol uptake has been another essential step to enhance the lipid lowering effects. Many investigations were discovered natural pigments of plant extracts to have efficacy for interrupting cholesterol uptake into Caco-2 cells (Duangjai et al., 2016; Feng et al., 2010; Nagaoka et al., 1999). Our data was earlier indicated that *Prunus domestica* L. showed much more influential to be positive for decreased in cholesterol uptake into the intestinal cells, and its concentration 0.1-0.5 mg/mL was safe to the living Caco-2 cells. As far as we already known, review articles discussed the major content of anthocyanins containing in berries were cyanidins and peniodins groups, and Plums or *Prunus do mestica* L. was highly confirmed that the major component found in its flavonoids was anthocyanins (Fujii et al., 2006). In this study, *Prunus domestica* L. was showed the first time to decrease cholesterol uptake into the intestinal cells. The explanation may be involved to anthocyanins (cyanidin and peonidin groups), since the potent of *Prunus domestica* L. (0.25 mg/mL) alone showed

inhibitory effect on the cholesterol uptake toward ezetimibe (0.1 mg/mL), as well. Previously, pure compound that could be identified in black rice anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) inhibited the cholesterol uptake by Caco-2 cells (Yao et al., 2013). The inhibitory result could be agreed to the previous report (Yao et al., 2013), in which the researchers have suggested that the mechanism may be partly due to anthocyanins. In addition, the mode of action of ezetimibe has worked with NPC1L1 protein recycles between the plasma cell membrane and endocytic recycling compartment (Garcia-Calvo et al., 2005). This drug performs effectively when the extracellular cholesterol concentration is high because cholesterol is incorporated into the cell membrane by cell surface–localized NPC1L1. Generally, NPC1L1, the cholesterol transporter located in enterocyte, is important for cholesterol uptake into the cell. The uptake of cholesterol is then internalized together through clathrin/AP2-mediated endocytosis and transported along microfilaments to the endocytic recycling compartment in vesicles (Betters & Yu, 2010). To inhibit the cholesterol uptake into the enterocyte, ezetimibe come to impede the interaction of the NPC1L1/cholesterol complex with the AP2-clathrin complex, leading to decrease in cholesterol level (Yu et al., 2006). Our results were also found out the combinatory treatment between *Prunus domestica* L. (0.1 mg/mL) together with ezetimibe (0.05 mg/mL) demonstrated additive effect. The mixed treatment overcame single treatment of either the extract or ezetimibe (0.1 mg/mL and 0.05 mg/mL) alone. Recently, *Pirogyra neglecta* extract (SN), an edible freshwater macroalga, could inhibit cholesterol absorption into the Caco-2 cells (Duangjai et al., 2016). This SN extract was observed further in the combined dose with ezetimibe (40 mg/mL), and their results were reported the additive effect on inhibition of cholesterol uptake into Caco-2 cells. Based on the existed evidences, it may be inferred that *Prunus domestica* L., in particular its anthocyanins, probably shared the inhibition of cholesterol uptake with ezetimibe. This possibility is slightly to the suggestion in the study of SN extract

together with principle of clathrin-mediated function, which has have limited number based on AP-2 adapter (McMahon & Boucrot, 2011). This finding was also suggested that after considering to the adverse effects of treatment with ezetimibe (Hollingworth et al., 2017), the mix treatment between ezetimibe and *Prunus domestica* L. may help to decline the side effects along with the sufficient efficacy of lipid lowering treatment. However, anthocyanins were thought to have a very low bioavailability, with <1% of the ingested amount reaching the plasma (Liang et al., 2012). Hence, in case of mimic consumption of anthocyanins-rich *Prunus domestica* L. extract alone or together with ezetimibe *in vivo* situations to obtain that such those benefits may not be completely concluded. The metabolites and breakdown products of anthocyanins containing in *Prunus domestica* L. and its specific-active component are required to be investigated in the future.

CHAPTER VI

CONCLUSION

The present finding carry out to support the notion of Thai berries extracts including *Muntingia calabura* L., *Prunus domestica* L., *Syzygium nervosum* A. Cunn. Ex DC., *Antidesma bunius* (L.) *Spreng* and *Syzygium cumini* (L.) *Skeels* on anti-hyperlipidemic effect *in vitro*. Anthocyanins-rich Thai berries extracts decrease lipid digestion and absorption trough directly binding to both primary and secondary bile acids, hindering activities of pancreatic lipase and cholesterol esterase, and cholesterol micellization. Moreover, in particular anthicyanins-rich *Prunus domestica* L. extract decreased cholesterol absorption into the intestinal cells, and its ability showed additive effects when the extract combined to ezetimibe. Taken all together, five anthocyanins-rich Thai berries extracts have had benefits to be developed in food application, especially anthocyanins-rich in *Prunus domestica* L. extract. Although anthocyanins containing in *Prunus domestica* L. partly contribute to lower cholesterol absorption, other active compounds are need to be analyzed. The *Prunus domestica* L. extract can be counted as a new natural source for use as anti-hypercholesterolemia agent. Thus, this *in vitro* study should be further researched in identification of the specific-bioactive compounds of five anthocyanins Thai berries extracts and their therapeutic potent *in vivo* situation.

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APPENDIX

- Authentication of Thai berries

รายงานผลการตรวจระบุชื่อวิทยาศาสตร์ของพันธุ์ไม้และทำ voucher specimen

Collector Number	Herbarium Number	Scientific Name	Vernacular Name	Common Name	Family	หมายเหตุ
Netima Chamnansilpa 2	015910 (BCU)	*Syzygium cumini* (L.) Skeels	หว้า, ห้าขี้แพะ	Black plum, Jambolan	Myrtaceae	
Netima Chamnansilpa 3	015911 (BCU)	*Muntingia carabura* L.	ตะขบฝรั่ง, ตะขบ, ตานฝรั่ง	Bajelly tree, Jamaican cherry, Panama berry, Strawberry tree	Muntingiaceae	
Netima Chamnansilpa 4	015912 (BCU)	*Prunus domestica* L.	ลูกไหน, พลัม	Plum	Rosaceae	

แบบรายงานผล

เลขที่ 8 / 2560 วันที่ 15 พฤษภาคม 2560

รายการงานที่ทำ ตรวจระบุชื่อวิทยาศาสตร์ของพันธุ์ไม้ และทำ voucher specimen จำนวน 1 ตัวอย่าง

ผลการตรวจวิเคราะห์ / สืบค้นข้อมูล

ชื่อไทย : เม่าหลวง มะเม่าดง ม่าเม่าภูวชี เม่าช้าง หมงเม่าควาย

ชื่อสามัญ : Chinese laurel, Currentwood ชื่อวงศ์ : Phyllanthaceae (แยกมาจาก Euphorbiaceae)

ชื่อวิทยาศาสตร์ : Antidesma bunius (L.) Spreng.

Herbarium number : 015866 (BCU)

Collector number : Pattamaporn Aksornchu 2

ค่าบริการ 200 บาท (สองร้อยบาทถ้วน)

ลงชื่อ .....

(ปวิญญนุช กลิ่นร่วมน์)

ผู้ให้บริการ

แบบรายงานผล

เลขที่ 8 / 2561 วันที่ 10 พฤษภาคม 2561

รายการงานที่ทำ ตรวจระบุชื่อวิทยาศาสตร์ และทำ voucher specimen ของพันธุ์ไม้จำนวน 1 ชนิด

ผลการตรวจวิเคราะห์ / สืบค้นข้อมูล

ชื่อไทย : ชมเถื่อง

ชื่อวิทยาศาสตร์ : Syzygium nervosum A.Cunn. ex DC.

ชื่อวงศ์ : Myrtaceae

Herbarium number : 015918 (BCU)

Collector number : Netima Chamnansilpa 5

ค่าบริการ 200 บาท (สองร้อยบาทถ้วน)

ลงชื่อ .....

(นางปวิญญนุช กลิ่นร่วมน์)

ผู้ให้บริการ

- Bile acid test kit

GenWay Biotech, Inc.
6777 Nancy Ridge Drive
San Diego, CA 92121
Phone: 858.458.0866
Email: sales@genwaybio.com

Colorimetric Total Bile Acids Assay Kit

Catalog Number: GWB-BQK090

Configuration

The GenWay Colorimetric Total Bile Acids reagent is provided in the following kit configuration:

Configuration	Catalog No.	Kit Size
Universal	GWB-BQK090	R1 (diluent): 1 x 105 mL R2: 1 x 20 mL R3: 10 x 10 mL Cal: 1 x 2 mL

Intended Use

The assay kit is for determination of serum total bile acids (TBA).

Background

Total bile acids are metabolized in the liver and hence serve as a marker for normal liver function. Serum total bile acids are increased in research subjects with acute hepatitis, chronic hepatitis, liver sclerosis and liver cancer.

Assay Principle

In the presence of NAD, the enzyme 3-α hydroxysteroid dehydrogenase (3-α HSD) converts bile acids to 3-keto steroids and NADH. The NADH formed reacts with nitrotetrazolium blue (NBT) to form a formazan dye in the presence of diaphorase enzyme. The dye formation is monitored by measuring absorbance at 540nm and is directly proportional to the bile acids concentration in the serum sample.

$$NAD^+ \rightleftharpoons NADH + H^+$$

$$\text{Bile acids} \xrightarrow{\text{3-α HSD}} \text{Oxidized bile acids}$$

$$NADH + H^+ + NBT \xrightarrow{\text{diaphorase}} NAD^+ + \text{formazan}$$

Materials Required but not Provided

An analyzer capable of dispensing two reagents and of measuring absorbance at about 540 nm with temperature control (37°C).

Controls for validating the performance of the bile acid reagents are provided separately (GWB-BQK259).

Reagent Composition

	Active Ingredients
Reconstitution buffer (R1)	Phosphate buffer, EDTA
Reagent 2	3-α-HSD, Tris buffer
Reagent 3	Diaphorase, NAD^+, NBT, Oxamic Acid
Bile Acids Standard	35 µmole/L

Reagent Preparation

Transfer 10 mL of the contents of diluent **R1** to one bottle of **R3 (diaphorase)** and dissolve by swirling gently.

Reagent Stability and Storage

GenWay's Colorimetric Total Bile Acids Assay Kit, calibrators, and controls should be stored at 2-8°C. **DO NOT FREEZE**. The reagents, calibrators, and controls are stable when stored as instructed until the expiration date on the label. Do not mix reagents of different lots.

- **R3 is light and temperature sensitive**. **R3** should not be stored in temperatures above refrigerated conditions (2-8^{0}C) or exposed to light for extended periods of time.

The Reconstituted **R3** is stable for 1 week at 4^{0}C

Specimen Collection and Handling

Use fresh serum or EDTA treated plasma samples. Hemolysed or heparinized samples should not be used.

Precautions

1. For Research Use Only in the USA. Not for use in diagnostic procedures.
2. Avoid use haemolyzed samples and heparinized plasma as these interfere with the assay.
3. Inspect **R3** before use. Do not use **R3** if it is a dark color.
4. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.
5. Solutions 1 and 2 contain sodium azide. Avoid ingestion or contact with skin or mucous membranes. In case of skin contact, flush affected area with copious amounts of water.
6. Sodium azide reacts with lead and copper plumbing, to form potentially explosive azides. When disposing of such reagents, flush with large volume of water to prevent azide build up.
7. Additional safety information concerning storage and handling of this product is provided within the Material Safety Data Sheet for this product. To obtain an MSDS, please contact our customer service department at 858-458-0866.

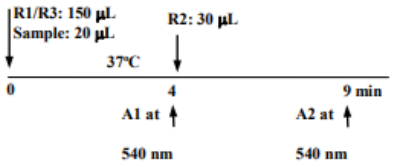
Assay Procedure

1. Reconstitute the contents of one bottle of **R3** (diaphorase) with 10 mL of reconstitution buffer **R1**. Reconstituted **R3** is stable for 1 week at 4^0C.
2. Pre-warm reconstituted **R1** and **R2** at RT.
3. To a cuvette add 150 µl of reconstituted **R3** and 20 µl of sample or standard, mix well, and incubate at 37^0C for 4 min.
4. Add 30 µl of **R2**, mix well, and immediately read the absorbance at 540 nm as A_1.
5. Incubate for 5 min, and read the absorbance at 540 nm as A_2.
6. Calculate ΔA540/5min for sample and standard by subtracting A_1 from A_2. ΔA540/5min = (A_2 - A_1).

7. Determine total bile acids concentration using the equation below:

Sample Bile Acids (µmole/L) =

$$\frac{\Delta A_{540sample}}{\Delta A_{540standard}} \text{ X standard (35µmole/L)}$$



Calibration

A single level of calibrator included is ready to use and are stable up to expiration date when stored at 2-8°C.

1. This assay should be calibrated daily using the enclosed calibrator.
2. Construct a calibration curve by plotting the ΔA values of the calibrators against the corresponding concentrations.
3. The bile acid concentration of the sample is read from the calibration curve.

A Reagent blank may be performed by replacing sample or standard with distilled water.

Quality Control

Good laboratory practice recommends the use of control materials. Users should follow the appropriate federal, state and local guideline concerning the running of external quality control.

To ensure adequate quality control, normal and abnormal control with known values should be run as unknown samples.

Results

Results are printed out in µmol/L. Literature reports cite subjects having a TBA activity in the range of 0-10µmol/L[1,2].

Limitations

The assay is designed for use with fresh serum sample and EDTA treated plasma only.

Linearity is up to 200 µmole/L. Samples that exceeded the linearity limit should be diluted with an equal volume of 0.9% saline. Multiply the result by two.

References

1. LaRusso, N.F. et al., Dynamics of Enterohepatic Circulation of Bile Acids, New Engl J M, 291, 689-692, (1974).
2. Skrede S. et al: Bile acids measured in serum during fasting as a test for liver disease, Clin Chem 24: 1095-1099, 1978

- Total cholesterol test kit

CHOLESTEROL liquicolor

Método CHOD-PAP

Prueba enzimática colorimetrica para colesterol con factor aclarante de lípidos (LCF)

Presentación del estuche

REF	10017	4 x 30 ml	Estuche completo
	10019	3 x 250 ml	Estuche completo
	10028	4 x 100 ml	Estuche completo
	10015	9 x 3 ml	Estándar

IVD

Método

El colesterol se determina después de la hidrólisis enzimática y la oxidación. El indicador es la quinoneimina formada por el peróxido de hidrógeno y 4-aminoantipirina en presencia de fenol y peroxidasa.

Principio de la reacción

$$\text{Esteres de colesterol} + H_2O \xrightarrow{\text{CHE}} \text{colesterol} + \text{acidos grasos}$$

$$\text{Colesterol} + O_2 \xrightarrow{\text{CHO}} \text{colestene-3-ona} + H_2O_2$$

$$2\,H_2O_2 + \text{4-aminoantipirina} + \text{fenol} \xrightarrow{\text{POD}} \text{quinoneimina} + 4\,H_2O$$

Contenidos

RGT	**4 x 30, 3 x 250 ó 4 x 100 ml Reactivo enzimático**	
	Buffer fosfato (pH 6,5)	100 mmol/l
	4-aminoantipirina	0,3 mmol/l
	Fenol	5 mmol/l
	Peroxidasa	> 5 KU/l
	Colesterolesterasa	> 150 U/l
	Colesteroloxidasa	> 100 U/l
	Azida de sodio	0,05 %
STD	**3 ml Estándar**	
	colesterol	200 mg/dl ó 5,17 mmol/l

Preparación de reactivos

RGT y STD están listos para usar.

Estabilidad de los reactivos

Los reactivos son estables hasta la fecha de caducidad, aún después de abrir, cuando se almacenan de 2...8°C o por 2 semanas de 15...25°C.
Una vez abiertos, debe evitarse la contaminación.

Muestras

Suero, plasma con heparina ó EDTA.
Nota: Muestras lipémicas usualmente producen turbidez cuando se mezcla la muestra con el reactivo generando resultados elevados falsos. La prueba CHOLESTEROL **liquicolor** evita estos resultados elevados falsos por medio del factor aclarante de lipidos (LCF). El LCF aclara totalmente la turbidez causada por las muestras lipémicas.

Ensayo

Longitud de onda:	500 nm, Hg 546 nm
Paso de luz:	1 cm
Temperatura:	20...25°C ó 37°C
Medicion:	Frente a un blanco de reactivo. Sólo se requiere un blanco de reactivo por serie.

Esquema de pipeteo

Pipetar en las cubetas	Blanco de reactivo	Muestra ó STD
Muestra/STD	—	10 µl
RGT	1000 µl	1000 µl
Mezclar, incubar 10 minutos de 20...25°C o por 5 minutos a 37°C. Medir la absorbancia de la STD y de muestra frente al blanco de reactivo antes de 60 minutos (ΔA).		

Cálculo

1. Con factor

Longitud de onda	C [mg/dl]	C [mmol/l]
Hg 546 nm	840 x ΔA	21,7 x ΔA
500 nm	553 x ΔA	14,3 x ΔA

2. Con estándar

Usar solamente el estándar recomendado por HUMAN (incluído en el estuche ó en el REF 10015).

$$C = 200 \times \frac{\Delta A_{\text{muestra}}}{\Delta A_{\text{STD}}} \quad [\text{mg/dl}]$$

ó

$$C = 5{,}17 \times \frac{\Delta A_{\text{muestra}}}{\Delta A_{\text{STD}}} \quad [\text{mmol/l}]$$

Características de la prueba

Linearidad

La prueba es lineal hasta concentraciones de colesterol de 750 mg/dl ó 19,3 mmol/l. Diluir las muestras con concentraciones más altas de colesterol 1 + 2 con solución salina fisiológica (NaCl 0,9%) y repetir la determinación. Multiplicar el resultado por 3.

Los datos típicos de ejecución de la prueba pueden ser encontrados en el informe de verificación, accesible vía
www.human.de/data/gb/vr/su-chol.pdf y
www.human-de.com/data/gb/vr/su-chol.pdf

Interpretación clínica

Sospechoso:	sobre	220 mg/dl	ó	5,7 mmol/l
Elevado:	sobre	260 mg/dl	ó	6,7 mmol/l

La Sociedad Europea De Aterosclerosis recomienda disminuir los niveles de colesterol a aproximadamente 180 mg/dl para adultos menores de 30 años y a 200 mg/dl para adultos mayores de 30 años.

Control de calidad

Pueden emplearse todos los sueros controles con valores determinados por este método.
Nosotros recomendamos el uso de nuestro suero de origen animal HUMATROL ó nuestro suero de origen humano SERODOS para control de calidad.

Automatización

Proposiciones para la aplicación de los reactivos sobre analizadores están disponibles sobre demanda. Cada laboratorio tiene que validar la aplicación en su propia responsabilidad.

Notas

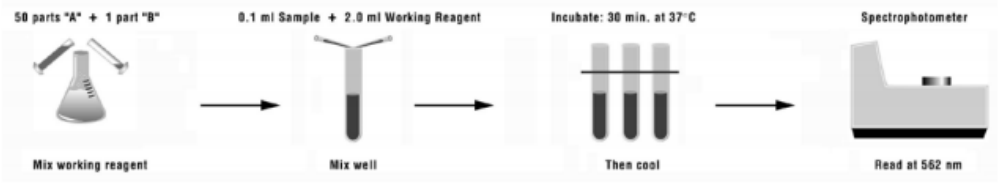
1. La prueba no es influenciada por valores de hemoglobina de hasta 200 mg/dl ó por valores de bilirrubina de hasta 5 mg/dl.
2. Los reactivos contienen azida de sodio como preservante (0,05%). No ingerirlos. Evitar el contacto con la piel y membranas mucosas.

Literatura

1. Schettler, G. and Nüssel, E., Arb. Med. Soz. Med. Präv. Med. **10**, 25 (1975)
2. Richmond, W., Clin. Chem. **19**, 1350 (1973)
3. Röschlau, P. *et al.*, J. Clin. Chem. Clin. Biochem. **12**, 403 (1974)
4. Trinder, P., Ann. Clin. Biochem. **6**, 24 (1969)

■ BCA Protein Assay Kit

Procedure Summary (Test-tube Procedure, Standard Protocol)



Test-tube Procedure (Sample to WR ratio = 1:20)

1. Pipette 0.1mL of each standard and unknown sample replicate into an appropriately labeled test tube.
2. Add 2.0mL of the WR to each tube and mix well.
3. Cover and incubate tubes at selected temperature and time:
   - Standard Protocol: 37°C for 30 minutes (working range = 20-2000µg/mL)
   - RT Protocol: RT for 2 hours (working range = 20-2000µg/mL)
   - Enhanced Protocol: 60°C for 30 minutes (working range = 5-250µg/mL)

   Notes:
   - Increasing the incubation time or temperature increases the net 562nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
   - Use a water bath to heat tubes for either Standard (37°C incubation) or Enhanced (60°C incubation) Protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.
4. Cool all tubes to RT.
5. With the spectrophotometer set to 562nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

   Note: Because the BCA assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562nm absorbance measurements of all tubes are made within 10 minutes of each other.
6. Subtract the average 562nm absorbance measurement of the Blank standard replicates from the 562nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (Sample to WR ratio = 1:8)

1. Pipette 25µL of each standard or unknown sample replicate into a microplate well (working range = 20-2000µg/mL) (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).

   Note: If sample size is limited, 10µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125-2000µg/mL.
2. Add 200µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.

- **Laboratory instruments and devices**

	Company/Country
Spectrofluorometer	Perkin Elmer (MA, USA)
Spectrophotometer	Perkin Elmer (MA, USA)
pH meter	Thermo Scientific (MA, USA)
Laboratory refrigerator	Sanden intercool (Thailand)
Vortex mixer	Gemmy industrial corp. (Taiwan)
Autoclave	Amegax Intruments, Inc. (USA)
CO_2 incubator	Skadi Europe B.V. (Netherland)
Cell culture plate	Thermo Scientific (USA)
Centrifuge tube	Sterilin (UK)
Cryotube	Thermo Scientific (USA)
Filter paper (0.45 μm)	Whatman (UK)
Glass bottle	Duran (Germany)
Microcentrifuge tube (1.5, 2 mL)	Hyclone (UK)
Pipette tip (20, 200, 100 μL)	Axygen (USA)
Freezer (-20 °C)	Sanyo (Japan)
Light microscope	Olympus (Japan)
Magnetic stirrer	IKA (Germany)
Rotary evaporator (R-215)	Bochi (Switzerland)
Vacuum pump	Bochi (Switzerland)
Water bath	Memmert (Germany)

VITA

Miss Netima Chamnansilpa was born on October, 7th, 1989 in Phare province, in the north of Thailand. She graduated Bachelor degree of Public Health (Nutrition and Dietetics) from faculty of Public Health, Mahidol University in 2012. She used to work at Phrae Provincial Public Health Office as a project coordinator, nutritional guest speaker, and guest lecturer for Boromarajonani College of Nursing Uttaradit in 2014-2015.