The Inhibitory Effects of Anthocyanin-rich Thai Berry Extracts on Carbohydrate Digestion, Glycation and Adipogenesis

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ฤทธิ์ของสารสกัดผลไม้ไทยกลุ่มเบอร์รี่ที่มีแอนโธไซยานินสูงต่อการยับยั้งการย่อยคาร์โบไฮเดรต กระบวนการไกลเคชั่น และการสร้างเซลล์ไขมัน

น.ส.ปัทมาภรณ์ อักษรชู

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ปัทมาภรณ์ อักษรชู : ฤทธิ์ของสารสกัดผลไม้ไทยกลุ่มเบอร์รี่ที่มีแอนโธไซยานินสูงต่อการยับยั้งการย่อยคาร์โบไฮเดรต กระบวนการไกลเคชั่น และการสร้างเซลล์ไขมัน. (The Inhibitory Effects of Anthocyanin-rich Thai Berry Extracts on Carbohydrate Digestion, Glycation and Adipogenesis) อ.ที่ปรึกษาหลัก : ผศ. ดร. สถาพร งามอุโฆษ

การศึกษาในอดีตพบว่าเบอร์รี่ที่มีสารแอนโทไซยานินสูงมีส่วนช่วยในการส่งเสริมสุขภาพร่างกายให้ดีขึ้นอย่างไรก็ตามยังไม่มีหลักฐานทางวิทยาศาสตร์แสดงถึงฤทธิ์ของเบอร์รี่ไทยต่อการชะลอการดูดซึมน้ำตาล การต้านอนุมูลอิสระ ต้านการเกิดไกลเคชั่น และการยับยั้งการสร้างและสะสมไขมันในเซลล์ไขมัน งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของสารแอนโทไซยานินในเบอร์รี่ไทย ได้แก่ มะเม่า (*Antidesma bunius)* มะหวด (*Lepisanthes rubiginosa*) และมะเกี๋ยง (*Syzygium nervosum*) ต่อการยับยั้งการย่อยและการดูดซึมน้ำตาลและไขมัน การต้านอนุมูลอิสระ การยับยั้งการเกิดไกลเคชั่น และการยับยั้งการสร้างและสะสมไขมันในเซลล์ไขมัน จากการศึกษาพบว่า ปริมาณโดยรวมของสารประกอบฟีโนลิค และแอนโทไซยานิน ของสารสกัดที่จากเบอร์รี่พบว่า สารสกัดจากเบอร์รี่ไทยทั้ง 3 ชนิด มีปริมาณรวมของสารประกอบฟีโนลิค เทียบเท่ากับ 237.90-300.91 มิลลิกรัมของกรดแกลลิคต่อกรัมของผงสารสกัดและมีปริมาณแอนโทไซยานินรวมเท่ากับ 32.45-66.86 มิลลิกรัมของไซยานิดิน-3-กลูโคไซด์ต่อกรัมของผงสารสกัด และมีปริมาณไซยานิดิน-3-กลูโคไซด์อยู่ในช่วง 27.19-39.96 มิลลิกรัมต่อกรัมของผงสารสกัด นอกจากนี้สารสกัดจากมะเม่าและมะหวดมีปริมาณเดลฟินิดิน-3-กลูโคไซด์เท่ากับ 21.65 และ 0.93 มิลลิกรัมต่อกรัมของผงสารสกัดตามลำดับนอกจากนี้ยังพบว่าสารสกัดดังกล่าวมีฤทธิ์ในการยับยั้งการทำงานของเอนไซม์มอลเตสและซูเครส โดยมีค่า IC_{50} อยู่ในช่วงความเข้มข้น 0.79-1.52 มิลลิกรัมของสารสกัดต่อมิลลิลิตรและ 1.34-1.65 มิลลิกรัมของสารสกัดต่อมิลลิลิตรตามลำดับ สารสกัดจากมะเม่ามีฤทธิ์ในการต้านอนุมูลอิสระสูงกว่ามะหวดและมะเกี๋ยง สารสกัดจากมะเม่าจึงถูกนำมาศึกษาความสามารถในการต้านการเกิดไกลเคชั่นของโปรตีนจากการเหนี่ยวนำด้วยน้ำตาล ฟรุคโตสและกลูโคสพบว่า สารสกัดจากมะเม่า (ความเข้มข้น 0.25 มิลลิกรัมต่อมิลลิลิตร) ลดการเกิดผลิตภัณฑ์สุดท้ายของปฏิกิริยาไกลเคชั่น โดยลดผลิตภัณฑ์ฟลูออเรสเซนต์และไม่ใช่ฟลูออเรสเซนต์อย่างมีนัยสำคัญทางสถิติ อีกทั้งยังสามารถป้องกันการเกิดโปรตีนออกซิเดชัน โดยลดระดับของการเกิดโปรตีนคาร์บอนิล (protein carbonyl) ยับยั้งการตกตะกอนของโปรตีนโดยลดระดับของการสร้างโครงสร้างเบต้าอะไมลอยด์ (β-amyloid structure) นอกจากนั้นสารสกัดจากมะเม่าที่ความเข้มข้น 16 ไมโครกรัมต่อมิลลิลิตรป้องกันการเปลี่ยนแปลงของเซลล์ 3T3-L1 และลดการสะสมไตรกลีเซอไรด์ภายในเซลล์ โดยการยับยั้งการแสดงออกของ mRNA ของ adipogenic transcriptional factors C/EBPα และ PPARγ receptor ซึ่งสำคัญต่อกระบวนการสร้างเซลล์ไขมัน และลดการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างไขมันได้แก่ Acetyl CoA carboxylase (*ACC)* Fatty acid synthase (*FASN*) และ Lipoprotein lipase (*LPL*) จึงอาจสรุปได้ว่าสารสกัดเบอร์รี่ไทย โดยเฉพาะมะเม่าอาจนำมาพัฒนาต่อยอดเป็นผลิตภัณฑ์ที่ดีต่อสู่สุขภาพเพื่อช่วยชะลอการย่อยคาร์โบไฮเดรต ลดการเกิดไกลเคชั่นที่ถูกเหนี่ยวนำด้วยน้ำตาลโมเลกุลเดี่ยว ลดการเกิดออกซิเดชั่นและลด การเกาะกลุ่มของโปรตีน รวมถึงยับยั้งการสะสมไขมันในเซลล์ไขมันได้

สาขาวิชา	อาหารและโภชนาการ	ลายมือชื่อนิสิต .....
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Pattamaporn Aksornchu : The Inhibitory Effects of Anthocyanin-rich Thai Berry Extracts on Carbohydrate Digestion, Glycation and Adipogenesis . Advisor: Asst. Prof. SATHAPORN NGAMUKOTE, Ph.D.

Several studies have reported the benefits of anthocyanin-rich berries on improving human health. However, the effect of Thai berries on delaying carbohydrate digestion, antioxidant properties, glycation inhibition, and anti-adipogenesis have not been investigated. Therefore, the present study aimed to determine the potential of anthocyanin-rich Thai berry extracts from *Antidesma bunius or Mamao* (ABE), *Lepisanthes rubiginosa* or Mahuat (LRE) and *Syzygium nervosum or Makiang* (SNE) on the inhibition of carbohydrate digestive enzymes, the antioxidant activity, anti-glycation, and anti-adipogenic property. In this study, total phenolics, total anthocyanins, and cyanidin-3-glucoside (C3G) contents of the anthocyanin-rich Thai berry extracts were 237.90-300.91 mg GAE/ g extract, 32.45-66.86 mg C3G/ g extract, and 27.19-39.96 mg/ g extract, respectively. Besides, ABE and LRE also contained delphinidin-3-glucoside with values of 21.65 and 0.93 mg/ g extract, respectively. All extracts demonstrated inhibitory activity against intestinal maltase and sucrase with IC_{50} values of 0.79 -1.52 mg extract/ml and 1.34-1.65 mg extract/ ml, respectively. It was found that ABE exhibited better antioxidant properties than LRE and SNE. Therefore, ABE was further investigated on the antiglycation and antiadipogenic properties. It was found that ABE (0.25 mg/ml) significantly reduced the formation of fluorescence and non-fluorescence AGEs (N^{ε}-carboxymethyl lysine, N^{ε}-CML) in fructose and glucose-mediated protein glycation. ABE prevented protein oxidation by reducing the protein carbonyl content and inhibiting protein aggregation by decreasing the β-amyloid cross structure formation. Furthermore, ABE (16 µg/ml) prevented 3T3-L1 cell differentiation. It reduced the intracellular triglyceride accumulation by the inhibition of adipogenic transcription factor expression, C/EBPα. Pparγ receptor contributed to reducing the mRNA expression of acetyl-CoA carboxylase (*ACC*), fatty acid synthase (*FASN*), and lipoprotein lipase (*LPL*). These findings suggested that Thai berry extract in this study, especially ABE, can be useful as a promising natural compound for delaying carbohydrate digestion, decreasing the monosaccharide-induced protein glycation and oxidative protein damage, protein aggregation, and preventing adipogenesis.

Field of Study:	Food and Nutrition	Student's Signature .....
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CHAPTER I

INTRODUCTION

1.1 Background and significance of this study

Diabetes mellitus (DM) is a group of metabolic disorders characterized by prolonged hyperglycemia, dyslipidemia, and abnormal protein metabolism caused by the deficiency of insulin secretion or resistance to insulin action in target cells (Sarkar et al., 2016). Normal carbohydrate consumption relates to increasing blood sugar level or hyperglycemia (Sheard et al., 2004). Dietary carbohydrates are digested into monosaccharides by the carbohydrate digestive enzymes, including α-amylase and intestinal α-glucosidase (Aisa, Gao, Yili, Ma, & Cheng, 2019; Baynes, 1991). The absorption of monosaccharides into the bloodstream leads to the increasing postprandial blood glucose level (Edirisinghe & Burton-Freeman, 2016). Besides, chronic hyperglycemia is a major risk for diabetes progression and vascular complications, especially retinopathy, nephropathy, neuropathy (Yeh, Hsia, Lee, & Wu, 2017). Several factors are involved in developing vascular complications, specifically, advanced glycation end-products or AGEs (Spínola, Llorent-Martínez, & Castilho, 2019).

Advanced glycation end-product (AGEs) is non-enzymatic glycation. This reaction involves amino residues of proteins interact with carbonyl groups of reducing sugars such as glucose and fructose, resulting in an unstable Schiff' s base formation. After that, the Schiff' s base rearranges to form a more stable ketoamine, called Amadori products such as fructosamine and hemoglobin A1C (Kato et al., 1989;

Peppa, Uribarri, & Vlassara, 2003). Amadori products are consequently dehydrated or oxidized, and converted to dicarbonyl compounds. These dicarbonyls then reactively undergo a formation of irreversible AGEs, including fluorescence cross-link AGEs and non-fluorescence crosslink AGE and non-crosslink AGEs (Aramsri Meeprom, Weerachat Sompong, Catherine B. Chan, & Sirichai Adisakwattana, 2013; Wu, Huang, Lin, & Yen, 2011). Moreover, the high level of endogenous fructose in diabetic patients resulted from glucose conversion through the polyol pathway (Chayaratanasin, Barbieri, Suanpairintr, & Adisakwattana, 2015). Scientific evidence demonstrated that fructose exhibited the rate of AGEs formation about 10 times faster than glucose (Aragno & Mastrocola, 2017). In general, the binding of AGEs and receptors for AGEs causes the vascular damage through the increased reactive oxygen species (ROS), the producing inflammatory cytokines, and the macrophage-platelet interaction (Yamagishi, 2011). The ROS could interact with macromolecules including protein, lipid, and DNA contributing to the pathogenesis of diseases such as Alzheimer's disease, cardiovascular disease, and cancer (R. Singh, Barden, Mori, & Beilin, 2001). Therefore, controlling postprandial blood glucose levels to be in normal range may reduce glycation formation, contributing to preventing or delaying hyperglycemia-induced vascular complications in diabetes patients. At present, acarbose is an anti-diabetic agent that has been used to reduce the postprandial blood glucose level in diabetic patients (Adisakwattana, Ruengsamran, Kampa, & Sompong, 2012; Paul J. Thornalley, 2003). Aminoguanidine (AG) is also a potent anti-glycating agent, however, it exhibited severe adverse effect such as flu-like symptoms, anemia, myocardial infarction, congestive heart failure, and arterial

fibrillation. Hence, it was terminated in a clinical trial (Friedman, 2010; R. Singh et al., 2001).

Obesity is a metabolic syndrome caused by excessive energy intake leading to adverse effects on health such as cardiovascular disease, hypertension, and atherosclerosis (Chayaratanasin et al., 2019; Li et al., 2016). Furthermore, obesity is a risk factor for type 2 diabetes through insulin resistance and hyperglycemia (Martyn, Kaneki, & Yasuhara, 2008; Ye, 2013). The characteristic of obesity is involved in the adipogenesis process. This process consists of the increasing adipocyte cell numbers (hyperplasia) and adipocyte cell size (hypertrophy). Consequently, the fibroblast-like shape cells change to mature adipocyte cells (Li et al., 2016). Adipogenesis consists of cell proliferation and cell differentiation processes. These processes are regulated by the CCAAT/enhance binding protein α (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ). The expression of these proteins could activate the lipogenic genes. The lipogenic genes expression increases adipocyte differentiation, fatty acid transportation, and lipid synthesis (lipogenesis). The lipogenesis pathway includes triglyceride synthesis and lipid accumulation into adipocytes (Kongthitilerd et al., 2020). Therefore, the management of obesity may relate to the inhibition of adipocyte differentiation through the decreasing fat cell size and number (Li et al., 2016).

Nowadays, plant-derived products containing phenolic compounds have been of interest in preventing or delaying metabolic disorders by inhibiting carbohydrate digestion, preventing AGEs formation, and reducing adipogenesis. Anthocyanins, flavonoids compound, are the natural pigment that presents red, blue, or purple color in plants such as onion, eggplant, grape, cherry, and berry (Fang, 2015). In *in vitro*

studies, plants containing anthocyanins exhibited the inhibitory effect of pancreatic α-amylase and intestinal α-glucosidase (Mojica, Berhow, & Gonzalez de Mejia, 2017; Sui, Zhang, & Zhou, 2016) and decreased fructose-induced protein glycation and protein aggregation (Ma et al., 2018). Moreover, anthocyanin-enriched Rice berry rice inhibited adipogenesis in 3T3-L1 preadipocyte cells through decreasing lipogenic gene expression (Kongthitilerd et al., 2020). In an animal study, the fortification of defatted soybean flour with anthocyanins-rich blueberry extract reduced blood glucose levels in diabetic C57BL/6 mice fed with a very high-fat diet (Roopchand, Kuhn, Rojo, Lila, & Raskin, 2013). A clinical study in men and postmenopausal women showed that, drinking of blackcurrant extract containing anthocyanin decreased the postprandial blood glucose level in the first 30 minutes (Castro-Acosta et al., 2016).

Antidesma bunius (L) spreng (Mamao) is grown in the Northeast region of Thailand. The fruit extract of *Antidesma bunius* contained gallic acid, epicatechin, and cyanidin-3-glucoside, which have been reported for the antioxidant activity in various models (Jorjong, Butkhup, & Samappito, 2015). Moreover, this fruit prevented hypertension in N^{ω}-nitro-L-arginine methyl ester-induced hypertensive rats by reducing superoxide radical scavenging, inhibiting lipid and protein oxidation, and elevating eNOS expression (Kukongviriyapan et al., 2015). The fruit extract of *Antidesma bunius* decreased the gene expression of lipogenic enzymes, triglyceride content, and inflammation in high fat diet-induced rats (Ngamlerst et al., 2019).

Syzygium nervosum A. Cunn. ex DC. (synonym *Cleistocalyx nervosum var paniala*; Ma-kiang) is cultivated in Northern Thailand. The fruit extract of *Cleistocalyx nervosum* contained cyanidin-3-glucoside. This fruit extract exhibited

antioxidant activity in various models (Charoensin, Taya, Wongpornchai, & Wongpoomchai, 2012). The fruit extract of *Cleistocalyx nervosum var paniala* showed the lowering of malondialdehyde in serum and liver. Moreover, it enhanced the activities of glutathione peroxidase and catalase in the liver, resulting in the prevention of hepatocarcinogenesis in rats (S. Taya, Punvittayagul, Inboot, Fukushima, & Wongpoomchai, 2014). *Cleistocalyx nervosum var paniala* exhibited neuroprotective and antioxidant activity by reducing ROS production and increasing the antioxidant enzyme mRNA expressions (Sukprasansap, Chanvorachote, & Tencomnao, 2017).

Lepisanthes rubiginosa (Roxb.) Leenh, Mahuat, is cultivated in northeastern Thailand. The essential oil from fruits of *Lepisanthes rubiginosa* (Roxb.) Leenh inhibited the growth of microorganisms, including *Trichophyton mentagophyte, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* (Chuangbunyat, Teerawutgulrag, Pyne, Liawruangrath, & Liawruangrath, 2011). Although these berries show various biological properties, their inhibitory effects against carbohydrate digestive enzymes, protein glycation, and adipogenesis remain unknown. Therefore, it would be interesting to investigate these berries effects on carbohydrate hydrolysis enzymes, monosaccharide-induced protein glycation, and adipocyte differentiation *in vitro* model.

1.2 The objective of the present study

- To investigate the effect of Thai berry extracts on pancreatic α-amylase and intestinal α-glucosidase and antioxidant capacities.

- To examine the effect of *Antidesma bunius* extract (ABE) on glucose or fructose-induced AGEs formation, protein oxidation, and protein aggregation in the BSA model.
- To investigate the inhibitory effect of *Antidesma bunius* extract (ABE) adipogenesis in 3T3-L1 preadipocyte cells.

1.3 Hypotheses of the present study

- Thai berry extracts could inhibit the pancreatic α-amylase and intestinal α-glucosidase and reduce the free radical generation.
- *Antidesma bunius* extract (ABE) could prevent the glucose-, fructose-induced BSA glycation by reducing advanced glycation end-products formation, protein oxidation, and protein aggregation.
- *Antidesma bunius* extract (ABE) could decrease 3T3-L1 preadipocyte cell differentiation into mature adipocytes by suppressing adipogenic transcriptional factors and lipogenic genes.

CHAPTER II

LITERATURE REVIEW

2.1 Diabetes

2.1.1 Prevalence

Diabetes is a health problem which is the 10th leading cause of death in the world. The prevalence of diabetes was approximately 463 million worldwide, and it will be up to 700 million by 2045 (Saeedi et al., 2019). In Thailand, the prevalence of diabetes in adults was 4.28 million or 8.3% of total adults (International Diabetes Federation, 2019) Diabetes and its complications, including neuropathy, nephropathy, retinopathy, and cardiovascular diseases, impact life and socioeconomics among population globally (American Diabetes Association, 2014).

2.1.2 Characteristic and types of diabetes

Diabetes is a chronic disease that is involved in the abnormality of carbohydrate metabolism. Diabetes is characterized by a high blood glucose level, known as hyperglycemia (International Diabetes Federation, 2017). The long-term hyperglycemia resulting from inadequate insulin production, insulin resistance in target cells or both, leads to the increased blood glucose level (American Diabetes Association, 2014; International Diabetes Federation, 2017). Diabetes can be divided into three main groups as followings:

(1). Type I diabetes is involved in the damage of β-cells by a cellular-induced autoimmune, resulting in inability to produce insulin completely.

(2). Type II diabetes is the main type of diabetes, with approximately 90% of all diabetes types. This type is caused by the impairment of the target cell's insulin response (insulin resistance) or inadequate insulin production.

(3). Gestational diabetes mellitus (GDM) occurs during pregnancy, resulting from the placenta hormones, including estrogen, cortisol, and human placental lactogen, which then decreases insulin action (American Diabetes Association, 2014).

2.1.3 Carbohydrate digestion and hyperglycemia

Dietary carbohydrates are a major substance for energy production in the body. Carbohydrates can be categorized into monosaccharides, disaccharides, and polysaccharides, such as simple sugars and starch (Kwon, Jang, & Shetty, 2006). Moreover, the composition of starch is amylose (15%) and amylopectin (85%). These components consist of glucose units in their structure (Williamson, 2013). Recently, scientific evidence has reported that sugary drinks and refined carbohydrates' consumption increased diabetes development (International Diabetes Federation, 2017; Sarkar et al., 2016). A previous study has reported that the amount and type of dietary carbohydrate influenced the blood glucose response by 85-94% (Wolever & Bolognesi, 1996). Besides, other factors, including physical property (e.g., juice, whole fruit), ripeness, and cooking method, can affect postprandial blood glucose response (Pi-Sunyer, 2002). In general, carbohydrate consumption raises the postprandial glucose level or causes postprandial hyperglycemia (Aisa et al., 2019; Barik et al., 2020).

Dietary carbohydrates are digested by pancreatic α -amylase and intestinal α-glucosidase, resulting in the release of glucose absorbs by the small intestine to the hepatic portal vein (Sarkar et al., 2016; Williamson, 2013). As shown in Figure 1,

pancreatic α-amylase locates in the small intestine. This enzyme hydrolyzes the α-1,4-glycosidic bond in starch and other polysaccharides. The enzyme reaction produces a mixture of maltose, maltotriose, and branched oligosaccharides containing 6-8 glucose units (Holmes, 1971; Sales, Souza, Simeoni, & Silveira, 2012). Intestinal α-Glucosidase is a membrane-bound enzyme at the epithelium of the small intestine. This enzyme includes maltase, sucrase, glucoamylase, and isomaltase, which are involved in the late carbohydrate digestion stage. These enzymes hydrolyze the terminal non-reducing 1,4 linked α-glucose leading to monosaccharide releasing such as glucose absorbing into the bloodstream (Trinh, Staerk, & Jäger, 2016). Therefore, both pancreatic α-amylase and intestinal α-Glucosidase are an important factor for postprandial hyperglycemia.

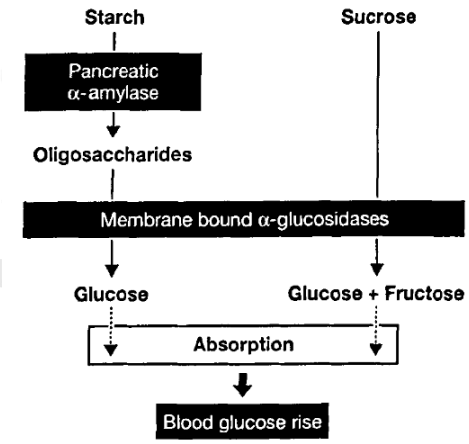


Figure 1 Schematic diagram of carbohydrate digestion by pancreatic α-amylase and intestinal α-glucosidase (Bischoff, 1994).

2.1.4 Hyperglycemia and oxidative stress

Oxidative stress is defined as an imbalance between oxidants and antioxidants. The cellular metabolism generates reactive oxygen species (ROS) such as superoxide

radical (O_2^-), which can convert to hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$), and alkoxyl radicals ($RO^{\cdot}$) (Matough, Budin, Hamid, Alwahaibi, & Mohamed, 2012). Recent studies have reported that the high consumption of carbohydrate as well as the high levels of glucose in intracellular and extracellular leading to the increasing of oxidative stress, then further to the progression of diabetic complications and chronic diseases such as cardiovascular disease (Sarkar et al., 2016; Surapon, 2015). According to a previous study, the high concentration of glucose-induced ROS production in endothelial cells by 250% within 24 hours increased lipid peroxidation by 330% within 168 hours (Araki & Nishikawa, 2010). High ROS level damaged the intracellular antioxidant enzymes: superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase (Patel, Chen, Das, & Kavdia, 2013). The generation of ROS is influenced by several pathways, including the auto-oxidation of glucose, protein glycation, protein kinase C, polyol pathway, and mitochondria ROS production, as shown in Figure 2. (Araki & Nishikawa, 2010; Matough et al., 2012; Surapon, 2015).

In the glucose auto-oxidation, the high blood glucose level modulates NADH generation, leading to activate NADH dehydrogenase activity. NADH dehydrogenase is an enzyme in the electron transport chain of mitochondria that produces superoxide radicals. This free radical can convert to hydroxide radicals and hydrogen peroxide (A. Ceriello & Motz, 2004). The high blood glucose level also increases the protein glycation through the reaction between the protein and carbonyl group of reducing sugar. The increased ROS level occurs during the glycation formation. Moreover, the binding between the glycated protein and the receptor of advanced glycation end products (RAGE) modulates NADPH dehydrogenase activity and superoxide radical

production (Vincent, Russell, Low, & Feldman, 2004). Under the hyperglycemia condition, the ROS can produce through the protein kinase C pathway. This pathway involves the diacylglycerol synthesis in vascular cells by NADH oxidase activation, leading to generate superoxide radicals and damage endothelial cells (Araki & Nishikawa, 2010; Surapon, 2015). In the polyol pathway, the blood glucose induces oxidative stress by two steps: the attenuation of glutathione regeneration and the overproduction of superoxide radicals by NADH oxidase activity (Bonnefont-Rousselot, 2002). Furthermore, the mitochondrial electron transport chain elevates the superoxide radicals that can change to hydroxyl radicals and hydrogen peroxide.

As abovementioned, the high blood glucose level induces a high amount of ROS, resulting in oxidative stress. Scientific evidence has indicated that the oxidative stress damages macromolecules (i.e., protein, lipids, and DNA) and also induces insulin resistance leading to the progression of diabetic complications, including macrovascular and microvascular complications (Asmat, Abad, & Ismail, 2016; Matough et al., 2012; Sarkar et al., 2016)

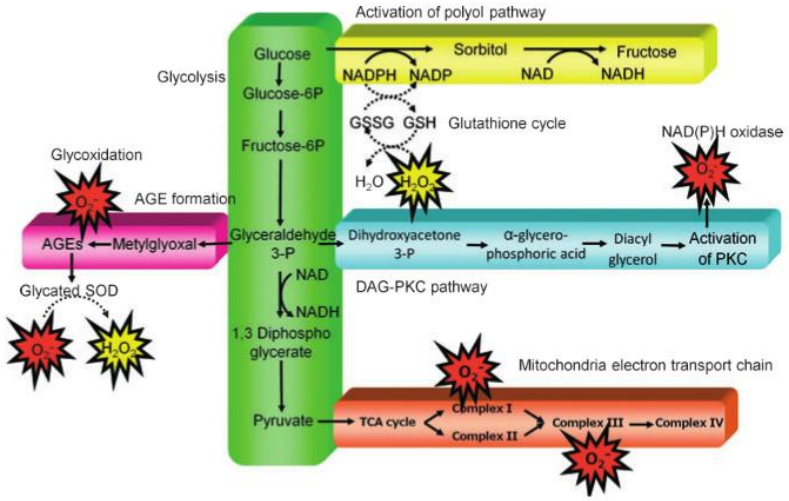


Figure 2 The proposed mechanisms of hyperglycemia-induced oxidative stress. AGE, advanced glycation end-products; DAG, diacylglycerol; GSH, glutathione;

GSSG, glutathione disulfide (oxidized glutathione); NADPH, nicotinamide adenine dinucleotide phosphate; PKC, protein kinase C; SOD, superoxide dismutase; TCA, tricarboxylic acid (Araki & Nishikawa, 2010).

2.1.5 Diabetes therapeutic strategies

Because of chronic hyperglycemia-induced diabetes and its complication, therapeutic strategies focus on lowering blood glucose levels. There are many mechanisms followed by:

1) The inhibition of α-amylase and α-glucosidase activity

The digestion of carbohydrate in the small intestine involves the hydrolysis of the polysaccharides by α-amylase (via endohydrolysis of α-1,4-glycosidic linkage) to produce oligosaccharides and disaccharides, which are degraded to monosaccharides by α-glucosidase enzyme via exohydrolysis of α-1,4-glycosidic linkage. Thus, the inhibition of these enzymes leads to decreasing postprandial hyperglycemia and subsequent glucose absorption (Oh et al., 2015). Acarbose is a diabetic therapeutic agent that has pseudo-carbohydrate property. It inhibits glucosidase activity via competitive inhibition. The drug can also increase gastric emptying time and modulate the secretion of insulin, CCK, and PYY. Therefore, acarbose is able to decrease postprandial glucose level. However, acarbose absorption into the bloodstream has adverse effects on the gastrointestinal tract, such as flatulence, abdominal distension, diarrhea, and dyspepsia. Furthermore, the limitation of acarbose therapy is found in patients with diabetic ketoacidosis, inflammation bowel disease, colonic ulceration, or intestinal obstruction (DiNicolantonio, Bhutani, & O'Keefe, 2015; K. He, Shi, & Mao, 2014; Joshi et al., 2014; Standl, Theodorakis, Erbach, Schnell, & Tuomilehto, 2014).

2) The improvement of insulin resistance

Insulin resistance is the inefficiency of insulin function, which reduces glucose uptake into target cells such as adipocytes and muscles. Besides, obesity can induce adipocyte dysfunction, decreasing adiponectin level, suppressing insulin signaling, and reducing glucose uptake. Thiazolidinediones (TZDs) is a diabetic therapeutic agent that can reduce insulin resistance, increase glucose uptake, and suppress hepatic gluconeogenesis. TZDs act as PPARγ agonists that lead to adipocyte differentiation and improve insulin sensitivity in fat cells. Moreover, TZDs increase insulin sensitivity in skeletal muscle indirectly via adipocytes function (Ross, Gulve, & Wang, 2004). However, TZDs have adverse effects, including weight gain, edema, increased bone fracture risk, and cardiovascular disease (Matsukawa, Inaguma, Han, Villareal, & Isoda, 2015; Scazzocchio et al., 2011).

3) The inhibition of AGEs formation.

The AGEs inhibition may involve in several pathways, including the inhibition of amino acid on protein, the inhibition of carbonyl group on reducing sugar, Amadori products or dicarbonyl intermediates (e.g., 3-DG, MGO), the suppression of Amadori product synthesis, the prevention of free radicals-derived glycation, the suppression of amadoriase activity, and the blockage of AGES and RAGE binding to inhibit the inflammation. An example of agents used for inhibition of AGES formation is Aminoguanidine. This drug showed the preventive effect on the glycation through several mechanisms: the reaction between guanidine residue and dicarbonyl group (i.e., methylglyoxal, glyoxal, 3-deoxyglucosone), inhibition of diamine oxidase activity, and prevention of AGEs and RAGE linkage. However, aminoguanidine's side effects include gastrointestinal disturbance, abnormality of

liver function, and fever-like symptoms (Khalifah, Chen, & Wassenberg, 2005; Paul J. Thornalley, 2003; Webster et al., 2005).

2.2 Advanced glycation end products (AGEs)

2.2.1 Definition of AGEs

Advanced glycation end products (AGEs) are non-enzymatic glycation. AGEs are derived from the reaction between aldehyde of ketone groups of reducing sugars and amino groups of proteins. As shown in Figure 3, the glycation process consists of 3 steps to complete the reaction (V. P. Singh, Bali, Singh, & Jaggi, 2014; Yeh et al., 2017). In the early phase, the carbonyl groups of reducing sugars react with amino acid residues of the protein. This reaction generates a Schiff base (an unstable and reversible structure). Then, the Schiff base rearranges to form Amadori products (stable keto-amines). This stage is reversible, and the substrate concentration and exposure time influence it. During this stage, the degradation of Schiff base and Amadori products results in the generation of free radicals and dicarbonyl compounds such as 3-deoxyglucosone, glyoxal, and methylglyoxal. (Sri Harsha, Lavelli, & Scarafoni, 2014). Later, Amadori products are dehydrated or oxidized by metal ions or oxygen that produce methylglyoxal, glyoxal, and 3-deoxyglucosone. These dicarbonyl compounds react with the arginine and lysine residues of proteins to form AGE adducts such as crossline, pentosidine, and N^{ε}-carboxymethyl-lysine (Yeh et al., 2017). Moreover, these reactions are modulated with hyperglycemia, contributing to diabetic complications (Dyer, Blackledge, Thorpe, & Baynes, 1991).

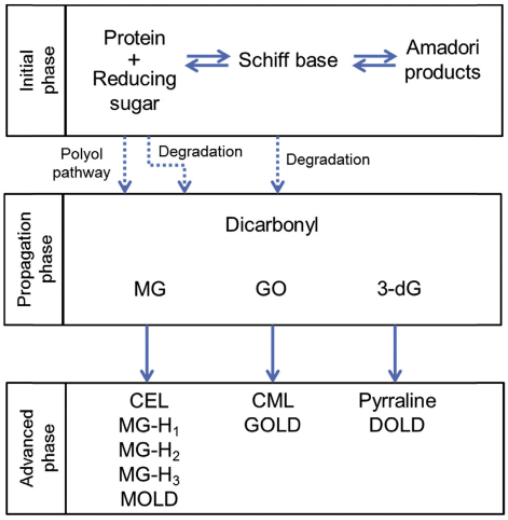


Figure 3 The formation of advanced glycation end products (AGEs).MG: methylglyoxal; GO: glyoxal; 3-dG: 3-deoxyglucosone; CEL: N^{ε}-carboxyethyl-lysine; CML: N^{ε}-carboxymethyl-lysine; MG-H: MG-derive-hydroimidazalone; MOLD: methylglyoxal-lysine dimer; GOLD: glyoxal-lysine dimer; DOLD: 3-deoxyglucosone lysine dimer (Yeh et al., 2017).

2.2.2 AGEs precursors

The AGEs formation is synthesized by the various precursors such as monosaccharides, trioses, and dicarbonyl compounds (Ramkissoon, Mahomoodally, Subratty, & Ahmed, 2016).

1) Glucose

The protein glycation by glucose is involved in glucose reaction with amino acid residues of the protein, such as lysine. Under physiological conditions, the human serum albumin (HSA) reacts with glucose to form the Schiff base (an acyclic structure) with an exposure time of about 2.5 hours. The Schiff base's rearrangement leads to generate Amadori products, including fructosamine and hemoglobin A1C (Peppa et al., 2003; P. J. Thornalley, Langborg, & Minhas, 1999). The fructosamine

level is 140 μM in healthy subjects, whereas it is increased about 2-3-fold in diabetic patients (Kato et al., 1989). Furthermore, the superoxide radicals are generated during the Schiff base and Amadori products rearrangement (Mullarkey, Edelstein, & Brownlee, 1990; Suji & Sivakami, 2004). Consequently, the condensation and fragmentation of Amadori products induce the formation of dicarbonyl compounds (P. J. Thornalley et al., 1999). Similarly, the spontaneous oxidation of glucose with oxygen and metal ion can also produce the dicarbonyl compounds, as shown in Figure 4 (P. J. Thornalley et al., 1999). These compounds react with lysine and arginine residues of proteins, resulting in the formation of AGEs such as N^{ε}-carboxymethyl-lysine, N^{ε}-carboxyethyl-lysine, and pyrraline that are presented in Fig.3(Peyroux & Sternberg, 2006; P. Thornalley, 1999).

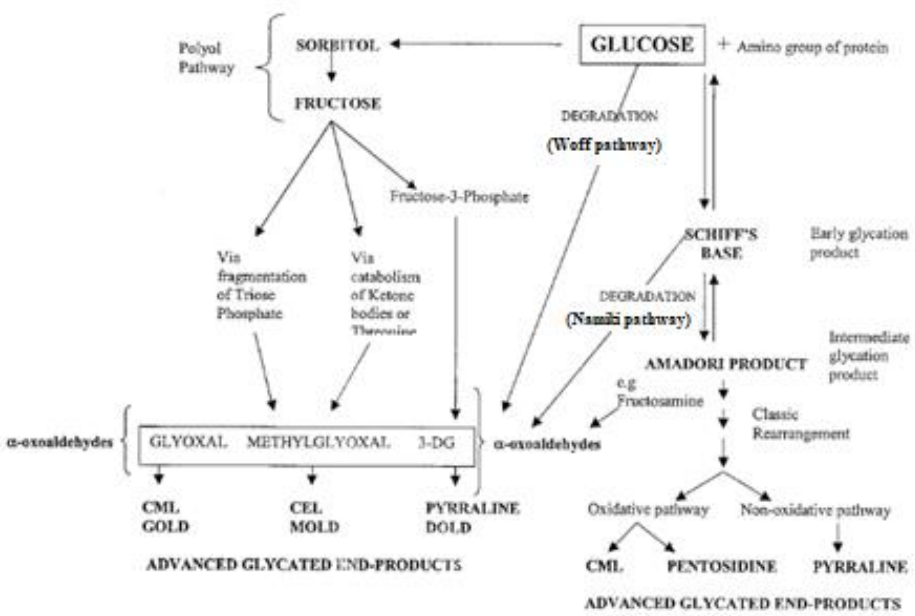


Figure 4 The formation of AGEs induced by glucose and fructose. 3-DG: 3-deoxyglucosone; CML: N^{ε}-carboxymethyl-lysine; CEL: N^{ε}-carboxyethyl-lysine; MOLD: methylglyoxal-lysine dimer; GOLD: glyoxal-lysine dimer; DOLD: 3-deoxyglucosone lysine dimer (Singh et al., 2001).

2) Fructose

Fructose is a reducing sugar containing honey, juice, table sugar, and high-fructose corn syrup. The scientific evidence demonstrates that fructose relates to the progression of diseases such as type 2 diabetes, cardiovascular and neurodegenerative diseases. (Semchyshyn, 2013). Therefore, the increased consumption of fructose may be potent to the high accumulation of fructose in the tissue and increase the glycation. The polyol pathway synthesizes the intracellular fructose. The intracellular accumulation of fructose is found in the ocular lens, peripheral nerves, and kidney (Gugliucci, 2017; W. Wang, Yagiz, Buran, Nunes, & Gu, 2011). Under the hyperglycemia condition, glucose converts to sorbitol by aldose reductase. Subsequently, the sorbitol oxidizes to fructose by sorbitol dehydrogenase, as shown in Figure 5 (W. H. Tang, Martin, & Hwa, 2012). The high sorbitol content leads to induce a cellular osmotic. In the meantime, the conversion of sorbitol to fructose increases NADH/NAD+ ratio, contributing to the overproduction of ROS and oxidative stress (Mathebula, 2015).

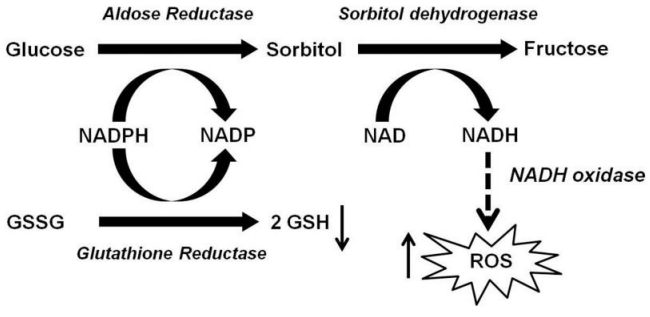


Figure 5 The polyol pathway. Glucose converts to fructose through aldose reductase and sorbitol dehydrogenase (Tang et al., 2012).

Furthermore, many studies indicate that fructose could modulate the protein glycation, and it shows a higher reactivity than glucose (Bunn & Higgins, 1981; Suárez, Rajaram, Oronsky, & Gawinowicz, 1989; Thilavech, Ngamukote, Abeywardena, & Adisakwattana, 2015). The rate of sugar-mediated protein glycation depends on its structure. As shown in Figure 6, glucose is an aldohexose which expresses the stable ring structure. Meanwhile, fructose contains a ketone group in its structure and has an open-chain form leading to higher reactivity than glucose (Bunn & Higgins, 1981; C. G. Schalkwijk, Stehouwer, & van Hinsbergh, 2004; Takagi et al., 1995).

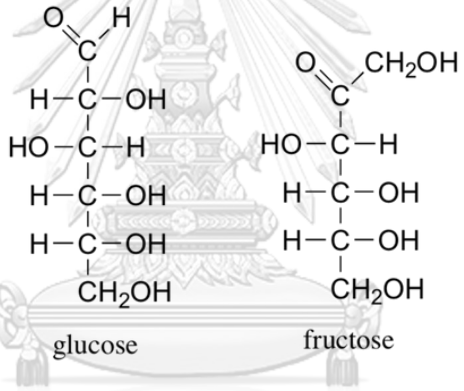


Figure 6 The open chain structures of glucose and fructose (Bunn & Higgins, 1981).

The fructation is the reaction between the carbonyl group of fructose and the amino group of protein, then undergoes to generate the Schiff base form. The Schiff base rearranges to Heyns products that are a stable structure. As shown in Figure 7, Heyns compound can oxidize and fragment to generate dicarbonyl compounds and reactive oxygen species (e.g., superoxide, hydrogen peroxide, and hydroxyl radicals). Moreover, fructose can be spontaneously oxidation resulting in the increased dicarbonyl compounds (Figure 7). In the last phase of glycation, the dicarbonyl compounds and Heyns adducts react with amino acids, sulfhydryl and guanidine of

protein, nucleic acids, and aminophospholipid, leading to the formation of (Semchyshyn, 2013).

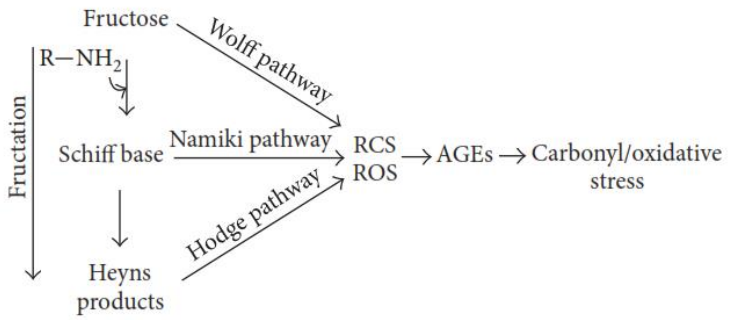


Figure 7 The formation of fructose-mediated AGEs increasing the carbonyl or oxidative stress (Semchyshyn, 2013).

3) Methylglyoxal

Methylglyoxal is one of the dicarbonyl compounds produced during the glycation and the oxidation of lipid and glucose (Li, Zheng, Sang, & Lv, 2014). Methylglyoxal has higher reactivity than glucose, approximately 20000-fold (Schalkwijk, 2015). It can react with lysine, arginine, and cysteine residues of the protein, increasing the AGEs formation such as carboxymethyl-lysine (CEL), methylglyoxal lysine dimer (MOLD), and hydroimidazolones (Peyroux & Sternberg, 2006). The interaction between amino acid residues and methylglyoxal modulates the generation of reactive oxygen species. Moreover, methylglyoxal can modify the protein and damage DNA, leading to an increased risk of cardiovascular disease, diabetes, and cancer (Thilavech, Ngamukote, Belobrajdic, Abeywardena, & Adisakwattana, 2016). Methylglyoxal level is decreased through the glyoxalase system that is presented in Figure 8. The glyoxalase-I converts methylglyoxal to S-D-lactoylglutathione, which further catalyzes by glyoxalase-II to produce D-lactate.

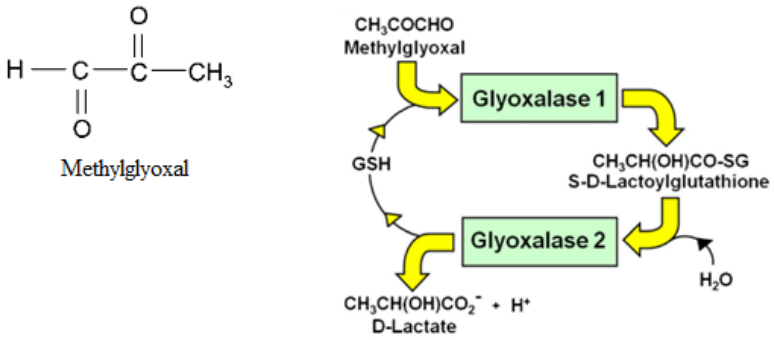


Figure 8 The conversion of methylglyoxal by the glyoxalase system (Masania et al., 2016).

2.2.3 AGEs and protein oxidation

Long-term hyperglycemia is a risk of diabetic complications through the modulation of AGEs formation leading to oxidative stress. During the protein glycation, free radicals are generated from all stages of glycation. Firstly, glucose oxidation in the presence of metal ions produces ketoaldehyde and hydrogen peroxide. The ketoaldehyde reacts with amino acid residues to generate Amadori products then undergo to form the protein carbonyl, which is a marker for cell oxidative damage (Arfin, Siddiqui, Naeem, & Moin, 2018; Wolff & Dean, 1987). Secondly, the oxidation of Amadori product with metal ions or oxygen leads to produce AGEs and superoxide radicals (Figure 9) (Mossine, Linetsky, Glinsky, Ortwerth, & Feather, 1999). Finally, the late-stage of glycation releases free radicals, including the hydroxyl radical, superoxide radical, and hydrogen peroxide (Ho, Wu, Lin, & Tang, 2010). Moreover, the methylglyoxal could induce superoxide dismutase glycation, leading to a reduced antioxidant system (Jung Hoon, 2003). In general, ROS or carbonyl interactions react with amino acids (lysine, arginine, and threonine)

or the sulfhydryl groups of cysteine residues, increasing carbonylated protein and the decreased thiol group content, respectively (Arfin et al., 2018).

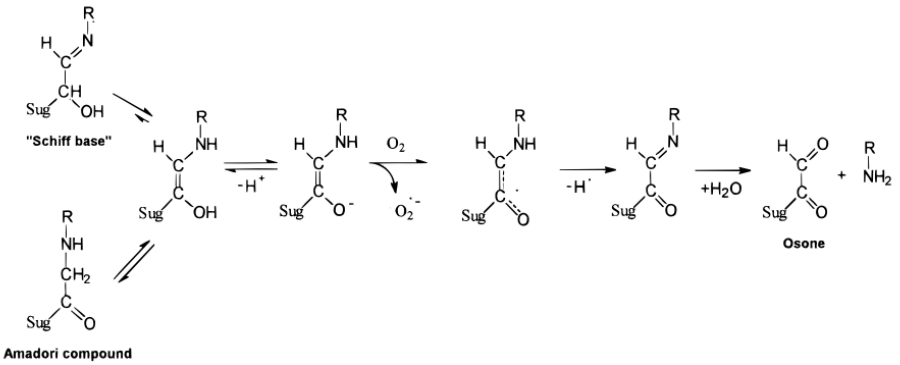


Figure 9 The proposed pathway of free radicals generated during the early stage of glycation (Mossine et al., 1999).

Furthermore, the binding of AGEs and its receptors (RAGEs) initiates the transcription factor NF-κB expression through the mitogen-activated protein kinase (MAPK) pathway, resulting in the modulation of pro-inflammatory cytokines including interleukin-1α (IL- 1α), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) (V. P. Singh et al., 2014). Also, the activation of TNF- α increases the ROS production leading to cell and DNA damage that effects on the development of diabetic complications, for example, cardiovascular diseases and neurodegenerative (Singh et al., 2014).

2.2.4 AGEs and protein aggregation

The glycation can modify the protein structure resulting in the increased protein aggregation. The glycation induces the refolding of globular proteins into amorphous or fibrillation that contains cross-β structure such as Aβ(1-42) and islet amyloid polypeptide (IAPP) (Bouma et al., 2003). The unfold of glycated protein is

involved in two pathways. Firstly, the interaction between sugar and amino acid residues induces the unfold of polypeptides. Secondly, the intramolecular or intermolecular of crosslink-AGEs modulates the unfold of globular protein structure. Consequently, the new contact between amino acids generates polypeptide comprising a cross-β structure that forms the amyloid fibril (Bouma et al., 2003). The accumulation of amyloid structure relates to degenerative diseases, including Alzheimer's disease, Parkinson's disease, and atherosclerosis (Adisakwattana, Thilavech, Sompong, & Pasukamonset, 2017). Moreover, the islet amyloid peptides lead to the destruction of β-cells and insulin secretion impairment (Bouma et al., 2003).

2.2.5 AGEs and diabetic complications

1) Diabetic retinopathy

Retinopathy has been found in diabetic patients, approximately 90%. The characteristic of retinopathy is the abnormal blood vessel, including the high blood vessel proliferation, vascular occlusion, angiogenesis, hemorrhage, and infarction (R. Singh et al., 2001). Moreover, the AGEs level elevation activates the fibroblast growth factor leading to Muller glial dysfunction (Ai, Liu, & Sun, 2013). The cross-link AGEs reduce pericyte adherence through the conversion of ECM proteins and expand vascular stiffness. The interaction between AGEs and RAGEs increases oxidative stress and modulates cytokines' expression, local growth factors, and adhesion molecules, leading to the pericytes damage. Besides, the AGEs activate the expression of vascular endothelial cell growth factor (VEGF) and increase the IL-6 secretion leading to angiogenesis (Singh et al., 2014).

2) Diabetic cataract

The glycated protein in the eye lens can cause blindness. The thiol groups of lens crystallins were oxidized to form the AGEs-crosslinking that undergoes protein aggregation (Ansari, Awasthi, & Srivastava, 1980). AGEs change the protein conformation between protein-protein and protein-water binding, resulting in light scattering and vision disruption (Beswick & Harding, 1987). Moreover, the binding of AGE and RAGE in the epithelium of lens induces free radicals, including superoxide radicals and hydrogen peroxide, that damage the antioxidant system and increase the oxidative stress (Ookawara, Kawamura, Kitagawa, & Taniguchi, 1992).

3) Diabetic nephropathy

Diabetic nephropathy involves decreasing glomerular filtration rate, increasing the basement membrane and mesangium associated with proteinuria in the kidney (Ahmed, 2005; Singh et al., 2014). Long-term hyperglycemia induces collagen glycation, leading to the conformation structure through the inter and intramolecular crosslinking, increasing the blood vessel stiffness (Mott et al., 1997). The AGEs accumulation on collagen increases the basement membrane's thickness and reduces the glomerular filtration (Ahmed, 2005). Moreover, the binding of AGEs and RAGE modulates the JAK/STAT expression increasing the profibrotic cytokines, platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF). The high level of CTGF induces ECM synthesis leading to angiogenesis (Twigg et al., 2001). The MCP-1 expression in mesangial cells is activated by AGEs contributing to the macrophage migration and inflammation in renal tissue (Nowotny, Jung, Höhn, Weber, & Grune, 2015).

4) Diabetic neuropathy

The characteristic of diabetes neuropathy includes the demyelination, peripheral neuron degeneration, and deceased nerve fiber (Ahmed, 2005). Chronic hyperglycemia induces the glycolytic and polyol pathway, which increases the glycated protein in the peripheral nerve. The glycation on myelin regulates macrophage protease secretion, leading to neuronal demyelination (Ahmed, 2005). The CML, a glycated protein, locates in vascular endothelial cells, pericytes, basement membrane, and on axons Schwann cells that may lead to cell death (V. P. Singh et al., 2014). The interaction of AGEs with neurofilament and tubulin could decrease the axonal transport resulting in the reduced nerve fiber synthesis and the progression of atrophy (Williams, Howarth, Devenny, & Bitensky, 1982). Besides, the glycation initiates the protein aggregation and amyloid fibrillation increasing the level of neurofibrillary tangles (NFTs) as a primary marker of Alzheimer's disease. The NFTs impair synapses and damage neuron cells contributing to the loss of cognitive and functional ability in patients (Gandy, 2005). Parkinson's disease has resulted from neuron cell damage in the Substantia Nigra (SN) and the accumulation of Lewy bodies and neuromelanin. α-synuclein is a neurofilament in Lewy bodies that could form the glycation and globular-like aggregation, causing oxidative stress (Chen, Wei, Wang, & He, 2010).

5) Diabetic cardiopathy

Diabetic cardiopathy is involved in myocellular hypertrophy and myocardial fibrosis that is a risk factor for heart failure. The AGEs could increase heart failure progression by binding AGEs and RAGE increased oxidative stress and inflammation, resulting in vascular and myocardial tissue damage(Bodiga, Eda, &

Bodiga, 2014). Besides, AGEs inhibit the fibrinolysis and increase the gene expression of MCP-1 and adhesion molecules, contributing to the dysfunction of endothelial cells (Nowotny et al., 2015)

2.2.6 Antiglycation mechanisms and inhibitors

1) Antiglycation mechanism

The accumulation of AGEs in tissue increases the protein damage and loss of function related to diabetic complications. The reduction or prevention of AGEs formation could decrease the development of diabetes and its complications. The proposed mechanisms to delay glycation formation are presented in Figure 10 (Ahmed, 2005; Wu et al., 2011).

- Preventing interaction between free amino groups of proteins and free sugars.
- The glycation process and AGEs formation could inhibit by blocking the carbonyl of reducing sugars, Schiff base, Amadori products, and dicarbonyl compounds (e.g., 3-deoxyglucosone, methylglyoxal)
- Blocking Amadori products with antibodies
- The metal ions regulate the autooxidation glycation and AGEs formation. Thus, the chelating of metal ions could decrease the glycation-derived free radicals
- Free radical scavenging activity. During the initial stage of glycation, both Schiff base and Amadori products oxidation generate reactive dicarbonyl compounds and free radicals, including hydroxyl radicals and superoxide radicals, contributing to the increased oxidative stress. Moreover, the glycated protein could induce free radicals generation. Therefore, the decreasing of reactive dicarbonyls and free radicals could reduce oxidative stress.

- The autooxidation of glucose or fructose in the presence of metal ions could generate the AGEs. Therefore, the inhibition of glycation formation is prevented by antioxidant activity
- Preventing the formation of late-stage Amadori products
- The Amadoriases enzyme could reduce the level of Amadori product or dicarbonyl compounds such as 3-deoxyglucosone.
- Breaking the formation of glycated-crosslink structure could prevent the progression of diabetic complications.
- The interaction of AGEs and RAGEs increases oxidative stress and inflammation. Thus, the blocking of RAGEs could prevent inflammation changing and diabetic complications.

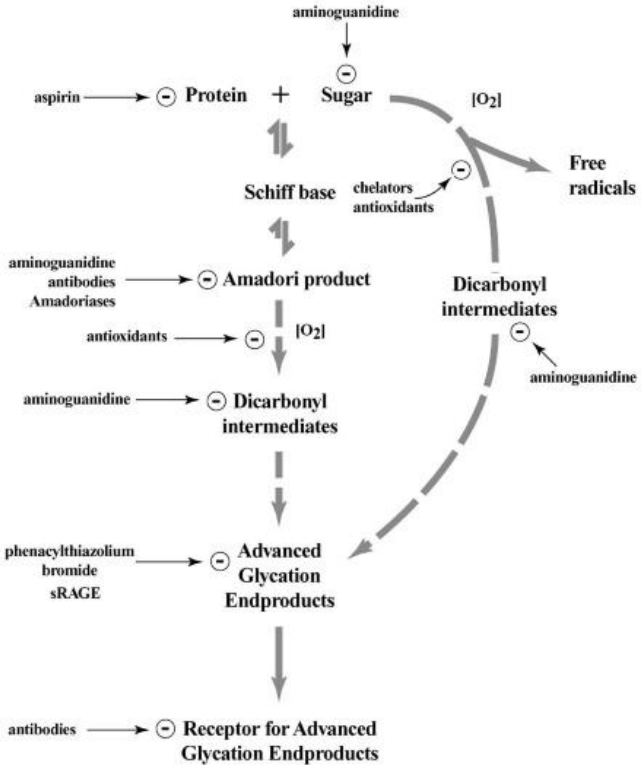


Figure 10 The proposed mechanism of antiglycation and AGE-induced protein damage (Ahmed, 2005).

2) Antiglycation inhibitors

❖ **Aminoguanidine**

Aminoguanidine is a nucleophilic hydrazine compound (Figure 11). The inhibitory effect of aminoguanidine on glycation formation is involved in the blocking carbonyl groups of reducing sugar and Amadori products (Edelstein & Brownlee, 1992; Lewis & Harding, 1990). In addition, aminoguanidine plays a role in the trapping dicarbonyl compounds such as methylglyoxal and the scavenging free radicals (Ou & Wolff, 1993). According to in vivo study, aminoguanidine reduced the AGEs levels in glomeruli and renal tubules. Also, it decreased the thickness of the glomerular basement in diabetic rats after 32 weeks of aminoguanidine administration. The proposed mechanism of AGEs inhibition may involve inhibiting collagen-linked fluorescence and cross-linking formation (Soulis, Cooper, Vranes, Bucala, & Jerums, 1996). In diabetic patients, the daily intake of aminoguanidine (50 mM) for 28 days decreased the formation of hemoglobin-derive AGEs (Makita et al., 1992). In phase III clinical trial, the randomized, double-blind ACTION I trial revealed that the aminoguanidine (300-mg twice-daily) could delay the development of diabetic nephropathy in Type I diabetic patients. However, this study found some patients who received high doses of aminoguanidine expressed autoimmune disease (Bolton et al., 2004). The investigation of aminoguanidine in the ACTION II trial was terminated because it showed the adverse effects in diabetic patients, including flu-like symptoms, gastrointestinal disturbances, anemia, glomerulonephritis, and myocardial infarction (Friedman, 2010).

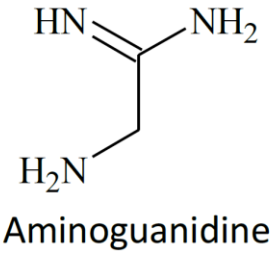


Figure 11 The chemical structure of aminoguanidine (Guilbaud et al., 2016).

❖ **Pyridoxamine**

Pyridoxamine or ((4-aminomethyl)-5-(hydroxymethyl)-2-methylpyridin-3-ol)) could prevent the generation of hydroxyl radicals from the oxidation of Amadori products. The proposed inhibition included the hydroxyl radical scavenging activity and the preventing metal-catalyzed reducing sugars or dicarbonyls degradation (Abbas et al., 2016). The chemical structure of pyridoxamine is presented in Figure 12.

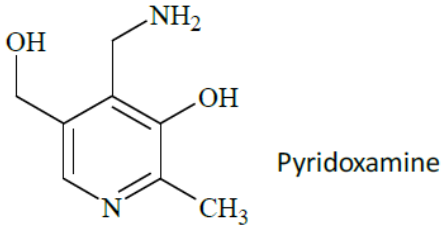
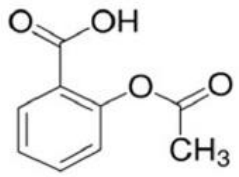


Figure 12 The chemical structure of pyridoxamine (Guilbaud et al., 2016).

❖ **Aspirin**

Aspirin is an acetylsalicylic acid (Figure 13) that uses as an analgesic. The scientific evidence suggested that aspirin inhibited the glycation process through the acetylation of amino acid residues and inhibited glycoxidation and AGE crosslinking formation (Baynes, 1991; Robert & Harding, 1992). Moreover, aspirin plays a role in scavenging free radicals, chelating metal ions, and preventing Amadori products

oxidation (Abbas et al., 2016). On the other hand, aspirin exhibited an adverse effect on the gastrointestinal tract (Ahmed, 2005).



acetyl salicylic acid (ASA)

Figure 13 The chemical structure of acetyl salicylic acid or aspirin (Ghazanfari-Sarabi et al., 2019).

❖ **Vitamin E**

In general, oxidative is one factor to increase diabetic complications such as cardiovascular diseases (Yang, Feng, Peng, Liu, & Fan, 2019). The daily intake of vitamin E (1200 mg) in diabetic patients for 2 months could reduce the glycated HbA1 and proteins (Antonio Ceriello et al., 1991). Furthermore, vitamin E (50IU) slightly inhibited the AGEs formation in diabetic rats after 4 weeks of supplementation (Aoki et al., 1992). The proposed antiglycation mechanism by vitamin E might relate to its antioxidant activity that inhibited the auto-oxidation of reducing sugars in the presence of metal ions (Aoki et al., 1992; Antonio Ceriello et al., 1991). The chemical structure of vitamin E is presented in Figure 14.

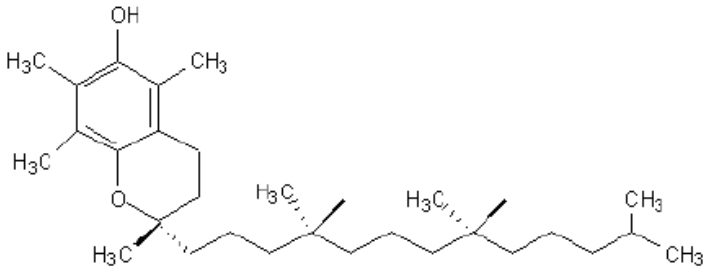


Figure 14 The chemical structure of vitamin E (Shanmugam et al., 2010).

❖ **Vitamin C**

According to an animal study, vitamin C feeding (1000 mg/day) decreased the serum glycated proteins after 4 weeks (Wu et al., 2011). The chemical structure of vitamin C is presented in Figure 15.

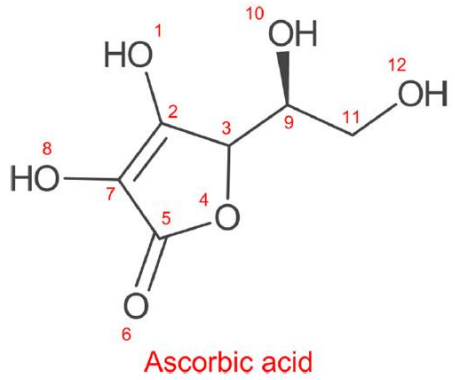


Figure 15 The chemical structure of vitamin C (Toccaceli et al, 2017).

2.3 Obesity

Obesity has become a global health issue. The World Health Organization reported that more than 650 million adults were obese, and 1.9 billion were overweight in 2016 (World Health Organization, 2016). The cause of obesity is an imbalance between energy intake and energy expenditure (Rosen & Spiegelman, 2006). Obesity is one risk factor for non-communicable diseases such as type 2

diabetes, hypertension, cardiovascular diseases, and cancer (Kopelman, 2000). According to prospective studies, the increasing weight gain or body mass index (BMI) is positively related to the risk for diabetes (Chan, Rimm, Colditz, Stampfer, & Willett, 1994; Colditz, Willett, Rotnitzky, & Manson, 1995). Besides, the characteristic of obesity is the high fat accumulation in adipose tissue through the adipogenesis. The adipogenesis involves in the proliferation of fibroblast preadipocyte and the differentiation of mature adipocytes contributing to the increasing of adipocyte cell number (hyperplasia) and adipocyte cell size (hypertrophy) (Aranaz et al., 2019).

2.3.1 Adipocyte differentiation process

Adipogenesis consists of growth arrest, clonal expansion (MCE), and terminal differentiation, resulting in cell morphology and the accumulation of lipid droplets as shown in Figure 16 (Niemelä, Miettinen, Sarkanen, & Ashammakhi, 2008). After post-confluence, the proliferation of fibroblast-like preadipocyte cells is terminated through the cell contact inhibition, known as the growth arrest phase. Following growth-arrested cells, the preadipocyte cells undergo mitotic clonal expansion by the adipogenic inducing agents (E. D. Rosen & Spiegelman, 2000). This phase induces the conversion of fibroblast-like preadipocyte cells to a spherical shape of adipocytes. After the clonal expansion phase, adipocyte cells further accumulate the lipid droplets inside cells, which is influenced by the expression of transcriptional factors and lipogenic genes (Niemelä et al., 2008).

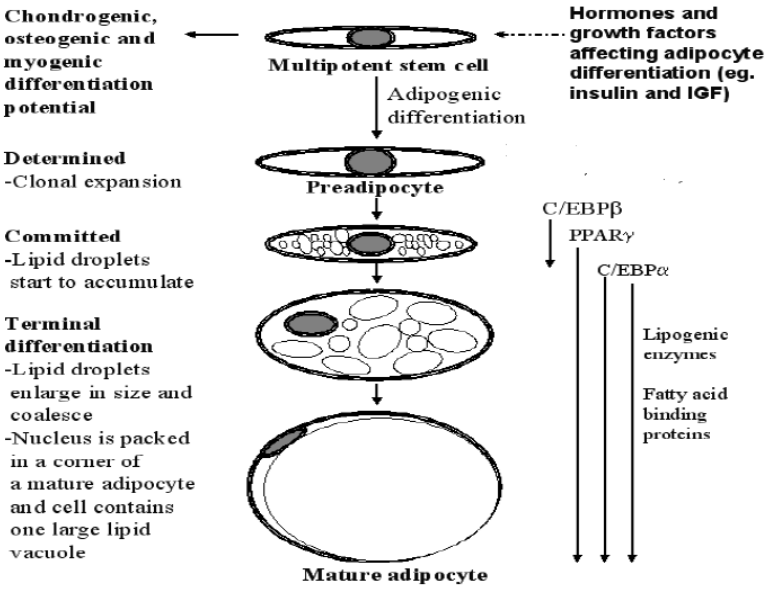


Figure 16 The adipocyte differentiation process . Multipotent stem cells could differentiate into various cell types, including adipocyte cells. In the presence of a differentiation cocktail, preadipocytes enter clonal expansion and undergo terminal differentiation leading to lipid accumulation in adipocyte cells. C/EBP: CCAAT/enhanced binding protein; IGF: insulin-like growth factor; PPARγ: peroxisome proliferator-activated receptor-γ (Niemelä et al., 2008).

2.3.2 Adipocyte differentiation signaling pathway

The adipocyte differentiation requires preadipocytes growth arrest by cell contact inhibition after cell confluence. In the early phase of adipogenesis, the growth-arrested preadipocytes re-enter to S phase of the cell cycle in adipocyte differentiation inducers leading to the clonal expansion. This process is involved in the modulation of C/EBPβ and C/EBPδ expression within 4 hours. Their expressions remain for 48 hours at the early phase of adipogenesis (Tang & Lane, 1999). The expression of C/EBPβ is activated by the cAMP/glucocorticoids-induced PKA/CREB

signaling pathway, as shown in Figure 17 (Wang & Hai, 2015). During the mid-phase adipocyte differentiation, both expressions of C/EBPβ and C/EBPδ trigger the expression of PPARγ and C/EBPα, which is a major of adipogenic transcription regulators. PPARγ and C/EBPα show the synergistic effect on the regulation of adipocyte-specific genes expression including aP2/FABP4 is an adipocyte-specific fatty acid bind protein), FAT/CD36 is a putative fatty acid transporter (Gregoire, Smas, & Sul, 1998; Rosen, Walkey, Puigserver, & Spiegelman, 2000). In the meantime, the C/EBPα exhibits the antimitotic effect on both C/EBPβ and C/EBPδ expression (Wang & Hai, 2015). During the late-phase adipocyte differentiation, the induction of lipogenesis occurs in adipocyte cells. The stimulation of lipogenic genes, including acetyl-CoA-carboxylase (ACC), stearoyl-CoA desaturase (SCD-1), and fatty acid synthase (FASN), are increased by 10- to 100-fold (Gregoire et al., 1998).

2.3.3 Adipocyte differentiation inducers

Based on in vitro studies, the induction of adipocyte differentiation is regulated by a DMI cocktail, including glucocorticoids, 3-isobutyl-1-methylxanthine (IBMX), and insulin to mediate adipocyte differentiation (Figure 17).

1) Glucocorticoids

Dexamethasone is a glucocorticoid that interacts with glucocorticoid receptor and induces CCAAT/enhanced binding protein-δ (C/EBPδ) during the early stage of adipocyte differentiation (Rosen & Spiegelman, 2000).

2) IBMX

3-isobutyl-1-methylxanthine (IBMX) is a phosphodiesterase inhibitor. The chemical structure of IBMX is presented in Figure 18. IBMX could activate the cAMP-dependent protein kinase pathway leading to the increased cAMP level.

Moreover, this compound stimulates PKA and the expression of C/EBPβ, which is a transcription factor for adipocyte differentiation (Wang & Hai, 2015).

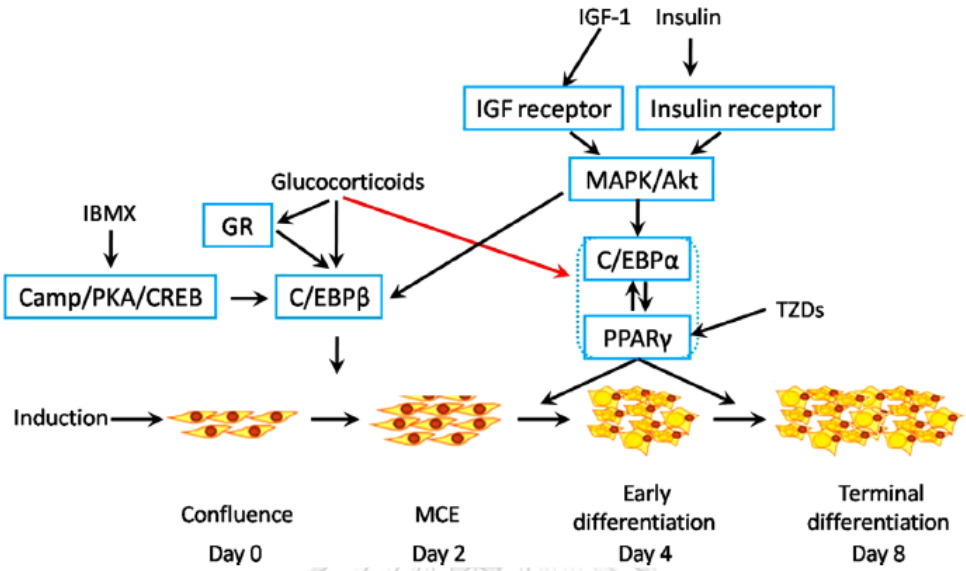


Figure 17 The MDI cocktail induces the adipogenesis process. The black arrow indicates activation. The red pointer indicates inhibition (Wang & Hai, 2015).

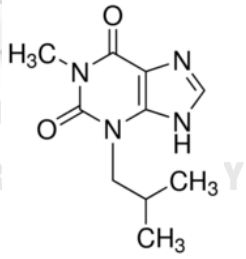


Figure 18 The chemical structure of 3-isobutyl-1-methylxanthine (IBMX).

3) Insulin

Insulin plays a role in the proadipogenic agonist. Insulin mediates the adipocyte differentiation through the stimulation of IGF-1 (insulin-growth like factor-1) receptor, resulting in the downstream of signaling pathways. Insulin and IGF-1 activate Ras, Raf, phosphoinositide-3-kinase (PI3K)/Akt, ERK1/2, andp38 MAPK,

leading to the expression of C/EBPα and PPARγ during the adipogenesis process (Rosen & Spiegelman, 2000; Wang & Hai, 2015).

2.3.4 Transcriptional controlling adipocyte differentiation

1) PPARγ

PPARγ is a nuclear hormone receptor that is mostly found in adipocyte cells. According to scientific evidence, the PPARγ strongly regulates adipogenesis and could mediate the cell growth arrest that contributes to fully adipocyte differentiation. The PPARγ is required to control adipocyte formation and maintain mature adipocytes (Evan D Rosen & MacDougald, 2006). In addition, the PPARγ is activated by thiazolidinediones, an antidiabetic agent, leading to adipose conversion (Gregoire et al., 1998).

2) C/EBPs

The C/EBPβ and C/EBPδ levels increased in the initial phase of differentiation but decreased in the late differentiation. The expression of C/EBPβ and C/EBPδ is induced by adipogenic stimuli, including dexamethasone, IBMX, and insulin (Gregoire et al., 1998). Regarding, the IBMX could increase the cAMP level, which further activates the expression of C/EBPβ. Meanwhile, the dexamethasone (glucocorticoid) associates with C/EBPβ stimulate the expression of C/EBPδ (Farmer, 2006). Besides, a high level of C/EBPα is found in the terminal differentiation. The C/EBPα is required to maintain the PPARγ expression in the mature adipocyte cells (Farmer, 2006).

2.4 Anthocyanins

Anthocyanins are water-soluble and natural coloring substances, commonly found in many plants, for example, some cereals, vegetables (cabbage, onions, radishes, etc.), fruits (cherries, berries, grapes, black currants, etc.). Anthocyanins structure consists of two aromatic rings and a heterocyclic ring called anthocyanidins or aglycone (Figure 19). The types of anthocyanins depend on the number of hydroxyl groups, nature, and the number of sugar-binding with their structures, the aliphatic or aromatic carboxylates in their structures. The most types of anthocyanins are cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). Moreover, the glycoside forms of cyanidin, delphinidin, and pelargonidin are found in leaves, fruits, and flowers, respectively (Castaneda-Ovando, de Lourdes Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). The stability of anthocyanins factors involved many factors, including pH, temperature, solvent extraction, co-pigmentation, light, and oxygen. Furthermore, the pH difference can divide anthocyanin into 4 major species: flavylium, pseudobase, chalcone, and quinonoidal base (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014).

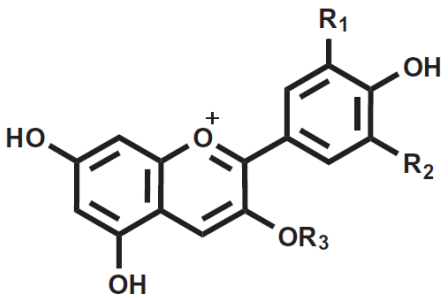


Figure 19 The general structure of anthocyanins (Fernandes et al., 2014)
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Several factors may affect anthocyanins bioavailability, including pH, food matrix, digestive enzymes, bile acids, microorganisms, the moving across the GI tract, anthocyanin, and their derivatives. However, only glycoside form can absorb into the bloodstream, hydrolyzed to aglycone form or phenolic acid, and excreted in urine (Fernandes et al., 2014).

2.4.1 Sources of anthocyanins

Anthocyanins are natural colorants found in many plants, including fruits (such as berries, cherry, grape), vegetables (such as onion, black bean, eggplant, purple corn, red cabbage). Anthocyanin-riched fruits are separated into three major groups that followed by their structures: pelargonidin group (i.e., strawberries), cyanidin or peonidin group (i.e., blackberries, cherries, mulberries, and raspberries), multiple anthocyanins group (i.e., acerola, blueberries, jambul, maqui, bilberries). In addition, both cyanidin/peonidin and multiple anthocyanins groups have more fruits than the pelargonidin group (Fang, 2015).

2.4.2 The effect of anthocyanins on diabetes

It has been reported that anthocyanins affect biological activities such as antioxidant activities involving radical scavenging activity and preventing lipid peroxidation, anti-inflammatory, anticancer, and anti-obesity (i.e., decreasing of fatty acid and triglyceride synthesis), and anti-diabetic activity (He & Giusti, 2010). Moreover, many investigations suggested that anthocyanin can lower blood sugar levels via several possible pathways, including:

1) The activation of insulin secretion

Because insulin plays a role in glucose homeostasis. The glucose homeostasis can be regulated by stimulating glucose uptake into muscle cells and adipocyte cells and suppressing gluconeogenesis in hepatic cells. Therefore, the insufficiency of insulin levels may induce hyperglycemia and progress to diabetes. The anthocyanin-riched chokeberry study was found that anthocyanin can stimulate insulin secretion, which is proportionated to anthocyanin contents (Rugină et al., 2015). There has been suggested that the possible mechanisms may involve the regulation of insulin gene expression and activation of voltage-gated calcium channels (Rugină et al., 2015).

2) The inhibition of intestinal α-glucosidase activity

The intestinal α-glucosidase locates on epithelial cells of the small intestinal, and its function is carbohydrate digestion. Therefore, the suppression of enzyme activity may decrease postprandial blood glucose. According to previous studies, anthocyanins could inhibit intestinal α-glucosidase. Muscadine's whole fruit extract inhibited intestinal α-glucosidase with IC_{50} value 1.50 mg/ml by competition inhibition (K_i= 0.56 mg/ml) of anthocyanidin. Therefore, anthocyanin's hydrolysis in the small intestine may influence enzyme inhibition (You, Chen, Wang, Luo, & Jiang, 2011). The anthocyanin-rich black mung bean exhibited the competitive inhibition of α-glucosidase activity (Yao et al., 2013). Similarly, blueberry peel extracts shown the capacity of α-glucosidase inhibition. These results indicated that the structure of anthocyanin and protein binding capacity between anthocyanin and α-glucosidase enzyme might involve the inhibitory process (Wang, Camp, & Ehlenfeldt, 2012).

3) Antioxidant activity

Because of the relation between oxidative stress correlated with hyperglycemia and hyperlipidemia. Many studies suggested that anthocyanins can alter free radical generation. For example, anthocyanin fraction (436.65-810 µg/g) of black mung bean was related to antioxidant activities (DPPH, ABTS and ORAC assays) and anti-glycation activities (via BSA-glucose and methylglyoxal induction) that caused by the inhibition of ROS generation, which may reduce AGEs formation and prevent cellular protein modification. Moreover, anthocyanins' free radical scavenging efficiency depended on the hydroxyl group's number and position and anthocyanins glycosylation (Yao et al., 2013).

4) Antiglycation activity

According to previous studies, wild berry extracts inhibited glycated bovine serum albumin with IC_{50} value higher than 50 µg/ml and decreased the CML-BSA production with dose-dependently (Cory S Harris et al., 2014). Moreover, red grape skin extract suppressed the AGEs formation, reduced fructosamine generation, and prevented protein oxidation, reducing carbonyl contents and thiol group levels (Jariyapamornkoon, Yibchok-anun, & Adisakwattana, 2013).

2.4.3 The effect of anthocyanins on obesity

Obesity is one of the risk factors that can promote the progression of diabetes. Many investigations suggested that anthocyanins can ameliorate obesity. Anthocyanins-enriched black soybean extract decreased adipose tissue mass and lipid accumulation in 3T3-L1 preadipocyte cells through the downregulation of glyceraldehyde-3-phosphate dehydrogenase activity and PPARγ expression. Conversely, this extract can increase glycerol releasing via lipolysis modulation (Kim

et al., 2012). Grape-containing anthocyanins exhibited the reduction of lipid accumulation in 3T3-L1 preadipocyte cells in a dose and time-dependent manner. Moreover, this extract suppressed the expression of C/EBPα, PPARγ, LXRα, and SREBP-1c during adipocyte differentiation and the reduction of lipogenic enzyme activities, including FAS, SCD-1 (B. Lee, M. Lee, M. Lefevre, & H.-R. Kim, 2014). Blueberry peel extract showed the inhibitory effect of adipogenesis in adipocyte cells by suppressing C/EBPβ, C/EBPα, PPARγ expressions, and the reduction of phosphorylation of Akt. Moreover, this extract decreased lipid accumulation via the downregulation of lipogenic genes, including fatty acid-binding protein, fatty acid synthase, and lipoprotein lipase (Song et al., 2013). Besides, the inhibitory effect of cranberry extract on adipogenesis found that the extract lowers the 3T3-L1 preadipocytes proliferation and viability, attenuated the lipid accumulation adipocytes through the inhibiting transcriptional factors expression including C/EBPα, PPARγ, and SREBP-1c (Kowalska, Olejnik, Rychlik, & Grajek, 2014). The effect of delphinidin on adipogenesis revealed that delphinidin reduced transcriptional factors expression, including C/EBPβ and C/EBPδ in the early phase of adipocyte differentiation. Moreover, it could suppress C/EBPα and PPARγ expressions in the middle stage of adipogenesis (Rahman, Jeon, & Kim, 2016).

2.4.4 Toxicity of anthocyanin

According to the European food safety authority document, the glycoside form of anthocyanins (an extract from currants, blueberries, and elderberries) induces acute oral toxicity (expressed as LD_{50} value) at level 25000 and 20000 mg/kg BW in mice and rats, respectively. Moreover, most genotoxicity (*in vitro* assays) anthocyanins have no adverse effect at low doses. Simultaneously, pure anthocyanidins include

delphinidin, cyanidin, malvidin, pelargonidin, and peonidin that were slightly genotoxic concentrations ≥ 50μM. The JECFA committee (Joint Expert Committee for Food Additives) suggested an ADI of 2.5 mg/kg BW/day for anthocyanins from grape skin extracts (Aguilar et al., 2010).

2.5 Thai berries

2.5.1 Mamao

Antidesma bunius (L) spreng (Mamao, Mao, Mak Mao, or Mao-Luang) belongs to Euphorbiaceae's family. In Thailand, most of the cultivation of Mamao locates in the northeast region. *Antidesma bunius* fruits are acidic in taste, but it will be changed to sweet in the ripen state. The fruit of this plant can be produced in juice, wine, jam (Kukongviriyapan et al., 2015). According to a previous study, *Antidesma bunius* fruit extract contained phytochemical compounds such as quercetin-3-rutinoside, catechin, epicatechin, gallic acid, vanillic acid, chlorogenic acid, caffeic acid, cyanidin-3-glucoside, and cyanidin-3-rutinoside (Jorjong et al., 2015). In *in vitro* study, The fruit extract of *Antidesma bunius* exhibited the antioxidant capacities including through DPPH, FRAP, and TEAC assay with values of 103.04 mmol VCEAC/g DW, 35.35 mmol Fe(II)/g DW, and 46.37 mmol TE/ g DW, respectively (Jorjong et al., 2015). In the animal study, the oral administration of *Antidesma bunius* fruit extract in high fat diet-induced rats decreased the malondialdehyde level and increased antioxidant capacity. Besides, the fruit extract could attenuate the pro-inflammatory genes expression, which resulted in the prevention of cardiac tissue deterioration (Udomkasemsab et al., 2018). Moreover, *Antidesma bunius* fruit extract could enhance the fat metabolism in liver tissue of high fat diet-induced rats by

increasing antioxidant activity and anti-inflammatory and suppressing lipogenic gene expression in the liver (Ngamlerst et al., 2019). The effect of *Antidesma bunius* fruit extract in hypercholesterolemia rats found that the extract reduced blood glucose level, decreased LDL-cholesterol and triglyceride levels, improved antioxidant activity leading to prevent the progression of cardiovascular disease (Udomkasemsab et al., 2019).

Figure 20 Characteristic of *Antidesma bunius* (Mamao).

2.5.2 Mahuat

Lepisanthes rubiginosa (Roxb.) Leenh, Mahuat, is belonging to the Sapindaceae family. This plant is grown in northeastern Thailand. The mature ripening stage was a purple color. According to a previous study, the essential oil from fruits of *Lepisanthes rubiginosa* (Roxb.) Leenh inhibited the growth of microorganisms, including *Trichophyton mentagophyte*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* (Chuangbunyat et al., 2011). Furthermore, the ethanolic leave extract of *Lepisanthes rubiginosa* exhibited antioxidant activity through DPPH assay, inhibited the writhing reflex, reduced blood glucose level, and decreased locomotor activity in mice (Hasan, Hossain, Shamim, & Rahman, 2017).


Figure 21 Characteristic of *Lepisanthes rubiginosa* (Mahuat).

2.5.3 Makiang

Syzygium nervosum A. Cunn. ex DC. (synonym *Cleistocalyx nervosum var paniala*; Ma-kiang) is belonging to the Myrtaceae family. This plant is cultivated in northern Thailand. Fruits are red-purple at the ripening stage and can produce juice, wine, and jam (Charoensin et al., 2012). According to previous studies, the ripe fruit of *Cleistocalyx nervosum var paniala* contained polyphenols (269±3.0 mg GAE/100 g), cyanidin-3-glucoside, and antioxidant activities through oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) with values of 37 μmolTE/ and 28 μmolTE/g, respectively (Charoensin et al., 2012; Patthamakanokporn, Puwastien, Nitithamyong, & Sirichakwal, 2008). Moreover, several studies suggested that fruit extract of *Cleistocalyx nervosum var paniala* exhibited antimutagenic and anticlastogenic activities in Wistar rats (Charoensin et al., 2012). Moreover, the fruit extract stimulated heme oxygenase-1 and glutathione peroxidase activities and prevention of malondialdehyde generation in male Wistar rats (Taya, Punvittayagul, Chewonarin, & Wongpoomchai, 2009), the prevention of oxidative stress in cadmium-induced Wistar rats (Poontawee, Natakankitkul, & Wongmekiat, 2016). The fruit extract of *Cleistocalyx nervosum var paniala* showed

the lowering malondialdehyde in serum and liver and the enhancing glutathione peroxidase and catalase activities in the liver, resulting in the prevention of hepatocarcinogenesis in rats (S. Taya et al., 2014). According to a previous study, *Cleistocalyx nervosum var paniala* exhibited neuroprotective and antioxidant activity by reducing ROS production and increasing antioxidant enzyme mRNA expressions (Sukprasansap et al., 2017).

Figure 22 Characteristic of *Syzygium nervosum* (Makiang).

CHAPTER III

MATERIALS AND METHOD

3.1 Materials

Chemicals	Company
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich (St. Louis, MO, USA)
2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Sigma-Aldrich (St. Louis, MO, USA)
2,4-Dinitrophenylhydrazine	Ajax Finechem (Taren Point, Australia)
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	Sigma-Aldrich (St. Louis, MO, USA)
3,5-Dinitrosalicylic acid	Sigma-Aldrich (St. Louis, MO, USA)
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich (St. Louis, MO, USA)
6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)	Sigma-Aldrich (St. Louis, MO, USA)
Acarbose	Bayer AG Pharmaceutical (Berlin, Germany)
Acetic acid	Merck (Darmstadt, FR, Germany)
Acetonitrile (AR grade)	Merck (Darmstadt, FR, Germany)
Aminoguanidine hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Ascorbic acid	Ajax Finechem (Taren Point, Australia)
Bovine serum albumin fraction V (BSA)	Sigma-Aldrich (St. Louis, MO, USA)

Chemicals	Company
Cyanidin-3-glucoside	PhytoLab GmbH& Co. KG (Vestenbergsgreuth, Germany)
Delphinidin-3-glucoside	Sigma-Aldrich (St. Louis, MO, USA)
D-fructose	Ajax Finechem (Taren Point, Australia)
D-glucose	Ajax Finechem (Taren Point, Australia)
D-maltose	Ajax Finechem (Taren Point, Australia)
D-ribose	Sigma-Aldrich (St. Louis, MO, USA)
Deoxyribose	Sigma-Aldrich (St. Louis, MO, USA)
Dexamethasone	Sigma-Aldrich (St. Louis, MO, USA)
Ethanol	Merck (Darmstadt, FR, Germany)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (St. Louis, MO, USA)
Folin-Ciocalteu's phenol reagent	Sigma-Aldrich (St. Louis, MO, USA)
Formic acid	Merck (Darmstadt, FR, Germany)
Gallic acid	Sigma-Aldrich (St. Louis, MO, USA)
Guanidine hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Hydrochloric acid	Merck (Darmstadt, FR, Germany)
Insulin bovine pancreas	Sigma-Aldrich (St. Louis, MO, USA)
Iron (III) chloride hexahydrate	Ajax Finechem (Taren Point, Australia)

Chemicals	**Company**
Iron (II) sulfate	Ajax Finechem (Taren Point, Australia)
Isopropanol	Merck (Darmstadt, FR, Germany)
Methanol (HPLC grade)	Merck (Darmstadt, FR, Germany)
Oil Red O	Sigma-Aldrich (St. Louis, MO, USA)
Potassium sodium tartrate	Ajax Finechem (Taren Point, Australia)
Porcine pancreatic α-amylase (Type VI-B)	Sigma-Aldrich (St. Louis, MO, USA)
Rat intestinal acetone powder	Sigma-Aldrich (St. Louis, MO, USA)
Sodium acetate anhydrous	Ajax Finechem (Taren Point, Australia)
Sodium chloride	Ajax Finechem (Taren Point, Australia)
Sucrose	Ajax Finechem (Taren Point, Australia)
Thiobarbituric acid	Sigma-Aldrich (St. Louis, MO, USA)
Thioflavin T	Sigma-Aldrich (St. Louis, MO, USA)
Trichloroacetic acid	Merck (Darmstadt, FR, Germany)

Laboratory Equipment	**Company**
Autoclave	Amegax Instrument, Inc. (Concord, CA, USA)
Biosafety Cabinet (Class II)	Esco Micro Pte. Ltd (Singapore)

Laboratory Equipment	**Company**
CFX384 Touch™ Real-time PCR Detection system	Bio-Rad Laboratories (Hercules, CA USA)
CO_2 Incubator	Skadi Europe BV (Netherland)
Rotary Evaporator	Buchi Ltd. (Flawil, Switzerland)
Freeze dryer machine	GRT., Grisrianthong. Co. Ltd. (Samutsakorn, Thailand)
High-performance liquid chromatography (HPLC)	Agilent Technology (CA, USA)
High speed refrigerated micro-centrifuge	Hettich (Tuttlingen, Germany)
High-speed universal centrifuge	Hermle Labor Technik (GmbH, Germany)
Hot air oven	Conthem Scientific (New Zealand)
Hot plate	IKA-Works (Staufen im Greisgau, Germany)
Laboratory refrigerator	Sanden intercool (Thailand)
Mass spectrophotometer (MS)	Sciex (Massachusetts, USA)
Microplate reader	Tecan Trading (AG, Switzerland)
pH meter	Thermo Scientific, Inc. (Waltham, MA USA)
Spectrofluorometer	Thermo Fisher Scientific (Vantaa, Finland)
Strata-C18E solid-phase extraction	Phenomenex Inc. (CA, USA) Taiwan)

Laboratory Equipment	**Company**
Vortex mixer	Gemmy Industrial Corp. (Taipei,
Miscellaneous	**Company**
BCA assay kit	Thermo Fisher (Waltham, MA, USA)
C18 Inertsil ODS-2 column	Techno Quartz Inc. (Tokyo, Japan)
Dulbecco's Modified Eagles Medium With high glucose	GE Healthcare Life Science (South Logan, Utah, USA)
Gene-specific mouse primers	Integrated DNA Technologies, Inc., Coralville, IA, USA)
Glucose liquid color kit	HUMAN Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany)
iTaq^{TM} Universal SYBR green supermix	Bio-Rad Laboratories (Hercules, CA, USA)
Mouse 3T3-L1 preadipocytes (CL-173^{TM})	American Type Culture Collection (Manassas, VA, USA)
N^{ε}-(carboxymethyl)lysine ELISA kit	Cyclex Co., Ltd. (Nagano, Japan)
Reverse transcription system	Promega (Madison, WI, USA)
RQ1 DNase kit	Promega (Madison, WI, USA)
Triglyceride liquicolor GPO-POD kit	HUMAN Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany)
TRIzol^{TM}	Thermo Fisher (Waltham, MA, USA)

3.2 Methods

3.2.1 Plant materials

The dark-purple color fruit (mature ripened stage) of *Antidesma bunius* (Mamao) was collected during August-September 2017 from Sakon Nakhon province, Northeastern Thailand. The dark-purple color fruit of *Lepisanthes rubiginosa* (Roxb.) Leenh (Mahuat) was collected during March-April 2018, from Sakon Nakhon province. The red-color fruit of *Syzygium nervosum* A. Cunn ex. DC. (Makiang) was collected during March-April 2018 from Lampang province, Northern Thailand. The plants were authenticated by a taxonomist Parinyanoot Klinratana at Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The voucher specimen was 015866 (BCU), 015862 (BCU), and 015918 (BCU).

3.2.2 Sample preparation and extraction

The fruit extraction was performed according to a previous method with a slight modification (Grussu, Stewart, & McDougall, 2011). Each berry fruit (10 kg) was deseeded and blended with distilled water (20 L). The sample was filtered through a sieve cloth and centrifuged at 3500 rpm for 5 min at 4°C to remove the residue. Then the solution was filtered through Whatman no.1 filter paper under vacuum. The solution was kept at -20°C and lyophilized using a freeze-dryer at -40°C with 0.5 psi for 48 hours. The freeze-dried powder (1 g) was dissolved in distilled water (20 ml). The anthocyanin-rich fraction was purified using C_{18} solid-phase extraction (Strata-C18E, Phenomenex Inc., CA, USA) to remove free sugars and organic acids. The solid phase extraction was done by preconditioned with 0.2% formic acid in acetonitrile (6 ml) and then pre-equilibrated with 0.2% formic acid in

water (6 ml). Consequently, the sample (6 ml) was loaded into an extraction tube, washed with 0.2% formic acid in water (6 ml) and water (12 ml). After that, acetonitrile (80% v/v in water) was used to elute the anthocyanin-rich fraction. The fraction was evaporated at 50°C (BUCHI ROTAVAPOR, Flawil, Switzerland) to remove the solvent. The anthocyanin-rich fraction from fruit extract of *Antidesma bunius* (ABE), *Lepisanthes rubiginosa* (Roxb.) Leenh (LRE), and *Syzygium nervosum* A. Cunn ex. DC. (SNE) were kept at -20°C until use. In the experiment, Thai berry extracts were dissolved in distilled water.

3.2.3 Determination of phytochemical contents

3.2.3.1 Total phenolic content

The total phenolic content of ABE, LRE, and SNE were determined using the Folin-Ciocalteau method (Adisakwattana et al., 2012). In brief, 1 mg/ml anthocyanin-rich extracts (50 µl) were mixed with 50 µl Folin-Ciocalteu reagent (10-fold dilution with distilled water) and 50 µl Na_2CO_3 (10% w/v). After 30 min of incubation in the dark, the absorbance of solution was measured at 760 nm. The content of phenolic was expressed as mg gallic acid equivalent (GAE)/g extract.

3.2.3.2 Total anthocyanin content

The total anthocyanin content of ABE, LRE, and SNE were measured by the pH differential method (J. Lee, Durst, & Wrolstad, 2005). The anthocyanin-rich extract (1 mg/ml; 250 µl) was mixed with 750 µl of two different buffer systems, 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5). All sample solutions were incubated at room temperature for 20 min in the dark and monitored the absorbance at a wavelength of 520 nm and 700 nm. The absorbance of the

solution was calculated by the following formula: $A = (A_{520}-A_{700})pH_{1.0} - (A_{520}-A_{700})pH_{4.5}$. The anthocyanin content was obtained from:

$$\text{Anthocyanin content (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times \lambda \times c}$$

Where A is the absorbance of sample, MW is the molecular weight of cyanidin-3-glucoside (C3G), Ɛ is a molar extinction coefficient of C3G as 26,900 $L.mol^{-1}cm^{-1}$, λ is the path length (cm), c is the sample concentration (mg/l). Anthocyanin content was expressed as mg C3G equivalent/ g extract.

3.2.3.3 Quantification of individual anthocyanins

The individual anthocyanins in Thai berry extracts (ABE, LRE, and SNE) were quantified using LC-MS/MS system according to a previous method with modification (Huang, Wang, Williams, & Pace, 2009; Ramirez, Zambrano, Sepúlveda, Kennelly, & Simirgiotis, 2015). The HPLC system (Agilent 1290, Agilent Technologies, CA, USA) consisted of a binary pump and autosampler with a reverse-phase C-18 Inertsil ODS-2 column (250 x 4.6 mm, 5µm, 150 A°, Techno Quartz Inc., Tokyo, Japan). The mobile phase was formic acid:water (10:90 v/v; mobile phase A) and formic acid:water:acetonitrile:methanol (10:40:22.5:22.5 v/v/v/v; mobile phase B). The anthocyanin was separated by a linear gradient following: 15%-20% (B) in 5 min, 20%-27% (B) in 35 min, 27%-65% (B) in 45 min, 65%-100% (B) in 50 min and then back to 15% (B) until 60 min at a flow rate of 0.6 ml/min. The injection volume of the sample was 5 µl. The anthocyanin was quantified using a mass spectrophotometer with electrospray ionization (ABSciex QTRAP 5500, Sciex, Massachusetts, USA). The anthocyanin content was determined by the multiple reaction monitoring-enhanced product ion mode (MRM-EPI) under condition was set as follows: ion spray voltage 5.5kV, source temperature 500°C, curtain gas 25 psi,

collision energy 20eV. Data were analyzed using “ABSciex analyst” software (Sciex, Massachusetts, USA). Anthocyanins standard was cyanidin-3-glucoside (C3G) and delphinidin-3-glucoside (D3G).

3.2.4 The inhibition of carbohydrate digestive enzymes

3.2.4.1 The inhibition of pancreatic α-amylase

The inhibition of pancreatic α-amylase was performed according to a previous report (Adisakwattana et al., 2012). The various anthocyanin-rich extract concentrations (20 µl) and 4 g/l starch solution (75 µl) were mixed in a 0.1 M phosphate buffer, pH 6.9. After the addition of 75 µl pancreatic α-amylase (3 units/ml), the mixture was incubated at 37°C for 10 min. Then, 1% dinitrosalicylic acid (250 µl) was added to the mixture and heated for 10 min to stop the enzyme activity. The mixture was incubated with 40% potassium sodium tartrate (250 µl) and kept at room temperature. The absorbance was monitored at 540 nm. In this study, acarbose was used as a positive control. The inhibitory activity was expressed as a percentage of inhibition.

$$\% \text{ inhibition} = \left[\frac{\text{Abs control - Abs sample}}{\text{Abs control}}\right] \times 100$$

$Abs_{control}$ was absorbance without ABE or acarbose, and Abs_{sample} was the absorbance of ABE or acarbose.

3.2.4.2 The inhibition of intestinal α-glucosidase

The inhibition of intestinal α-glucosidase was determined following a previously published method (Adisakwattana et al., 2012). The rat intestinal acetone powder was dissolved in 0.9% NaCl solution at a concentration of 100 mg/3 ml and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected for further analysis. In the assay of maltase and sucrase activity, anthocyanin-rich extracts at the

various concentration (10 µl) were mixed with 86 mM maltose (30 µl) or 400 mM sucrose (40 µl) in 0.1 M phosphate buffer, pH 6.9, respectively. The reaction was incubated at 37°C for 30 min (maltase assay) or 60 min (sucrase assay). Then, the reaction was heated at 100°C for 10 min. The release of glucose was determined by the glucose oxidase method. The absorbance was measured at 500 nm. In this study, acarbose was used as a positive control. The results were expressed as IC_{50} values (the concentration required to the percentage of inhibition with 50% of the enzyme activity) calculated using the curve of logarithmic regression.

3.2.5 Determination of antioxidant activities

3.2.5.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The DPPH assay is involved the donation of an electron or hydrogen atom from antioxidant to DPPH radical undergo to form a non-radical(Tan, Chang, & Zhang, 2017). The DPPH radical scavenging activity was determined following the previous protocol (Jariyapamornkoon, Yibchok-anun, & Adisakwattana, 2013b). In brief, 100 µl of anthocyanin-rich extract at various concentrations was mixed with 100 µl of 0.2 mM DPPH in ethanol and incubated for 30 min at room temperature. The absorbance was measured at 515 nm. Ascorbic acid (5-30 µg/ml) was used as a positive control. The IC_{50} value was calculated using the curve of the percentage of DPPH scavenging activity corresponding to the various samples' concentration.

3.2.5.2 Trolox equivalent antioxidant capacity (TEAC)

TEAC was performed according to a previously published method (Suantawee et al., 2015). In brief, 2.45 mM potassium sulfate was mixed with 7 mM ABTS at a ratio of 1:1 and incubated at room temperature for at least 16 hours in the dark to generate $ABTS^{\cdot+}$ radicals. After incubation, the $ABTS^{\cdot+}$ solution was diluted with 0.1

M PBS, pH 7.4, to obtain the absorbance at 0.9-1.0 before analysis. The anthocyanin-rich extracts (20 µl) were incubated with 180 µl $ABTS^{\cdot+}$ solution and incubated at room temperature for 6 min. The reference standard was Trolox, which an analog compound of vitamin E. The antioxidant capacities of samples were calculated from the calibration curve of Trolox. TEAC values were expressed as millimole Trolox per gram extract (mmol Trolox/ g extract).

3.2.5.3 Ferric reducing antioxidant power (FRAP)

The FRAP value was determined according to a previous study (Jorjong et al., 2015). The FRAP reagent was prepared by mixing 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl with 20 mM $FeCl_3$ and 300 mM acetate buffer (1:1:10 v/v/v). The FRAP reagent was incubated at 37°C before analysis. The anthocyanin-rich extracts (20 µl) were mixed with 180 µl of FRAP reagent and incubated at 37°C for 30 min in the dark. The absorbance was measured at 595 nm. Iron (II) sulfate was used as a reference standard. The FRAP values were expressed as millimole Iron (II) per gram extract (mmol Fe (II)/ g extract).

3.2.5.4 Hydroxyl radical scavenging activity (HRSA)

The HRSA was analyzed through deoxyribose method as previously published (Suantawee et al., 2015). The reaction mixture will be freshly prepared by the mixing of 0.3mM iron (III) chloride, 0.6mM ascorbic acid, 1.2mM EDTA, 34mM hydrogen peroxide, and 17mM 2-deoxy-2-ribose at the volume ratio 2:2:1:1:1. The extract solutions will be added into the reaction and then will be incubated with shaking at 37°C for 30 min. After incubation, 1%(w/v) thiobarbituric acid or TBA in 0.05M NaOH and 2.8% (w/v) trichloroacetic acid or TCA will be added into the mixture and then will be heated at 100°C for 15 min. The solution will be cooled to room

temperature and the absorbance will be measured at 532 nm. Trolox will be used as a standard. The results will be expressed as milligram of Trolox equivalents per milligram extract.

3.2.5.5 Superoxide radical scavenging activity (SRSA)

The SRSA was determined as described by Suantawee et al. (2015) with some modification. Superoxide radicals were produced through the mixing of 0.3mM xanthine (75µl), 0.15mM NBT (50µl), 0.6mM EDTA (50µl) and 0.05 unit/ml of xanthine oxidase. The reaction mixtures were added with Thai berry extract solutions and was incubated for 40 min, at 37°C. The reduction of NBT was measured at 560 nm. Gallic acid was used as a reference standard and the SRSA was quantified using the standard curve of gallic acid. The results were expressed as milligram of gallic acid equivalent/ milligram of extract.

3.2.6 Identification of phytochemical compounds

The phytochemical compounds in ABE were characterized using LC-MS/MS according to a previously published method with a minor modification (Jiménez-Aspee et al., 2016). The HPLC system was equipped with a 6545 Quadrupole-TOF Mass spectrometer (Agilent Technologies, CA, USA). The phenolic compounds were separated using the Luna C_{18} column (150 x 2.0 mm, 3µm, Phenomenex Inc., CA, USA). The mobile phase consisted of (A) water:formic acid:acetonitrile (95:2:3 v/v/v) and (B) water:formic acid:acetonitrile (48:2:50 v/v/v). The flow rate was set at 0.20 ml/min. The gradient program was 5% (B), 25% (B) in 50 min, and 5% (B) in 5 min. The injection volume was 5 µl. The phenolic compounds were identified in negative and positive modes with a mass range of m/z 100 to 3000. The mass

spectrophotometer (MS) condition was followed by nitrogen gas temperature 325°C, gas flow 11 L/min, nebulizer gas was 35 psi, sheath gas temperature 350°C, sheath gas flow 11 L/min, capillary 3500 V, fragmentor voltage 125 V. The interpretation of MS/MS data were carried out using Respect, GNPS, and MassBank mass spectral libraries. The identification score of 80% was selected for mass verification.

3.2.7 The inhibitory effect of ABE on monosaccharide-induced protein glycation

3.2.7.1 Protein glycation

The protein glycation assay was performed following a published method (Thilavech et al., 2015). Briefly, 700 µl of BSA (10 mg/ml final concentration) was mixed with 560 µl of fructose or glucose (0.5 M final concentration) in 0.1 M phosphate buffer saline (PBS, pH 7.4) containing 0.02% sodium azide. The solution was mixed with 140 µl of ABE in 0.1 M PBS (0.025-0.25 mg/ml final concentration) or aminoguanidine (0.25 mg/ml final concentration) as shown in Table 1. The solution was incubated at 37°C in the dark for 4 weeks. The solution was collected each week to determine the effect of ABE on AGEs formation, protein oxidation, and protein aggregation

3.2.7.2 Determination of fluorescence AGEs formation

The fluorescent intensity of AGE formation was measured weekly using a spectrofluorometer (Fluoroskan Ascent, Thermo Fisher Scientific, Vantaa, Finland) at excitation and emission wavelength 355 nm and 460 nm, respectively. The following equation calculated the percentage inhibition:

$$\text{Inhibition of fluorescent AGEs (\%)} = [\ (F_C - F_{CB}) - (F_S - F_{SB}) \ / \ (F_C - F_{CB})] \times 100$$

Where F_C and F_{CB} were the fluorescent intensity of control with monosaccharide and blank of control without monosaccharide. F_S and F_{SB} were the sample's fluorescent intensity with monosaccharides and blank of the sample without monosaccharides.

Table 1 Chemicals were contained in bovine serum albumin (BSA) glycation.

Experimental groups	1.25M Fructose/ 1.25M Glucose (µl)	20 mg/ml BSA (µl)	2.5 mg/ml AG (µl)	0.25-2.5 mg/ml ABE (µl)	PBS (µl)
Blank					
○ Blank			-	-	1400
○ Negative	560	-	-	-	840
○ Positive	560	-	140	-	700
○ ABE	560	-	-	140	700
Test					
○ Blank	-	700	-	-	700
○ Negative	560	700	-	-	140
○ Positive	560	700	140	-	-
○ ABE	560	700	-	140	-

3.2.7.3 Determination of N^{ε}-CML

The level of N^{ε}-CML, a non-fluorescent AGEs, was analyzed by enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (Cyclex Co., Ltd., Nagano, Japan). The absorbance was measured at 450 nm. The level of N^{ε}-CML was calculated by the CML-human serum albumin (HSA) standard curve.

3.2.7.4 Determination of protein carbonyl

The content of protein carbonyl was determined using 2,4 dinitrophenylhydrazine (DNPH) assay according to a previous report (Thilavech et al., 2015). Briefly, the glycated BSA (100 µl) was mixed with 10 mM DNPH in 2.5 M HCl (400 µl) and incubated for 60 min in the dark. After incubation, the protein was precipitated by 20% (w/v) trichloroacetic acid (500 µl), kept on ice for 5 min, and centrifuged at 10,000 g for 10 min, at 4°C. The protein pellet was washed with methanol: ethyl acetate (1:1 v/v) for three times and dissolved in 250 µl 6 M guanidine hydrochloride. The absorbance was read at 370 nm. The carbonyl content was calculated using the molar extinction coefficient of 22,000 (DNPH). The results were expressed as nmol carbonyl/mg protein.

3.2.7.5 Determination of protein aggregation

The level of β-amyloid cross structure was analyzed by thioflavin T, according to a previous study (Thilavech et al., 2015). The glycated BSA (50 µl) was mixed with 64 µM thioflavin T (50 µl) in 0.1 M PBS, pH 7.4. The mixture was incubated at room temperature for 60 min. The β-amyloid cross structure level was measured using a spectrofluorometer at excitation and emission wavelength with 435 nm and 485 nm, respectively.

3.2.8 The effect of ABE on 3T3-L1 adipocyte differentiation

3.2.8.1 Cell culture and differentiation

The 3T3-L1 preadipocyte cells were cultured and differentiated following a previous report (Song et al., 2013). In brief, 3T3-preadipocyte cells were grown in high glucose (4.5 g/L) Dulbecco's Modified Eagles Medium (DMEM) that was supplemented with 10% (v/v) heat-inactivate fetal bovine serum (FBS), penicillin/streptomycin (100U/ml penicillin and 100 µg/ml streptomycin) at 37°C and 5% CO_2. ABE was dissolved in distilled water and directly diluted in DMEM medium to obtain the various concentration of ABE. For cell proliferation, cells (1.6 $x10^4$ cells/ml) were seeded into 24-well plates to confluence. After 24 hours of incubation, 3T3-L1 cells were treated with the various concentration of ABE (4-20 µg/ml) and incubated for 4 days. The culture medium was refreshed every 2 days. After 5 days of post-confluence (100%), preadipocytes were determined cell viability and MTT proliferation assay.

For differentiation, 3T3-preadipocytes ($3x10^4$ cells/ml) were seeded into 12-well plates. After post-confluence, cells were stimulated with the culture medium containing DMEM, 10% FBS, 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, and 2.5µg/ml insulin for 2 days. In the terminal phase of adipocyte differentiation, cells were maintained in DMEM with 10% FBS and 2.5µg/ml insulin. For treated cells, 3T3-L1 cells were cultured in DMEM containing the various concentration of ABE. On day 10 of differentiation, the adipocyte cells were analyzed cell viability, lipid droplets, triglyceride levels, and adipogenic genes expression. All experiments were performed with 3T3-L1 cells from passage 9-13. 3T3-L1 cells were cultured in DMEM medium without ABE which act as a control.

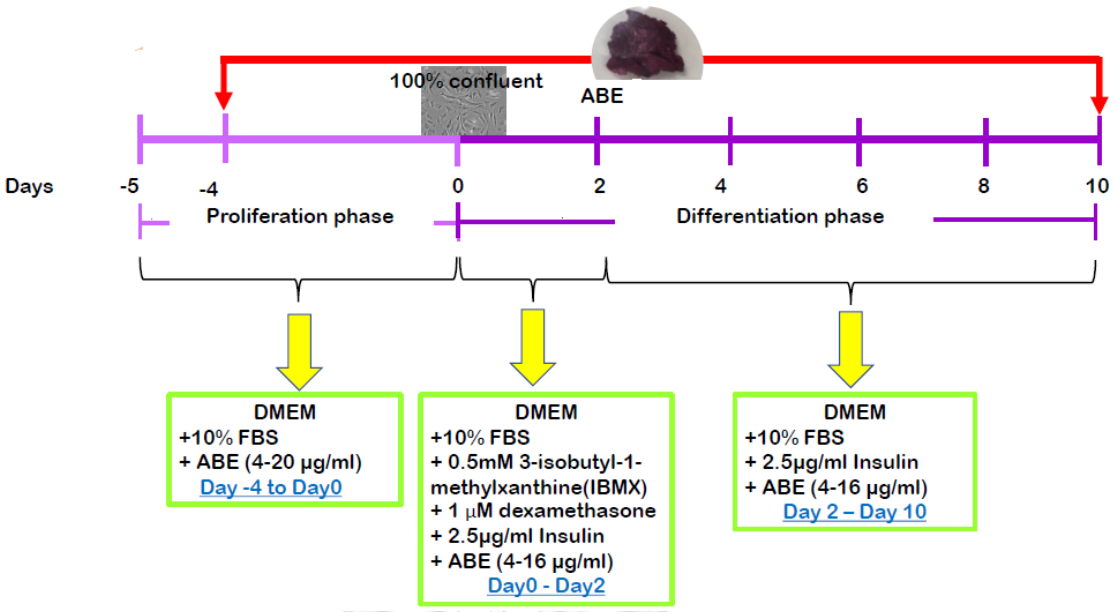


Figure 23 Schematic representation of 3T3-L1 differentiation into adipocyte cells.

3.2.8.2 Cell viability

The 3T3-L1 cell viability was determined by trypan blue assay. After day 4 and 8 of ABE treatment, cells were trypsinized and resuspended in DMEM (1 ml). Then cell suspension was mixed with 0.4% trypan blue in a ratio of 1:1 and counted using a hemocytometer. The cell viability was expressed as cell number (cells/ml) and a percentage of cell viability (% of control).

3.2.8.3 MTT proliferation assay

The MTT proliferation assay was determined following a previous study with minor modification (Drira, Chen & Sakamoto, 2011). The 3T3-L1 preadipocyte cells (1.6×10^4 cells/ ml) were seeded in to 24-well plates. After 24 hours, cells were treated with ABE (4-20 µg/ml) for 4 days. The culture medium was removed and replaced by a culture medium containing 0.5 mg/ml MTT. After 2.5 hours, the formazan crystals were dissolved in DMSO (250 µl) and absorbance was measured at 550 nm. The results were expressed as the percentage of viable cell compared to control.

3.2.8.4 Oil Red O staining

On day 10 of differentiation, adipocyte cells were washed twice with phosphate-buffered saline (PBS), and then cells were fixed by 10% formaldehyde. Cells were incubated with 60% isopropanol for 5 min. After that, cells were stained with filtered 60% Oil Red O staining solution for 20 min. The plate was rinsed with water for 5 times. An inverted microscope photographed the stained lipid droplets. The lipid accumulation in adipocytes was quantified by the addition of 100% isopropanol to stained lipid droplets and incubated for 10 min. The absorbance was

measured at 520 nm. The results were expressed as relative lipid content (% of control).

3.2.8.5 Determination of triglyceride level

The intracellular triglyceride accumulation in 3T3-L1 cells was analyzed according to a previous study with modification (K. K. Li et al., 2016). On day 10 of differentiation, the adipocytes were washed with PBS and incubated with cold lysis buffer for 3 min. The cell suspension was lysed by sonification for 5 min and centrifuged at 12,000 x g, 4 ° for 10 min. The supernatant (10 µl) was mixed with triglyceride reagent (200 µl) and kept for 10 min in the dark. The absorbance was measured at 500 nm. The protein concentration was determined by a BCA assay kit (Thermo Fisher, USA). The results were expressed as mg triglyceride per mg protein.

3.2.8.6 Quantification of gene expression

On day 10 of differentiation, total RNA was extracted from adipocytes using TRIzol™ reagent (Invitrogen, USA). The RNA level was quantified using NanoDrop 1000 spectrophotometer. Total RNA (200 ng/ml) was treated with RQ1 DNase enzyme (Promega, USA). According to the manufacturer's protocol, the DNase-treated RNA was reversed transcribed into cDNA using Reverse transcription system (Promega, USA). The cDNA was then amplified and quantified using a real-time quantitative PCR system (RT-qPCR) with iTaq™ Universal SYBR Green Supermix (Bio-Rad, USA). PCR mixture (10 µl) contained 5 ng of cDNA, 500nM of forward and reverse primers, and 5µl of SYBR. The gene-specific mouse primers to amplified cDNAs, as shown in Table 2. The RT-qPCR was carried out in a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The PCR reaction condition was following initial denaturation 95°C for 2 min, followed by 40PCR cycles: 95°C for 5s,

63.7°C for 30s, and 95°C for 5s. The purity of PCR product was analyzed using the melting curve analysis. The mRNA expression was normalized with β-actin and calculated the mRNA level using the $2^{-\Delta\Delta CT}$ method. The results were expressed as the relative mRNA expression.

3.2.9 Statistical analysis

All experiments were performed in triplicate (n=3). The values are presented as means ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA, followed by Duncan's post hoc test (SPSS version 17, SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Table 2 List of gene-specific primers for RT-qPCR.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
PPARγ	ACGTGCAGCTACTGCATGTGA	AGAAGGAACACGTTGTCAGCG	125
C/EBPα	GGAACTTGAAGCACAATCGATC	TGGTTTAGCATAGACGTGCACA	156
aP2	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC	133
FASN	AGGTGGTGATAGCCGGTATGT	TGGGTAATCCATAGAGCCCAG	138
ACC	CGGACCTTTGAAGATTTTGT	GCTTTATTCTGCTGGGTGAA	233
LPL	GGCTCTGCCTGAGTTGTAGAA	GGCATCTGAGAGCGAGTCTTC	112
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA	101

CHAPTER IV

RESULTS

4.1 Phytochemical contents of Thai berry extracts

4.1.1 Total phenolic contents

Thai berry extracts were determined the content of phenolic compounds using Folin's ciocalteau assay. As shown in Table 3, the results demonstrated that the phenolic contents were significantly different among Thai berry extracts. The highest total phenolic content was found in ABE (300.91 ± 3.25 mg gallic acid equivalent/g extract). Meanwhile, the lowest was found in LRE (237.90±2.26 mg gallic acid equivalent/g extract). However, the total phenolic contents did not a significant difference between SNE and LRE.

4.1.2 Total anthocyanins contents

The anthocyanins contents of each Thai berry extract were analyzed by the pH differential method. The result in Table 3 demonstrated that the anthocyanins contents of Thai berry extracts were in the range between 32.45-66.86 mg cyanidin-3-glucoside/ g extract). This study found that ABE had the highest anthocyanins contents 66.86 ± 1.32 mg cyanidin-3-glucoside/g extract following LRE and SNE. However, the content of anthocyanins of LRE did not make a significant difference with SNE.

Table 3 Phytochemical contents of the anthocyanin-rich fraction of Thai berry extracts.

Thai berry extracts	Total phenolic contents (mg GAE/ g extract)	Total anthocyanins contents (mg C3G/ g extract)	Cyanidin-3-glucoside content (mg/ g extract)	Delphinidin-3-glucoside content (mg/ g extract)
ABE	300.91±3.25[a]	66.86±1.32[a]	31.43±1.39[a]	21.65±0.79[a]
LRE	237.90±2.26[b]	39.93±2.70[b]	39.96±6.52[a]	0.93±0.15[b]
SNE	246.77±2.35[b]	32.45±0.82[b]	27.19±0.47[a]	ND

Data were expressed as mean ± SEM (n=4). The different superscripted letters in the same column are significantly different ($p < 0.05$). ABE: *Antidesma bunius* (L.) spreng; LRE: *Lepisanthes rubiginosa* (Roxb.) Leenh; SNE: *Syzygium nervosum* A. Cunn ex. DC; GAE: Gallic acid equivalents; C3G: cyanidin-3-glucoside; ND: Not detected.

4.1.3 Quantification of individual anthocyanins by LC-MS/MS

The anthocyanins in Thai berry extracts were quantified using LC-MS/MS. Cyanidin-3-glucoside (C3G) and delphinidin-3-glucoside (D3G) were used as a standard compound to confirm and quantify the anthocyanins contents. The MS/MS chromatogram of anthocyanin standard, ABE, LRE, and SNE are shown in Figure 24-27. The chromatogram demonstrated that ABE and LRE contained C3G and D3G, whereas SNE contained only C3G. The content of anthocyanins of ABE, LRE, and SNE was compared to standard curves. As shown in Table 3, this study found that cyanidin-3-glucoside contents of ABE, LRE, and SNE were 31.43, 39.96, and 27.19 mg/g extract, respectively. Meanwhile, the contents of delphinidin-3-glucoside in ABE and LRE were 21.65 and 0.93 mg/g extract, respectively. These results suggested that all cyanidin-3-glucoside was a major anthocyanin in Thai berry extracts.

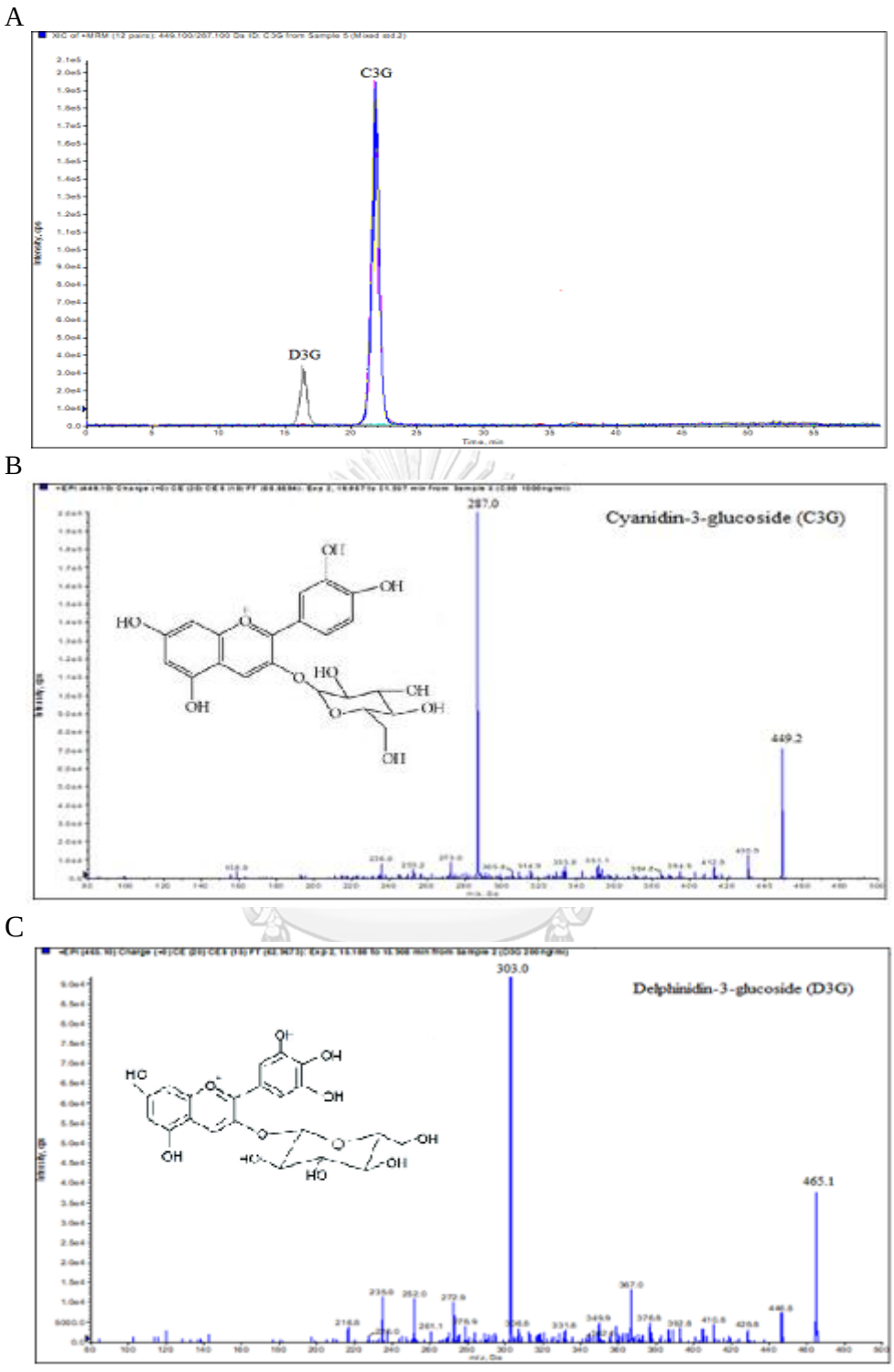


Figure 24 The LC-MS/MS chromatogram of individual anthocyanins (C3G and D3G) . (A): Chromatogram from multiple reaction monitoring mode; (B): EPI spectra of C3G; (C) EPI spectra of D3G.

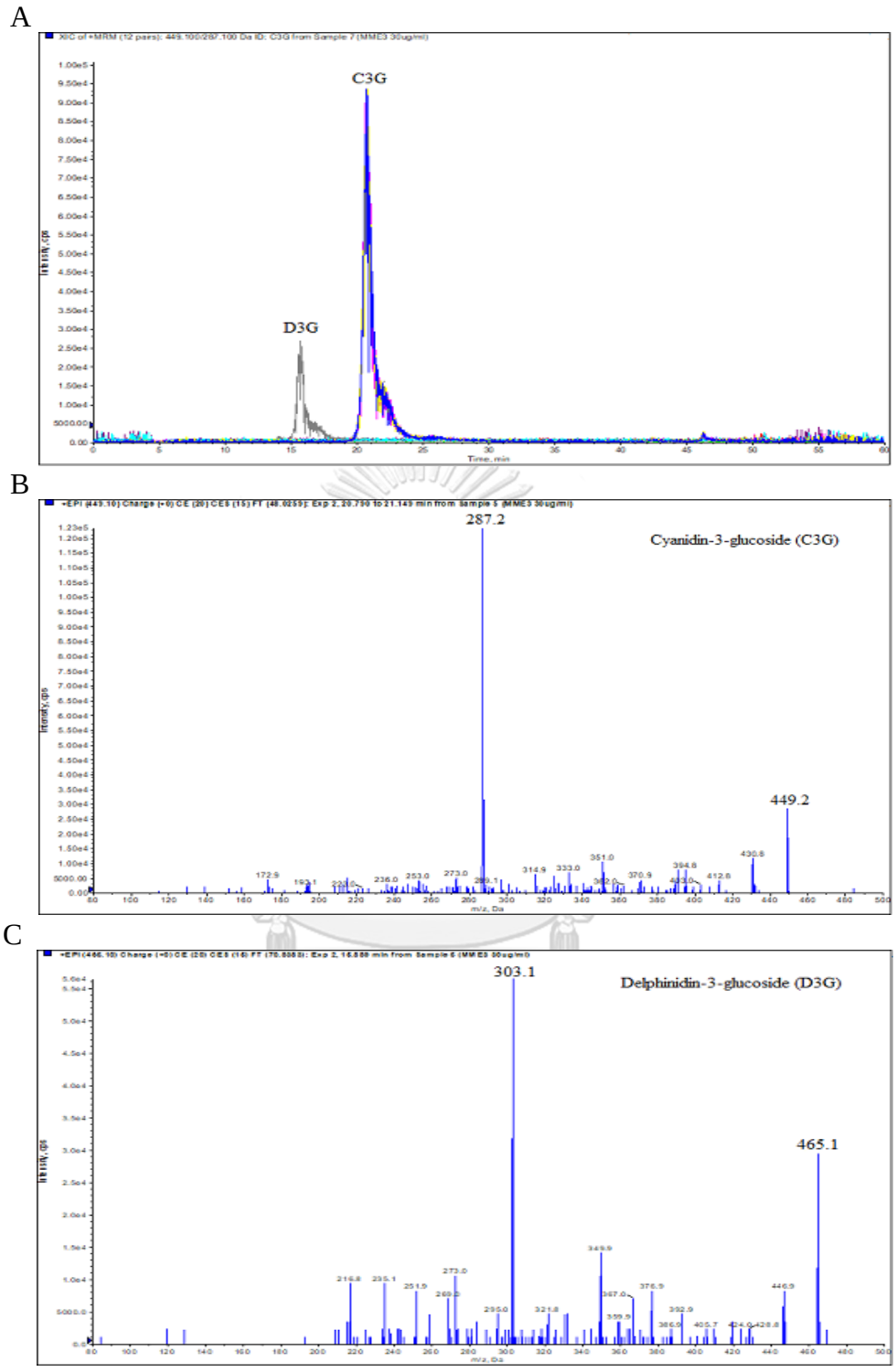
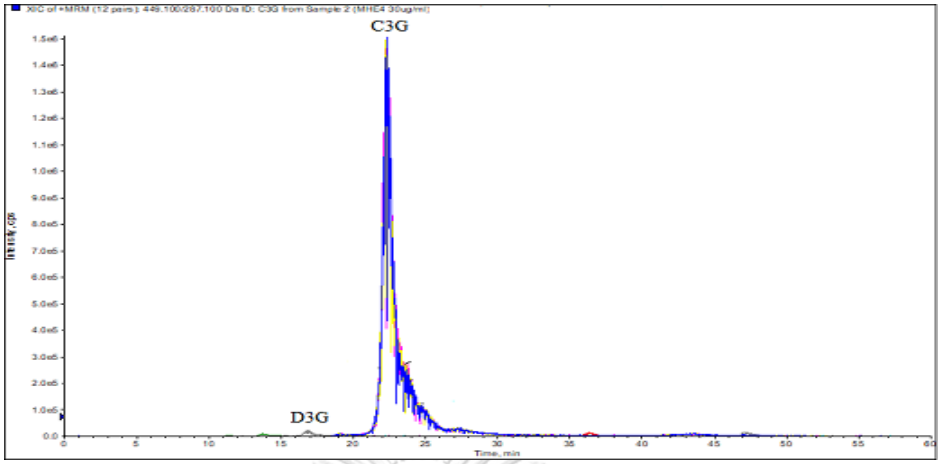
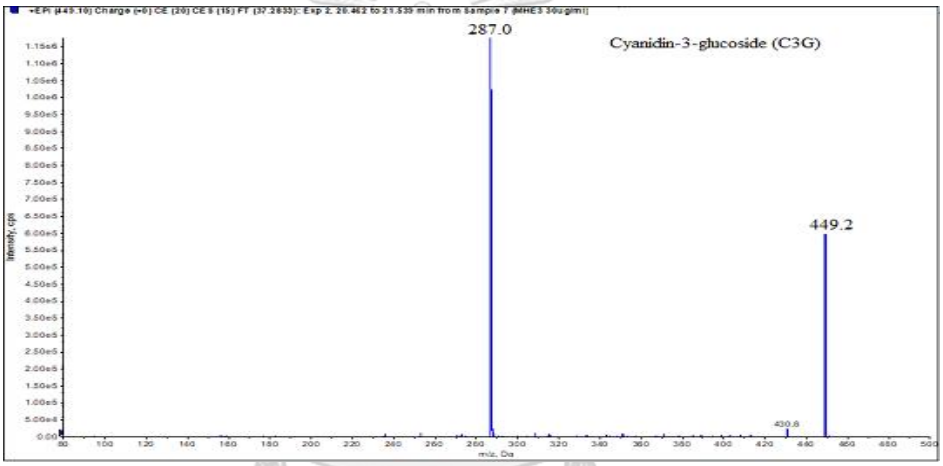


Figure 25 The LC-MS/MS chromatogram of individual anthocyanins in ABE . (A): Chromatogram from multiple reaction monitoring mode; (B): EPI spectra of C3G; (C) EPI spectra of D3G.

A



B



C

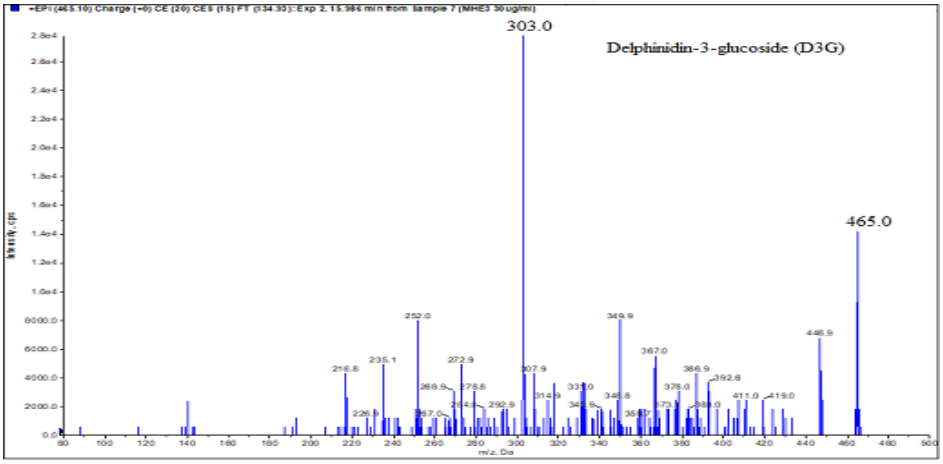
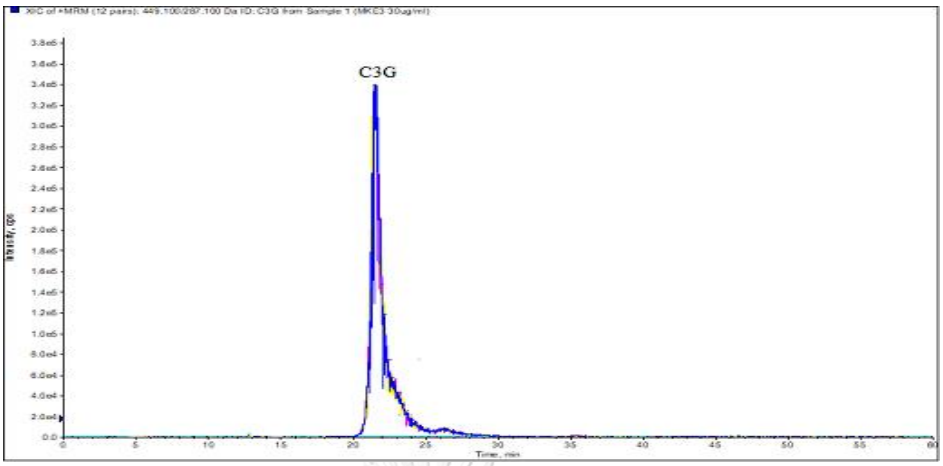


Figure 26 The LC-MS/MS chromatogram of individual anthocyanins in LRE . (A): Chromatogram from multiple reaction monitoring mode; (B): EPI spectra of C3G; (C) EPI spectra of D3G.

A



B

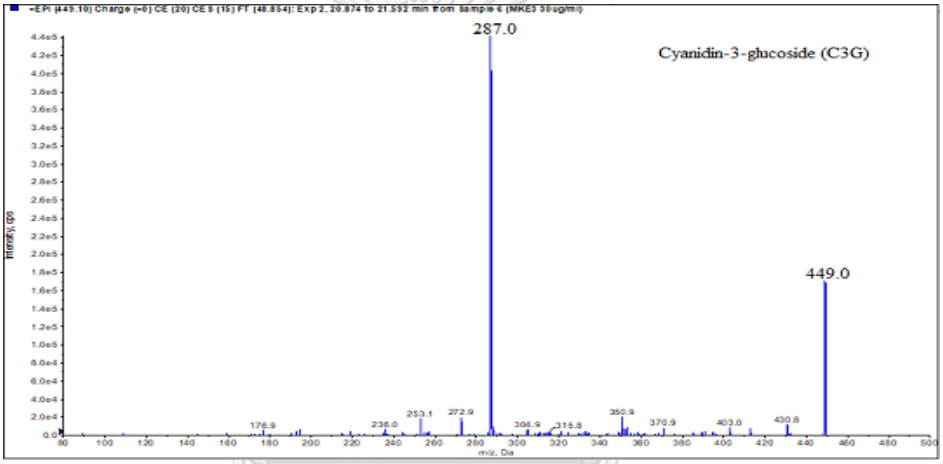


Figure 27 The LC-MS/MS chromatogram of individual anthocyanins in SNE. (A): Chromatogram from multiple reaction monitoring mode; (B): EPI spectra of C3G; (C) EPI spectra of D3G.

4.2 Inhibitory effect of Thai berry extracts on carbohydrate digestive enzymes

4.2.1 The investigation of pancreatic α-amylase inhibition

In this study, ABE and SNE (1-4 mg/ml) slightly inhibited the pancreatic α-amylase activity in range between 11.22% - 35.87% and 9.04% - 37.11%, respectively. Meanwhile, LRE exhibited the most potential of pancreatic α-amylase inhibition with an IC_{50} value of 0.19±0.01 mg/ml that is presented in Table 4. However, Thai berry extracts against pancreatic α-amylase were less potent than acarbose, showing the IC_{50} value with 34.62 ± 0.68 µg/ml.

4.2.2. The investigation of intestinal α-glucosidase inhibition

The present study found that anthocyanins-rich fraction Thai berry extracts (1 mg/ml) could inhibit intestinal maltase ranging from 40.56% to 57.26%. As shown in Table 2, the ABE showed the highest inhibitory effect on intestinal maltase with an IC_{50} value of 0.79 ± 0.04 mg/ml then followed by LRE (1.30 ± 0.04 mg/ml) and SNE (1.52 ± 0.03 mg/ml), respectively. Furthermore, the inhibitions of Thai berry extracts on intestinal maltase were less potent than acarbose (IC_{50} value = 0.89 ± 0.07 µg/ml).

Meanwhile, Thai berry extracts (1 mg/ml) inhibited the intestinal sucrase between 33.71% - 41.54%. The highest intestinal sucrase inhibition was found in ABE with an IC50 value of 1.34 ± 0.02 mg/ml, shown in Table 4. The IC_{50} values of LRE and SNE against intestinal sucrase were 1.63 ± 0.04 mg/ml and 1.65 ± 0.07 mg/ml, respectively. This study indicated that Thai berry extracts showed less intestinal sucrase inhibition than acarbose ((IC_{50} value = 8.48 ± 0. 07µg/ml). Moreover, it was noted that ABE had the most significant inhibition of intestinal α-glucosidase, including maltase and sucrase, among Thai berry extracts.

Table 4 The IC_{50} values of anthocyanins-rich fraction of Thai berry extracts against carbohydrate digestive enzymes.

Thai berry extracts	**IC_{50} values**		
	Pancreatic α-amylase	**Intestinal maltase**	**Intestinal sucrase**
ABE (mg/ml)	> 4	0.79 ± 0.04^{a}	1.34 ± 0.02^{a}
LRE (mg/ml)	0.19 ± 0.01	1.30 ± 0.04^{b}	1.63 ± 0.04^{b}
SNE (mg/ml)	> 4	1.52 ± 0.03^{c}	1.65 ± 0.07^{b}
Acarbose (μg/ml)	34.62 ± 0.68	0.89 ± 0.07	8.48 ± 0.07

Data are expressed as mean ± SEM (n=4). Results within the same column were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same column are significantly different among Thai berry extracts ($p < 0.05$). ABE: *Antidesma bunius* (L.) spreng; LRE: *Lepisanthes rubiginosa* (Roxb.) Leenh; SNE: *Syzygium nervosum* A. Cunn ex. DC**.**

4.3 Antioxidant activities of Thai berry extracts *in vitro* studies

4.3.1 The DPPH radical scavenging activity

As shown in Table 5, the DPPH radical scavenging activity had a significant difference among anthocyanin-rich fraction of Thai berry extracts. The IC_{50} values of all berry extracts were in the range of 15.59-31.97 µg/ml. The DPPH scavenging activity was in the following order, from highest to lowest: ABE > SNE > LRE. ABE showed the DPPH radical scavenging activity approximately 1.7-folds and 2.1-folds, respectively, compared to SNE and LRE. Furthermore, ascorbic acid scavenged DPPH radicals greater than Thai berry extracts except for ABE. ABE's scavenging activity did not significantly differ with ascorbic acid (IC_{50}= 11.83±2.78 µg/ml).

4.3.2 Trolox equivalent antioxidant capacity (TEAC)

The current finding demonstrated that the antioxidant capacity did not significant difference among berry extracts. The anthocyanins-rich fraction Thai berry extracts (1 mg/ml) exhibited TEAC values ranging from 4.01-4.55 µmol TE/ mg extract presented in Table 5. The highest TEAC value was found in ABE (4.55 ± 0.19 µmol TE/mg extract) following LRE and SNE, respectively.

4.3.3 Ferric reducing antioxidant power (FRAP)

The anthocyanins-rich fraction Thai berry extracts (1 mg/ml) had FRAP values varying from 3.58-5.32 µmol $FeSO_4$/mg extract, as shown in Table 5. The ABE exhibited the highest FRAP value among Thai berry extracts. Meanwhile, LRE and SNE did not a significant difference in their reducing ability, which were 3.58 ± 0.12 µmol $FeSO_4$/mg extract and 3.76 ± 0.20 µmol $FeSO_4$/mg extract, respectively.

These results indicated that ABE showed more FRAP capacity than both LRE and SNE, approximately 1.4-folds.

4.3.4 Hydroxyl radical scavenging activity (HRSA)

As shown in Table 5, the hydroxyl radical scavenging activity of Thai berry extracts was varied from 0.18 to 0.53 mg TE/mg extract. The lowest HRSA value was found in ABE (0.18 ± 0.02 mg TE/mg extract), whereas the LRE showed the hydroxyl radical scavenging activity as the same as SNE. Both LRE and SNE had a high HRSA value of about 2.9-folds compared to ABE.

4.3.5 Superoxide radical scavenging activity (SRSA)

The superoxide radical scavenging activity was significantly different among Thai berry extracts, as shown in Table 5. The SRSA values of Thai berry extracts were ranging from 0.19 – 0.42 mg GAE/ mg extract. The scavenging capacity of Thai berry extracts was in the following order: ABE > LRE > SNE. Moreover, the ABE had 1.8-folds and 2.2-folds higher potent than LRE and SNE, respectively.

Table 5 Antioxidant activity of anthocyanins-rich fraction of Thai berry extracts.

Thai berry extracts	**Antioxidant activity**				
	DPPH (IC_{50}; µg/ml)	**TEAC (µmol TE/mg extract)**	**FRAP (µmol $FeSO_4$/mg extract)**	**HRSA (mg TE/mg extract)**	**SRSA (mg GAE/mg extract)**
ABE	15.59 ± 0.25^{a}	4.55 ± 0.19^{a}	5.32 ± 0.15^{a}	0.18 ± 0.02^{a}	0.42 ± 0.03^{a}
LRE	31.97 ± 1.71^{b}	4.39 ± 0.36^{a}	3.58 ± 0.12^{b}	0.52 ± 0.02^{b}	0.23 ± 0.05^{b}
SNE	26.34 ± 1.74^{c}	4.01 ± 0.27^{a}	3.76 ± 0.20^{b}	0.53 ± 0.01^{b}	0.19 ± 0.02^{b}
Ascorbic acid	11.83 ± 2.78^{a}	NA	NA	NA	NA

Data are expressed as mean ± SEM (n=4). Results within the same column were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same column are significantly different ($p < 0.05$). ABE: *Antidesma bunius* (L.) spreng; LRE: *Lepisanthes rubiginosa* (Roxb.) Leenh; SNE: *Syzygium nervosum* A. Cunn ex. DC; DPPH: 2,2-diphenyl-1-picrylhydrazyl; TEAC: Trolox equivalent antioxidant capacity; FRAP: Ferric reducing antioxidant power; HRSA: Hydroxyl radical scavenging activity; SRSA: Superoxide radical scavenging activity; TE: Trolox equivalent; GAE: Gallic acid equivalent; NA: Not analyzed.

4.4 Identification and characterization of phytochemical compounds in ABE by LC-MS/MS

According to chromatogram obtained from LC-MS/MS (Figure 28), the ABE phenolic compounds were characterized using retention time, fragment ion, and comparing with mass spectral libraries. As shown in Table 6, The ABE contained phytochemical compounds including phenolic acids (quinic acid, gallic acid, 6-galloylglycose, ellagic acid), flavonols (quercetin-3-O-arabinoside, 2,5-dihydroxybenzoic acid, quercetin-3-galactoside, kaempferol-3-rhamnoside, kaempferol-3-glucoside, myricetin-3-galactoside), anthocyanins (delphinidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-glucoside).

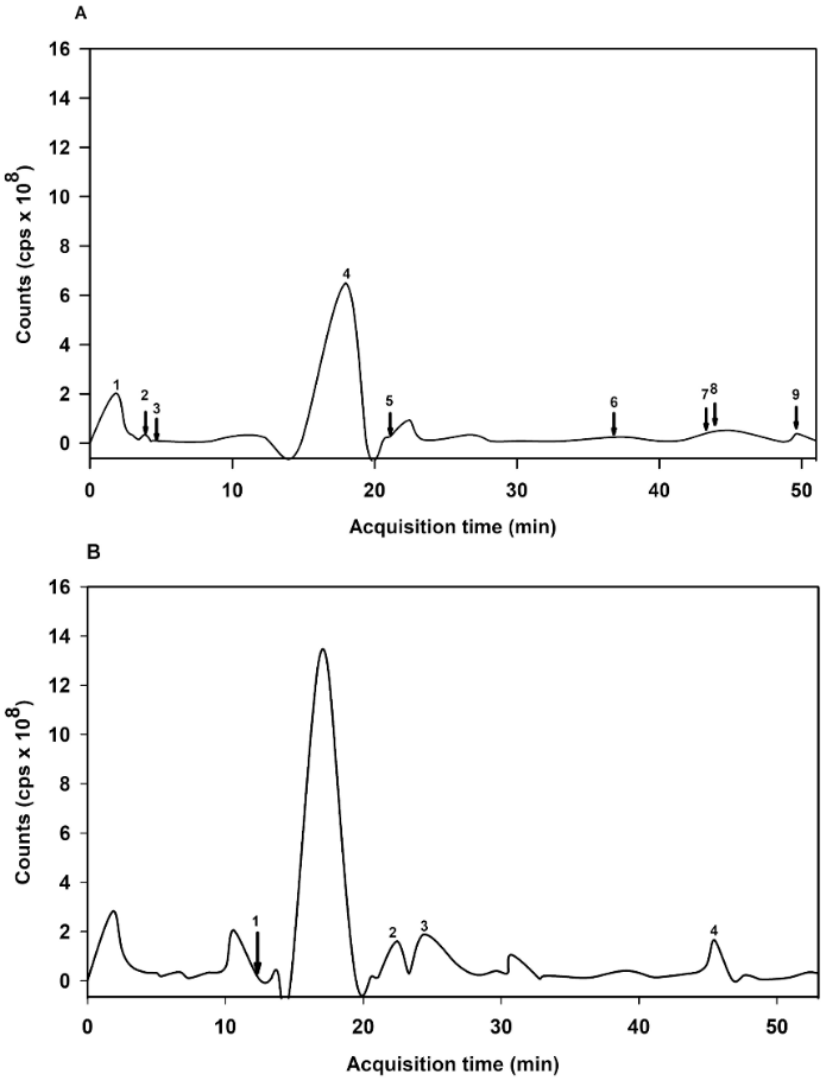


Figure 28 The chromatogram of proposed phytochemical compounds of anthocyanin-rich fraction in *Antidesma bunius* extract (ABE). (A): negative ionization mode; (b): positive ionization mode.

Table 6 Chromatographic MS and MS/MS data of proposed phytochemical compounds in *Antidesma bunius* extract (ABE).

Peak no.	Rt (min)	Proposed compounds	Product Ion
		Phenolic acids	
1	1.897	Quinic acid	192.0639 $[M-H]^-$, Calcd Mass: 191.0567
2	3.468	Gallic acid	170.0217 $[M-H]^-$, Calcd Mass 169.0144
3	4.076	6-Galloylglucose	332.0743 $[M-H]^-$, Calcd Mass: 331.0671
4	21.308	2,5-dihydroxybenzoic acid	154.0266 $[M-H]^-$, Calcd Mass: 153.0194
5	44.313	Ellagic acid	302.0063 $[M-H]^-$, Calcd Mass: 300.999
		Flavonols	
6	17.682	Quercetin-3-O-arabinoglucoside	596.1379 $[M-H]^-$, Calcd Mass: 595.1308, MS/MS: 300.0274
7	36.964	Quercetin-3-galactoside	464.0953 $[M-H]^-$, Calcd Mass: 463.0883, MS/MS: 301.0345
8	44.893	Kaempferol -3-rhamnoside	432.1052 $[M-H]^-$, Calcd Mass:431.098, MS/MS: 283.0605
9	49.761	Kaempferol-3-glucoside	448.1006 $[M-H]^-$, Calcd Mass: 447.0936, MS/MS: 285.4000
10	12.127	Myricetin-3-galactoside	480.1576 $[M-H]^+$, Calcd Mass: 481.1652, MS/MS: 319.1124
		Anthocyanins	
11	22.293	Delphinidin-3-glucoside	465.1811 $[M]^+$, Calcd Mss: 465.1715, MS/MS: 303.1166
12	24.556	Cyanidin-3-sambubioside	581.138 $[M]^+$, Calcd Mass: 581.1431, MS/MS: 287.0523
13	45.374	Cyanidin-3-glucoside	448.1714 $[M-H]^+$, Calcd Mass: 449.1802, MS/MS: 287.1236

4.5 The effect of ABE on advanced glycation end products formation induced by fructose and glucose *in vitro.*

4.5.1 The fluorescence AGE formation

The formation of fluorescence AGE was monitored weekly by measuring the fluorescence intensity of reducing sugar mediated BSA glycation for 4 weeks of incubation. As shown in Figure 29, fructose could increase the glycated BSA in the range between 11.29-folds to 45.42 folds compared to BSA throughout 4 weeks of incubation. The study revealed that the addition of ABE (0.25 mg/ml) into the BSA-fructose system significantly reduced the fluorescence AGE intensity in ranging from 48.95% - 57.95% during 4 weeks of the study period. Meanwhile, the glycated BSA containing AG (0.25 mg/ml) decreased fluorescence AGE formation with a percentage of 47.31% at week 4 of the experiment. Moreover, this finding demonstrated that ABE (0.25 mg/ml) showed the inhibitory effect of glycated BSA, similar to aminoguanidine (0.25 mg/ml) during the study period.

As shown in Figure 30, the formation of glycated BSA was induced by glucose, which showed the fluorescence AGE intensity ranging 3.42-folds to 20.52-folds comparing with non-glycated BSA during 4 weeks of the experiment period. The ABE (0.25 mg/ml) was added into the BSA-glucose system that exhibited the percentage reduction of AGEs formation by 25.29% - 34.49% throughout of incubation period. The decreasing glycated BSA was found in the addition of AG (0.25 mg/ml) with a value of 52.59%. These findings suggested that AG had a more potent than ABE in the inhibitory effect of AGEs formation at the same concentration (0.25 mg/ml). As the above-mentioned, fructose could induce higher glycation formation than glucose throughout 4 weeks of the incubation period.

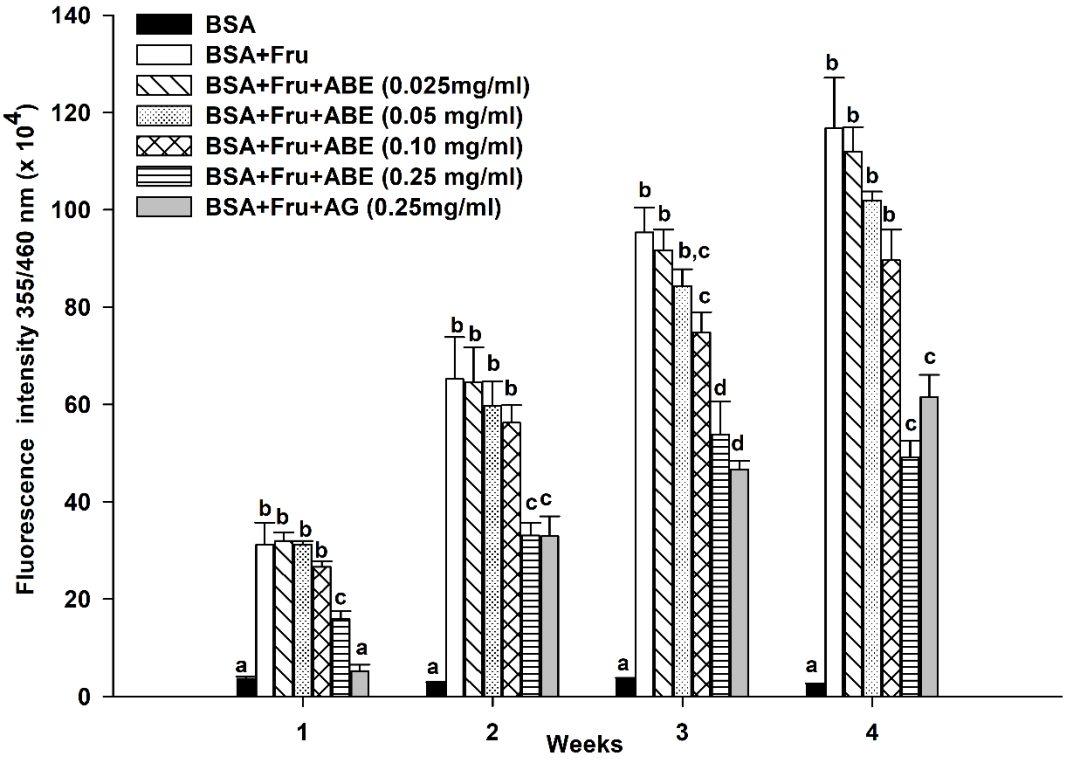


Figure 29 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the formation of fluorescence AGE in bovine serum albumin (BSA) with fructose (Fru; 0.5 M) as a glycating inducer during 4 weeks of study. Data are expressed as mean ± SEM (n=4). Results within the same week were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same week are significantly different ($p < 0.05$).

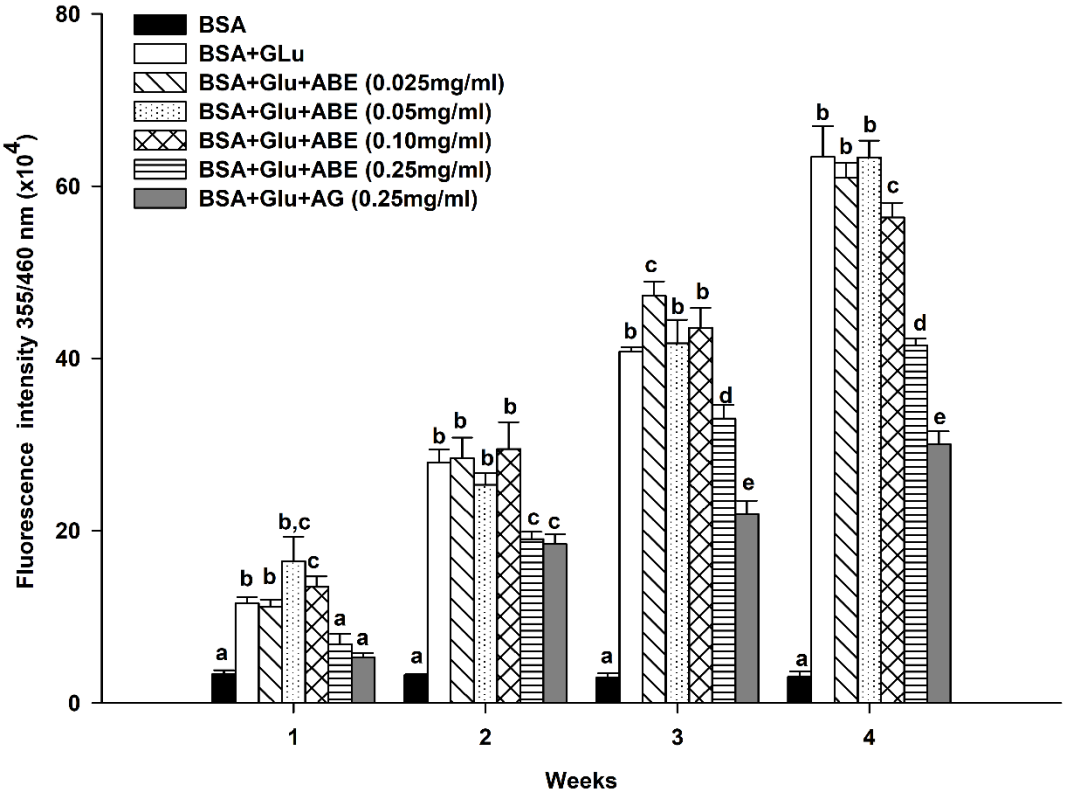


Figure 30 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the formation of fluorescence AGE in bovine serum albumin (BSA) with glucose (Glu; 0.5 M) as a glycating inducer during 4 weeks of study. Data are expressed as mean ± SEM (n=4). Results within the same week were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same week are significantly different ($p < 0.05$).

4.5.2 The formation of non-fluorescence AGEs N^{ε}-(carboxymethyl)lysine (N^{ε}-CML)

The level of N^{ε}-CML was measured after 4 weeks of incubation, as shown in Figure 31. According to the present study, the glycated BSA induced by fructose increased the level of N^{ε}-CML with 6.60-folds compared to BSA alone at week 4 of incubation. The addition of ABE (0.25 mg/ml) inhibited the formation of N^{ε}-CML by 35.01%, whereas aminoguanidine (0.25 mg/ml) reduced the N^{ε}-CML level by 28.24%. These results indicated that ABE showed the inhibitory effect of N^{ε}-CML formation similar to AG at the same concentration (0.25 mg/ml).

In the BSA-glucose system, the glucose-induced BSA glycation exhibited a 1.39-folds in the N^{ε}-CML formation compared to non-glycated BSA after 4 weeks of incubation, as shown in Figure 32. The presence of ABE (0.25 mg/ml) decreased the level of N^{ε}-CML by 26.19% in the BSA-glucose system in week 4. Similarly, the addition of AG (0.25 mg/ml) inhibited the formation of N^{ε}-CML by 35.46%. According to these results, ABE exhibited the inhibition of N^{ε}-CML formation similar potent to AG after week 4 of incubation at the same concentration (0.25 mg/ml). Moreover, the formation of N^{ε}-CML in BSA-glucose was lower level than in the BSA-fructose system.

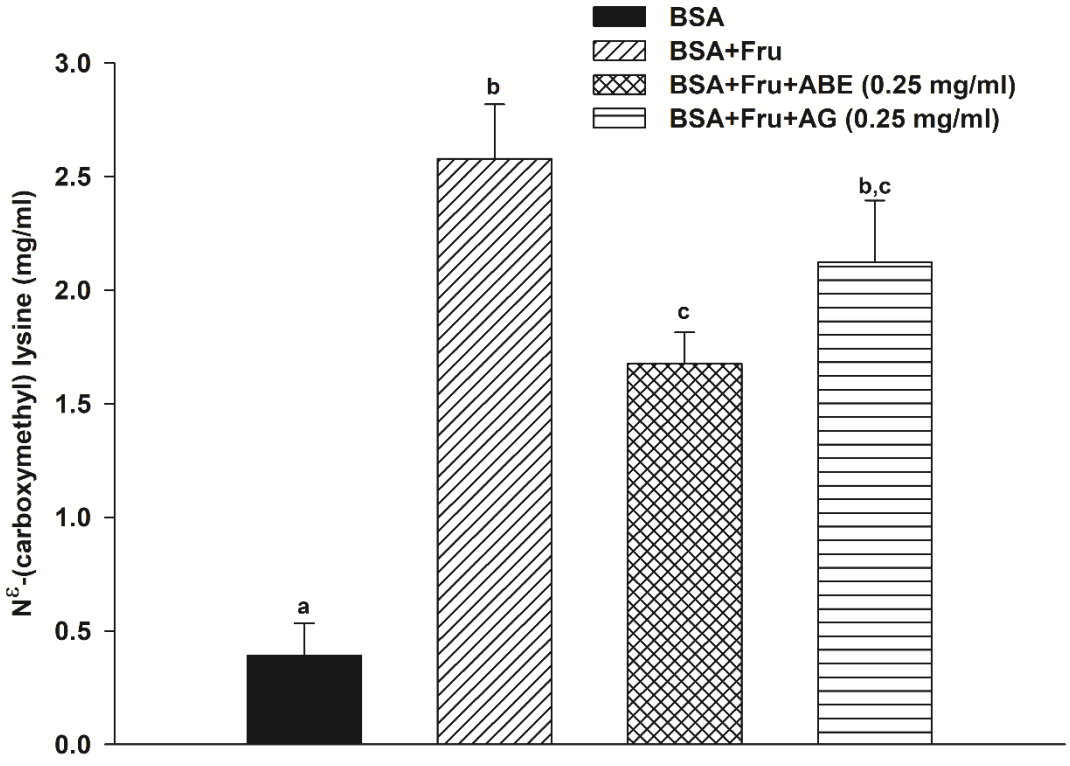


Figure 31 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the formation of N^{ε}-CML in bovine serum albumin (BSA) with fructose (Fru; 0.5 M) as a glycating inducer after 4 weeks of study. Data are expressed as mean ± SEM (n=3). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters are significantly different ($p < 0.05$).

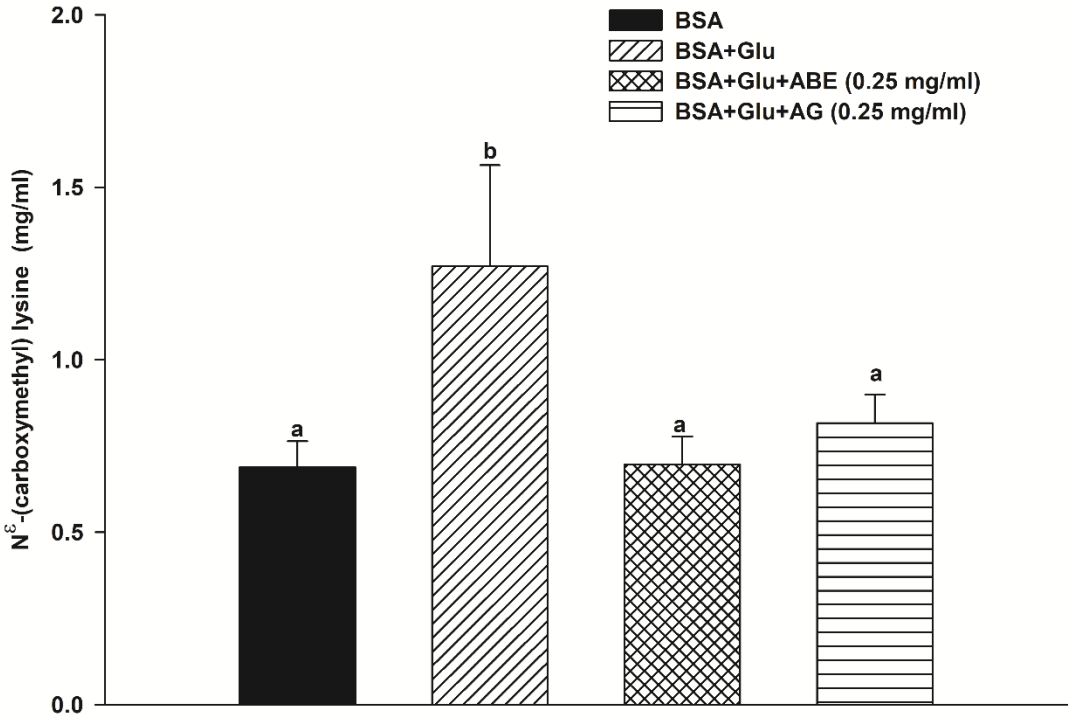


Figure 32 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the formation of N^{ε}-CML in bovine serum albumin (BSA) with glucose (Glu; 0.5 M) as a glycating inducer after 4 weeks of study. Data are expressed as mean ± SEM (n=3). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters are significantly different ($p < 0.05$).

4.5.3 Protein oxidation

The carbonyl content weekly determined the oxidation of protein in BSA glycation induced by reducing sugars. As shown in Figure 33, the carbonyl content of fructose-glycated BSA was between 4.86-folds to 11.78-folds compared to non-glycated BSA throughout the incubation period. The BSA-fructose system containing ABE (0.25 mg/ml) could reduce the protein carbonyl content by 4.60%, 24.80%, 24.67%, and 38.78% at weeks 1, 2, 3, and 4, respectively. Interestingly, the ABE (0.1 mg/ml) exhibited a reducing carbonyl content by 32.19% at week 4 of incubation. Meanwhile, the addition of AG (0.25 mg/ml) decreased protein carbonyl formation by 34.25% in fructose-glycated BSA at week 4. Based on these results, the addition of ABE (0.25 mg/ml) into the BSA-fructose system showed a similar potent as AG (0.25 mg/ml) for the decreasing protein carbonyl content.

The glucose-mediated BSA glycation could increase the protein carbonyl content in ranging 3.52-folds to 9.89-folds comparing with non-glycated BSA during 4 weeks of incubation period that are shown in Figure 34. The glucose-glycated BSA containing ABE (0.25 mg/ml) decreased the protein carbonyl level by 10.12%, 22.77%, 26.65%, and 17.46% at week 1, 2, 3, and 4, respectively. Moreover, AG was added into the BSA-glucose system to reduce the carbonyl content by 29.63% after week 4 of incubation. These findings demonstrated that ABE exhibited less potent than AG on the lowering carbonyl content at the same concentration (0.25 mg/ml).

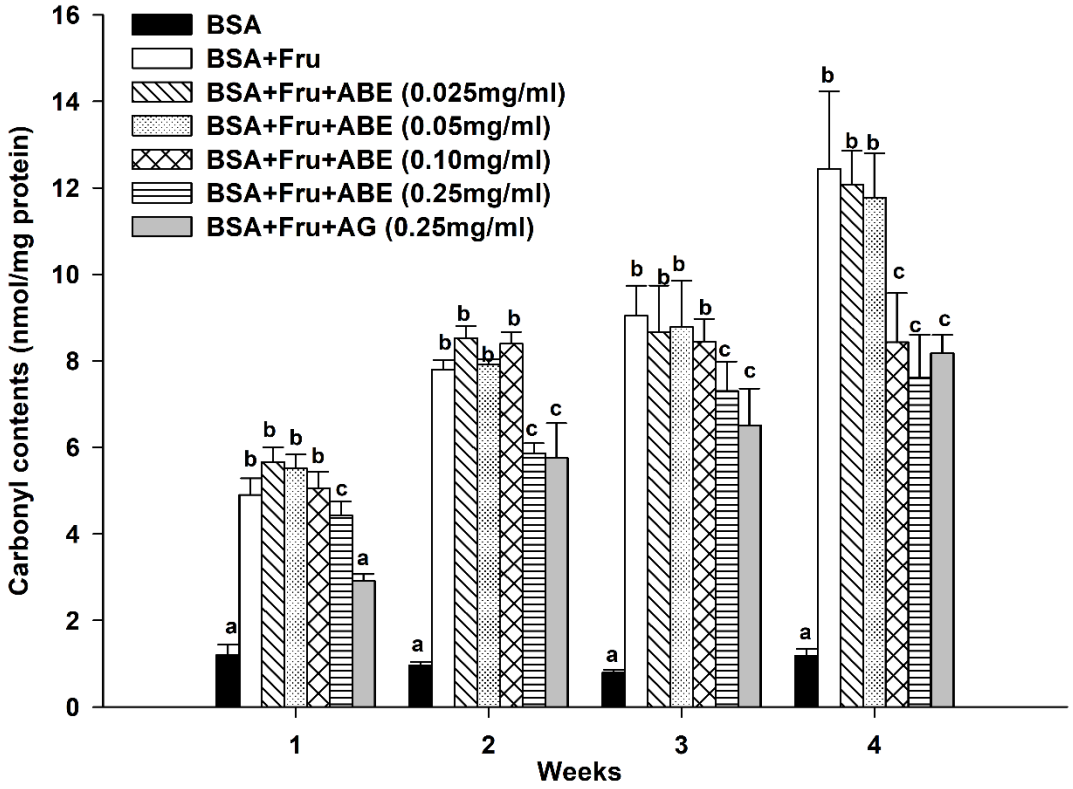


Figure 33 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the protein carbonyl content in bovine serum albumin (BSA) with fructose (Fru; 0.5 M) as a glycating inducer during 4 weeks of study. Data are expressed as mean ± SEM (n=3). Results within the same week were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same week are significantly different ($p < 0.05$).

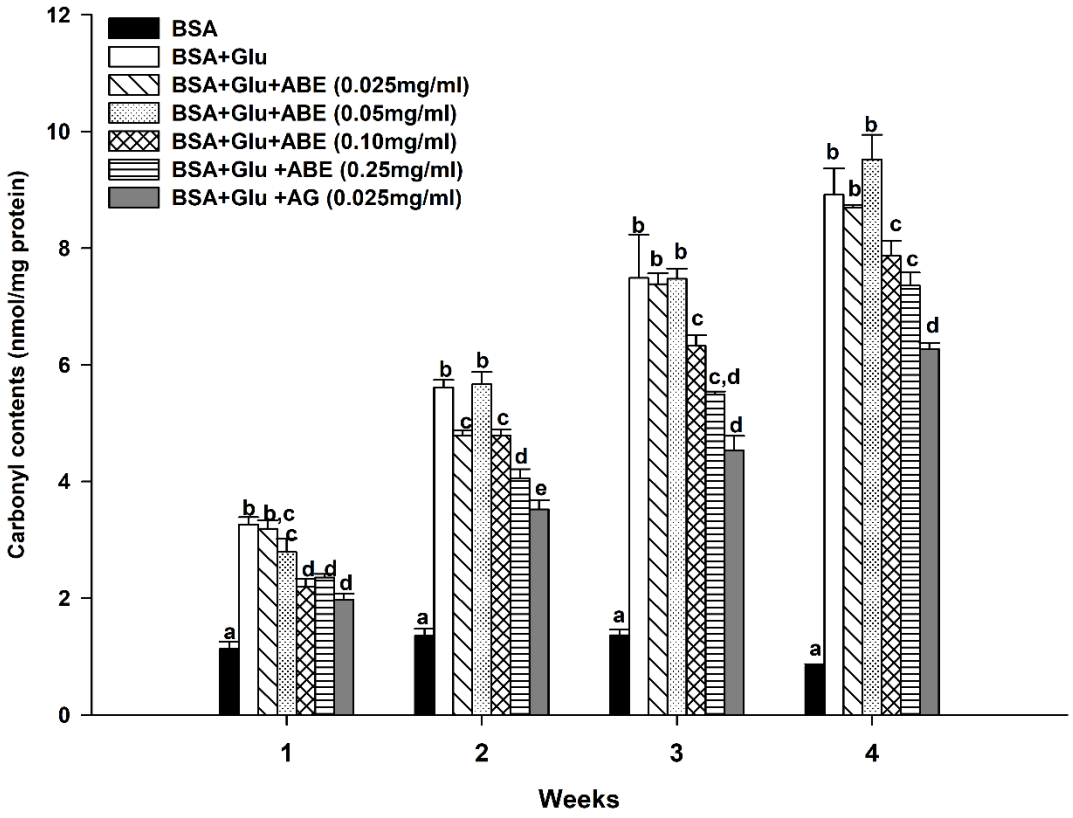


Figure 34 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the protein carbonyl content in bovine serum albumin (BSA) with glucose (Glu; 0.5 M) as a glycating inducer during 4 weeks of study. Data are expressed as mean ± SEM (n=3). Results within the same week were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same week are significantly different ($p < 0.05$).

4.5.4 Protein aggregation

The protein aggregation was determined by the level of β-amyloid cross structure. As shown in Figure 35, the fructose-glycated BSA elevated the β-amyloid conformation in ranging from 1.06-folds to 1.67-folds compared to BSA alone during the incubation period. The present study found that ABE (0.25 mg/ml) altered the formation of β-amyloid cross structure by 38.53% in fructose-induced BSA glycation after week 4 of incubation. In the meantime, AG (0.25 mg/ml) was added into the BSA-fructose system that reduced the amyloid cross- β structure by 23.14% after week 4 of incubation. According to these findings, ABE's inhibitory effect on the amyloid cross-β structure did not significantly differ with AG at the same concentration (0.25 mg/ml) at the end of the investigation.

As shown in Figure 36, the incubation of BSA with glucose raised the level of β-amyloid cross structure between 1.04-folds to 1.21-folds compared with non-glycated BSA during 4 weeks experimental period. At week 4 of incubation, the glucose-glycated BSA incubated with ABE (0.25 mg/ml) attenuated the β-amyloid cross structure formation by 17.91%. Similarly, the decreasing of β-amyloid cross structure was observed in AG (0.25 mg/ml) by 4.29%. However, the reduction of β-amyloid cross structure by ABE (0.25 mg/ml) did not significant difference with AG (0.25 mg/ml).

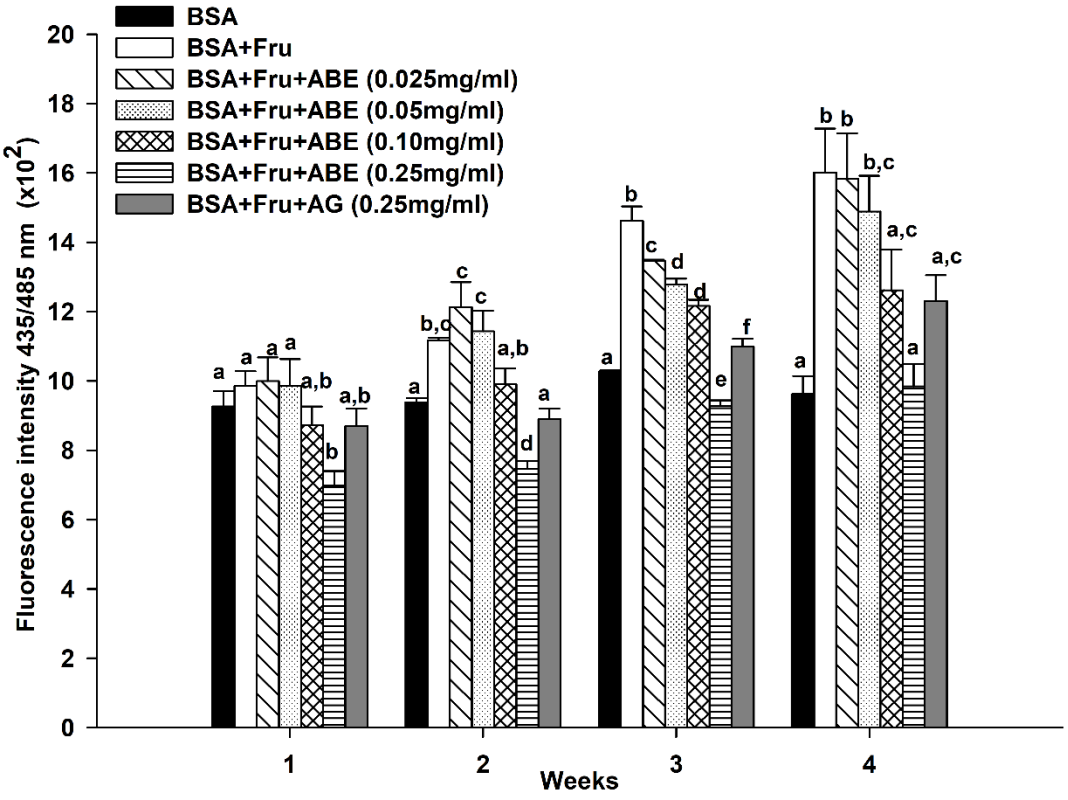


Figure 35 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the formation of β-amyloid cross structure in bovine serum albumin (BSA) with fructose (Fru; 0.5 M) as a glycating inducer during 4 weeks of study. Data are expressed as mean ± SEM (n=3). Results within the same week were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same week are significantly different ($p < 0.05$).

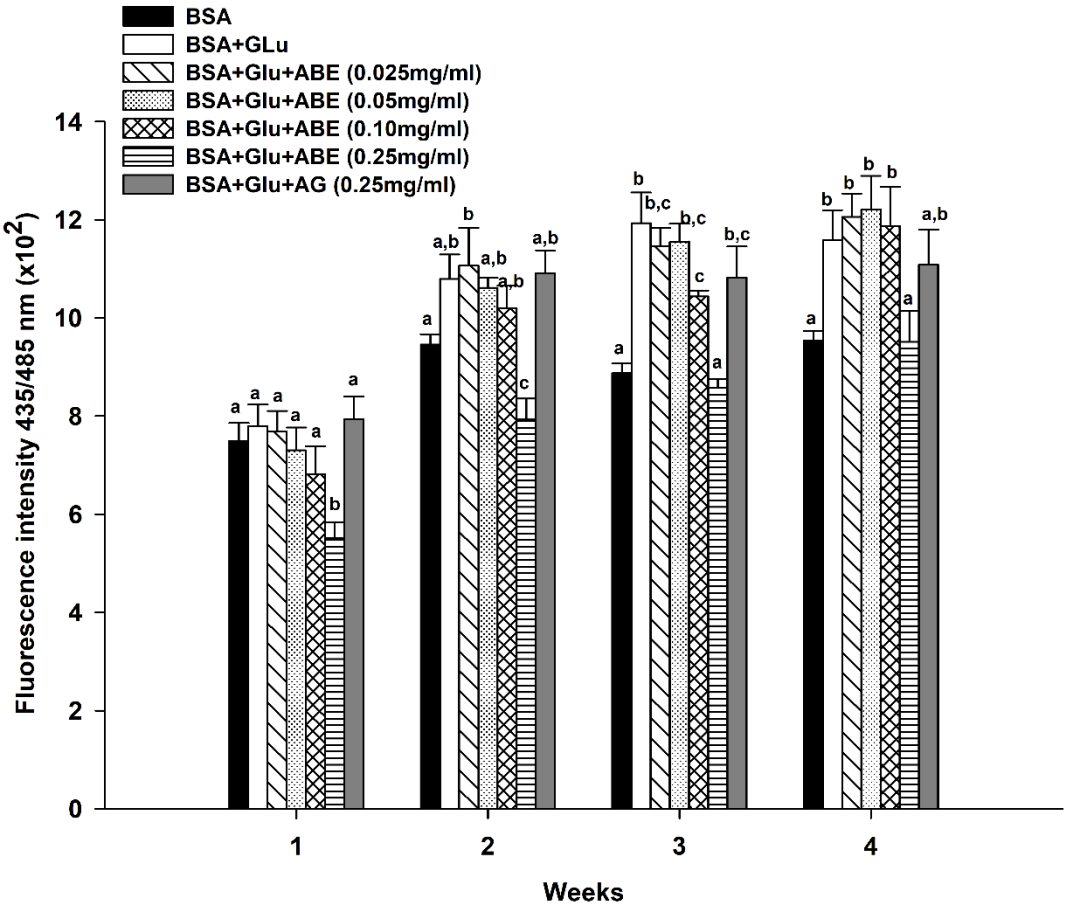


Figure 36 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) and aminoguanidine (AG) on the formation of β-amyloid cross structure in bovine serum albumin (BSA) with glucose (Glu; 0.5 M) as a glycating inducer during 4 weeks of study. Data are expressed as mean ± SEM (n=3). Results within the same week were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same week are significantly different ($p < 0.05$).

4.6 The effect of ABE on adipogenesis *in vitro.*

4.6.1 Cell viability of 3T3-L1 preadipocyte cells

The effect of ABE on the proliferation of 3T3-L1 cells was determined using trypan blue and MTT assay on day 4 after the ABE treatment. As shown in Figure 37, the ABE (12-20 µg/ml) decreased the 3T3-L1 cell number after day 4 of the ABE treatment. The highest concentration of ABE (20 µg/ml) significantly decreased the cell number by 46.05% compared with the control cultures containing medium without the ABE. In addition, the concentration of ABE at 4-16 µg/ml did not significantly difference with the control cultures containing DMEM medium without the ABE (Figure 38). In the meantime, ABE at 20 µg/ml showed the cell toxicity on 3T3-L1 preadipocytes. Therefore, the ABE at a concentration of 4-16 µg/ml was used to study further.

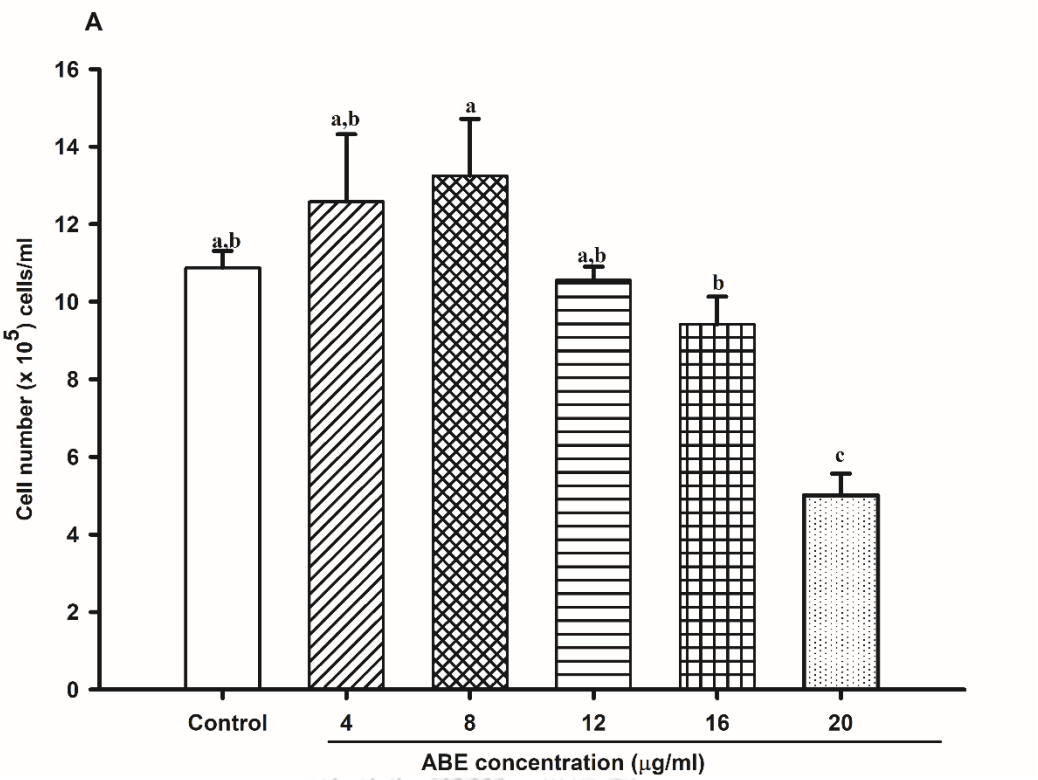


Figure 37 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on the 3T3-L1 preadipocyte cell number by trypan blue assay at day 4 of the ABE treatment (before adipocyte differentiation). Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters are significantly different ($p < 0.05$).

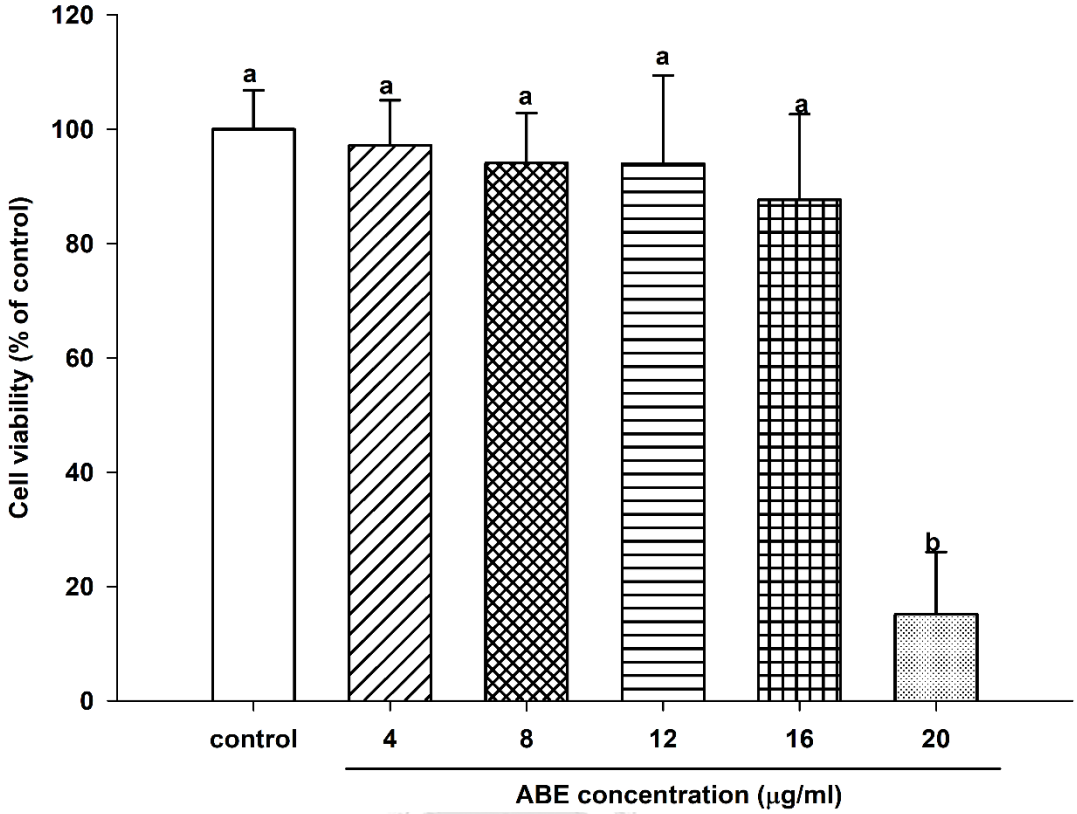


Figure 38 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on the 3T3-L1 preadipocyte cell viability by MTT proliferation assay at day 4 of the ABE treatment (before adipocyte differentiation). Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters are significantly different ($p < 0.05$).

4.6.2 Cell viability of adipocyte cells

After post-confluent 3T3-L1 cells were induced to the adipocyte differentiation using the differentiation medium, the 3T3-L1 cells were treated with differentiation medium containing ABE at various concentrations (4-16 µg/ml). After 10 days of treatment, the cytotoxicity effects of ABE were determined by the cell number using trypan blue assay. As shown in Figure 39, the ABE (4-16 µg/ml) did not reduce the adipocyte cell number throughout the incubation period. These results suggested that ABE (4-16 µg/ml) had no toxicity to adipocyte cells.

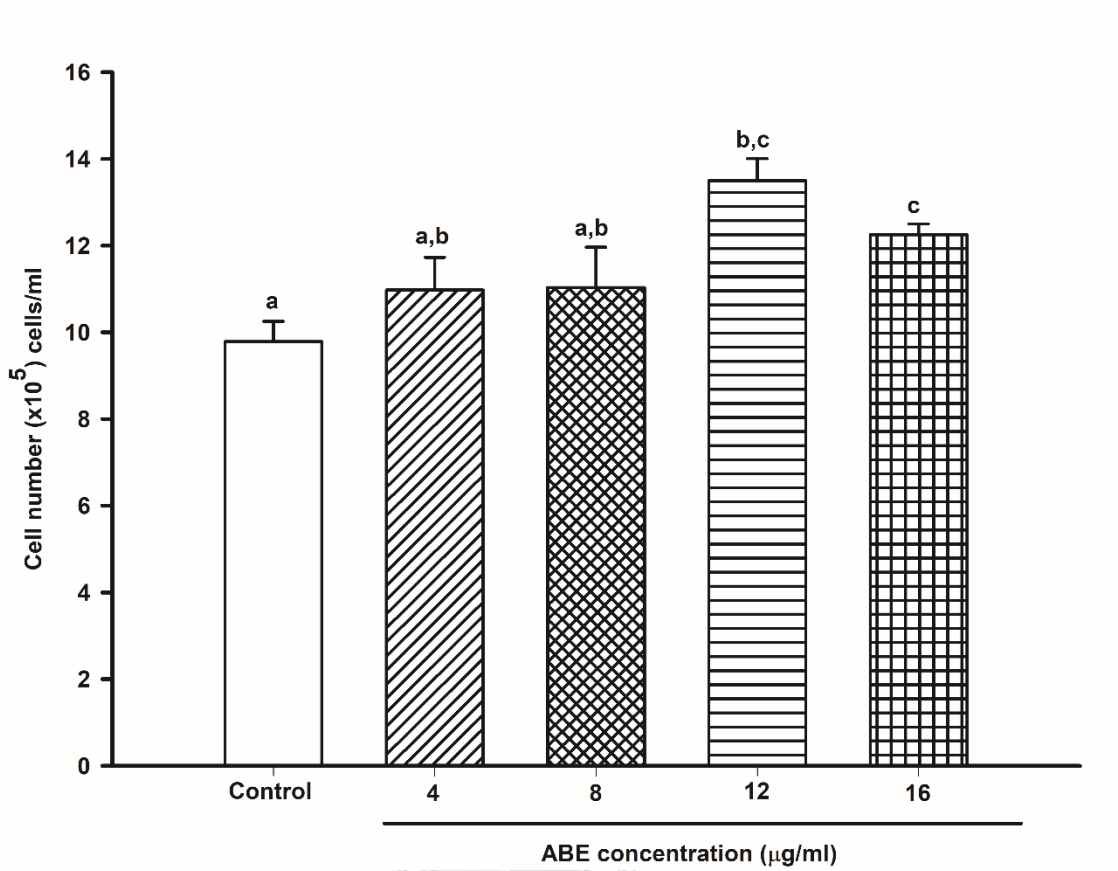


Figure 39 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on the 3T3-L1 cells number by trypan blue assay at day 10 differentiation stage (after adipocyte differentiation). Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$).

4.6.3 Lipid accumulation in differentiated 3T3-L1 cells

The intracellular lipid accumulation was analyzed in adipocyte cells using Oil Red O staining throughout the differentiation period (Figure 40). According to the microscopic results, the 3T3-L1 cell differentiated to adipocytes showed a round shape and consisted of intracellular lipid droplets. Whereas the ABE (16 µg/ml) maintained fibroblast-like cells and had fewer lipid droplets. Moreover, the amount of oil droplets was determined in both adipocyte cells and undifferentiated cells. As shown in Figure 41, the ABE (16 µg/ml) revealed lower lipid droplets by 51.11% compared with differentiated 3T3-L1 cells.

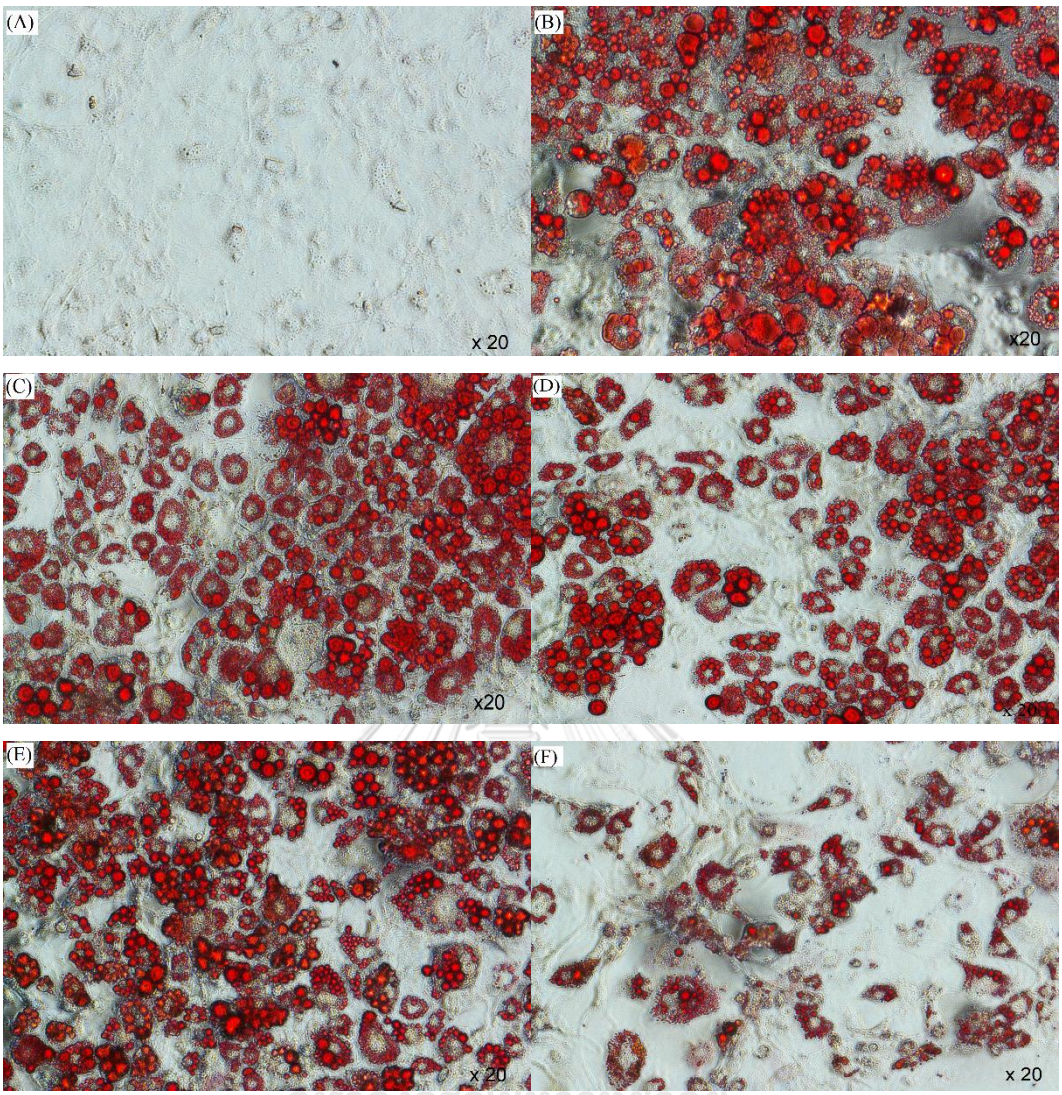


Figure 40 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on intracellular lipid accumulation in the 3T3-L1 cells 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (0-16 µg/ml). After 10 days of differentiation, adipocytes were stained with Oil Red O and photographed under the microscope (20x Magnification). (A): Undifferentiated cells; (B): Differentiated cells, (C): Differentiated cells in ABE 4 µg/ml; (D): Differentiated cells in ABE 8 µg/ml; (E): Differentiated cells in ABE 12 µg/ml; (F): Differentiated cells in ABE 16 µg/ml.

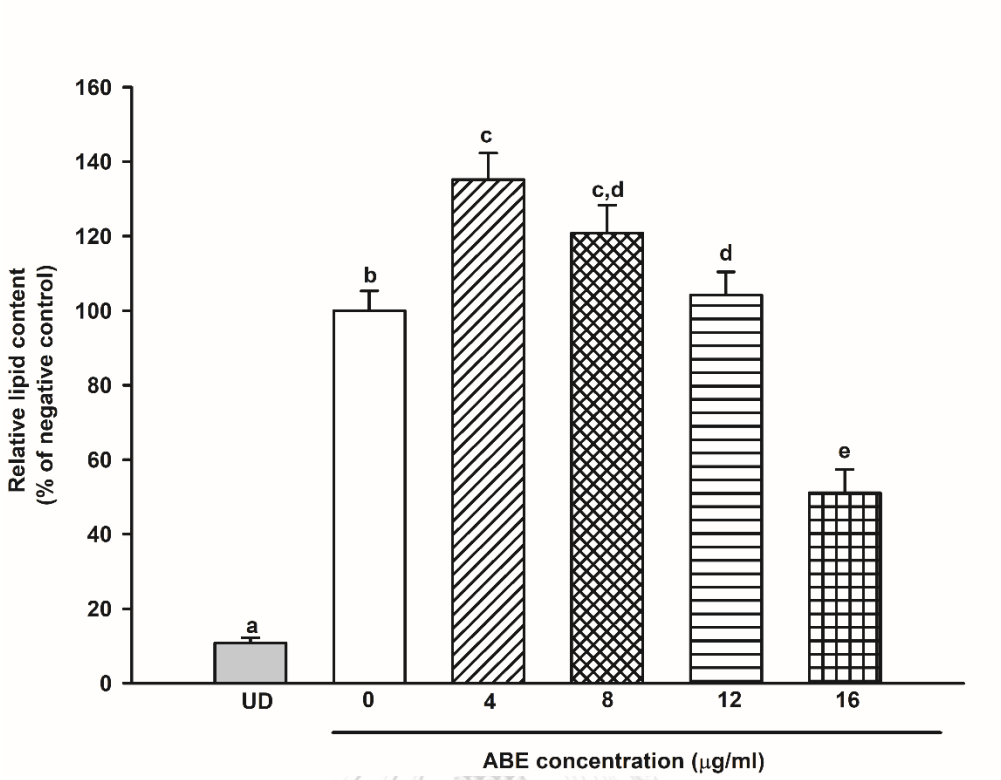


Figure 41 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on relative lipid content in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (0-16 μg/ml). After 10 days of differentiation period, the intracellular lipid droplets were quantified using Oil Red O assay. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

4.6.4 Triglyceride accumulation in differentiated 3T3-L1 cells

The intracellular triglyceride content in 3T3-L1 cells was measured on day 10 of the differentiation period. As shown in Figure 42, ABE at 4-12 µg/ml did not reduce intracellular triglyceride deposition comparing with the adipocytes group without ABE. However, the decreasing triglyceride content was found in the adipocytes in ABE (16 µg/ml) by 21.54% compared to the adipocytes group without ABE.

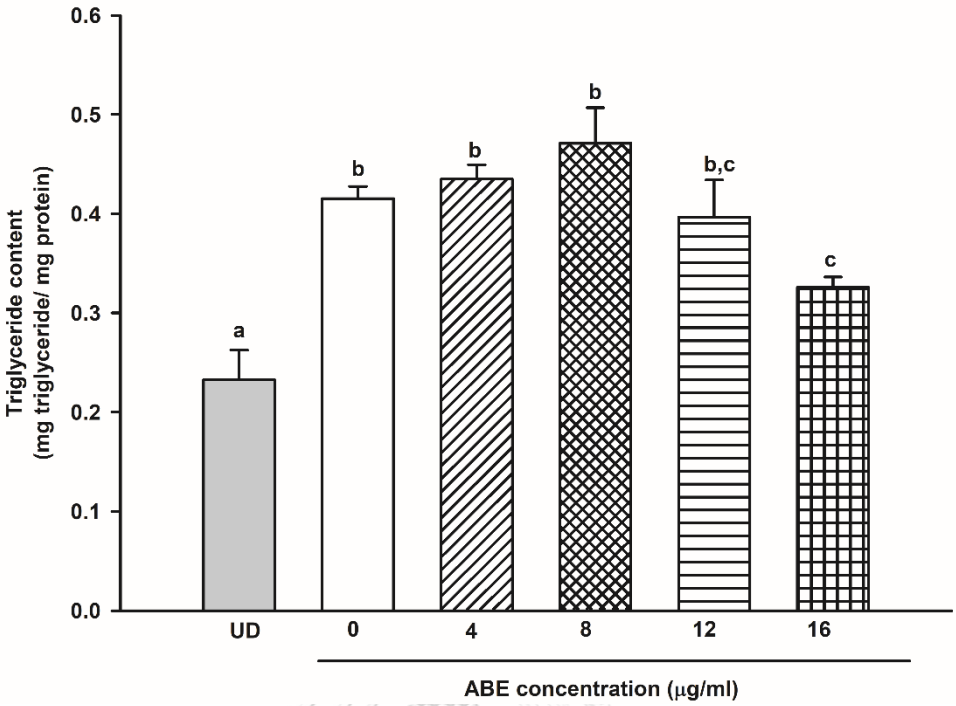


Figure 42 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on intracellular triglyceride content in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (0-16 μg/ml) after 10 days of differentiation. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

4.6.5 Adipogenic genes expression

The adipogenesis relates to the expression of the adipogenic transcription factor (C/EBPα) and PPARγ receptor. Adipogenic genes expression regulate the lipogenic genes to promote adipocyte differentiation. Therefore, the inhibitory effect of ABE on their expression was investigated. As shown in Figure 43-44, the mRNA expression of PPARγ and C/EBPα was significantly increased compared to undifferentiated cells during the differentiation period. In contrast, adipocytes were cultured in the differentiated media with ABE (16 µg/ml) that could reduce the PPARγ and C/EBPα m RNA expression by 63.44% and 54.56%, respectively comparing with the differentiated group without ABE.

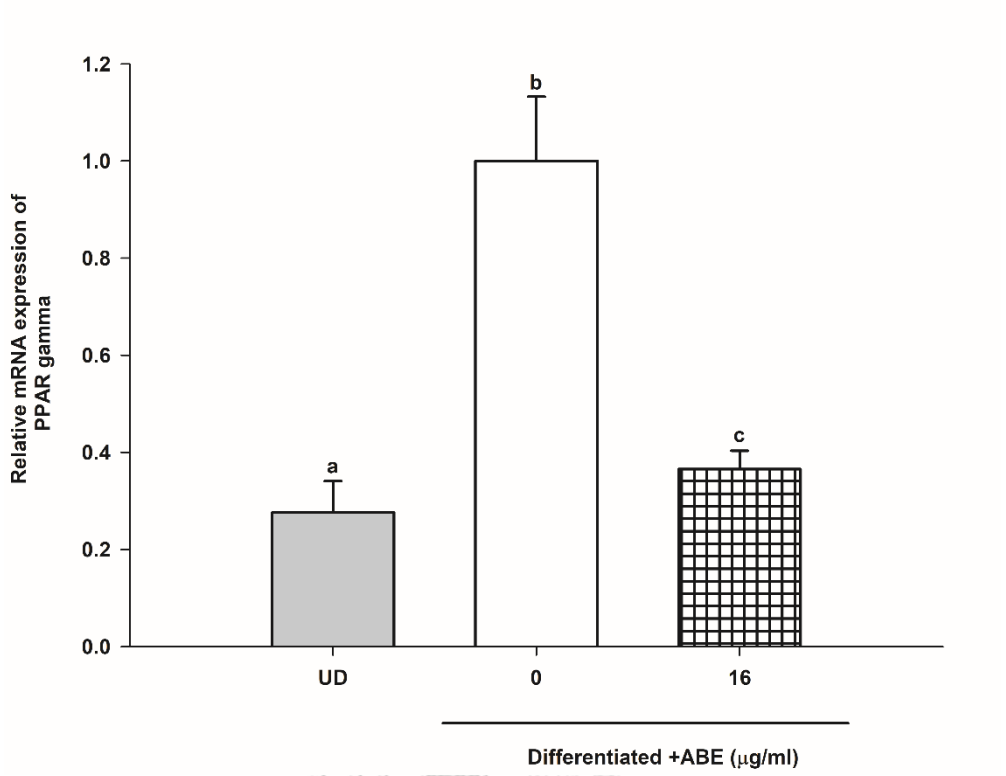


Figure 43 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on mRNA levels of PPARγ in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (16 μg/ml) after 10 days of differentiation. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

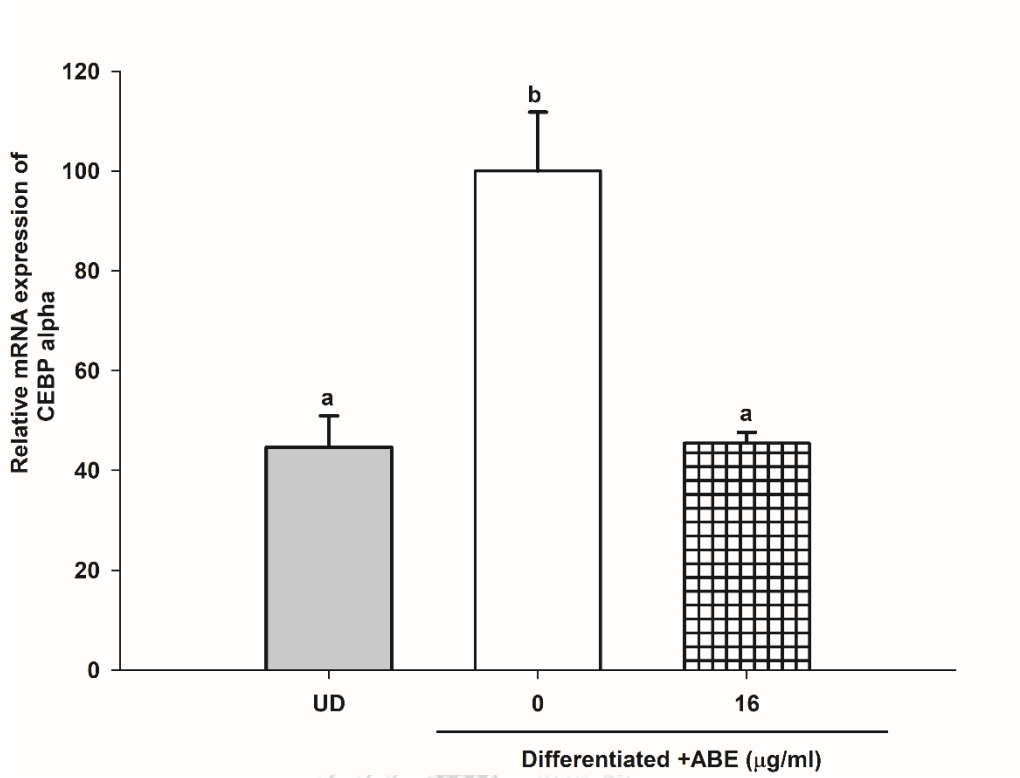


Figure 44 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on mRNA levels of *C/EBPα* in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (16 μg/ml) after 10 days of differentiation. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

4.6.5 Lipogenic genes expression

After the induction of adipocyte differentiation by a differentiation cocktail, the fatty acid and triglyceride synthesis has regulated by adipocyte-specific genes such as fatty acid-binding protein (*FABP/aP2*), acetyl CO-A carboxylase (*ACC*), fatty acid synthase (*FASN*), lipoprotein lipase (*LPL*). The effect of ABE on lipogenesis was investigated on the expression of the lipogenic genes, including *aP2, ACC FASN*, and *LPL*. As shown in Figure 45-48, the mRNA expression of *aP2, ACC FASN*, and *LPL* in the differentiated cells without ABE was higher than the undifferentiated cells after 10 days of the experimental period. Whereas the *ACC, FASN*, and *LPL* genes expression were significantly attenuated in the differentiated cells with ABE (16 µg/ml) by 54.55%, 37.24%, and 58.50%, respectively compared to the differentiated group without ABE. Moreover, ABE slightly decreased the mRNA expression of aP2 by 35.36% comparing with the differentiated group without ABE.

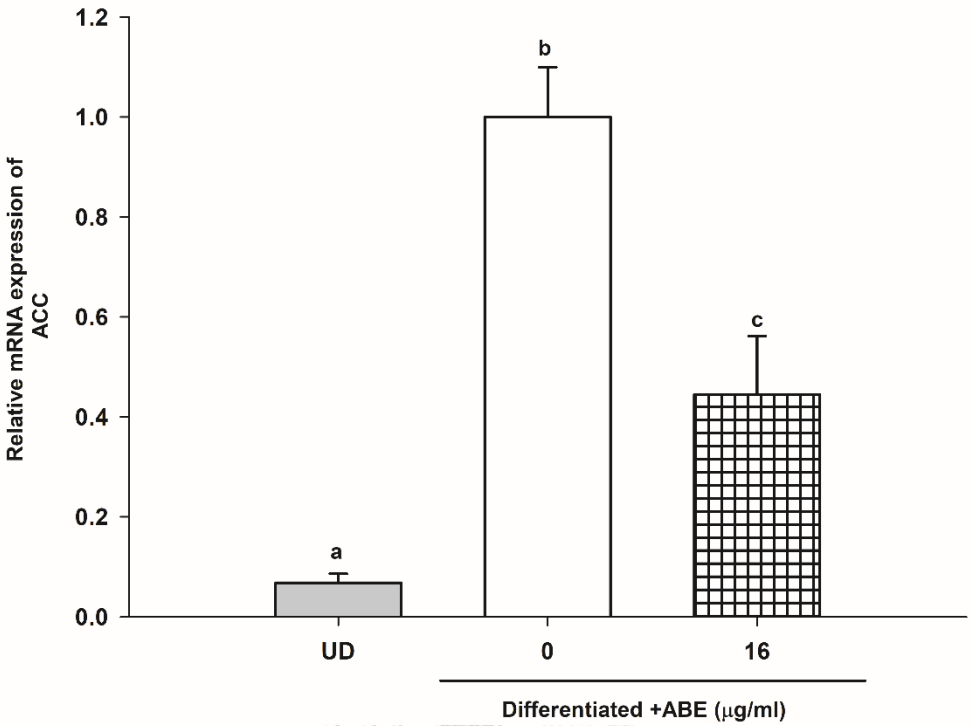


Figure 45 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on mRNA expression of acetyl-CoA carboxylase (ACC) in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (16 μg/ml) after 10 days of differentiation period. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

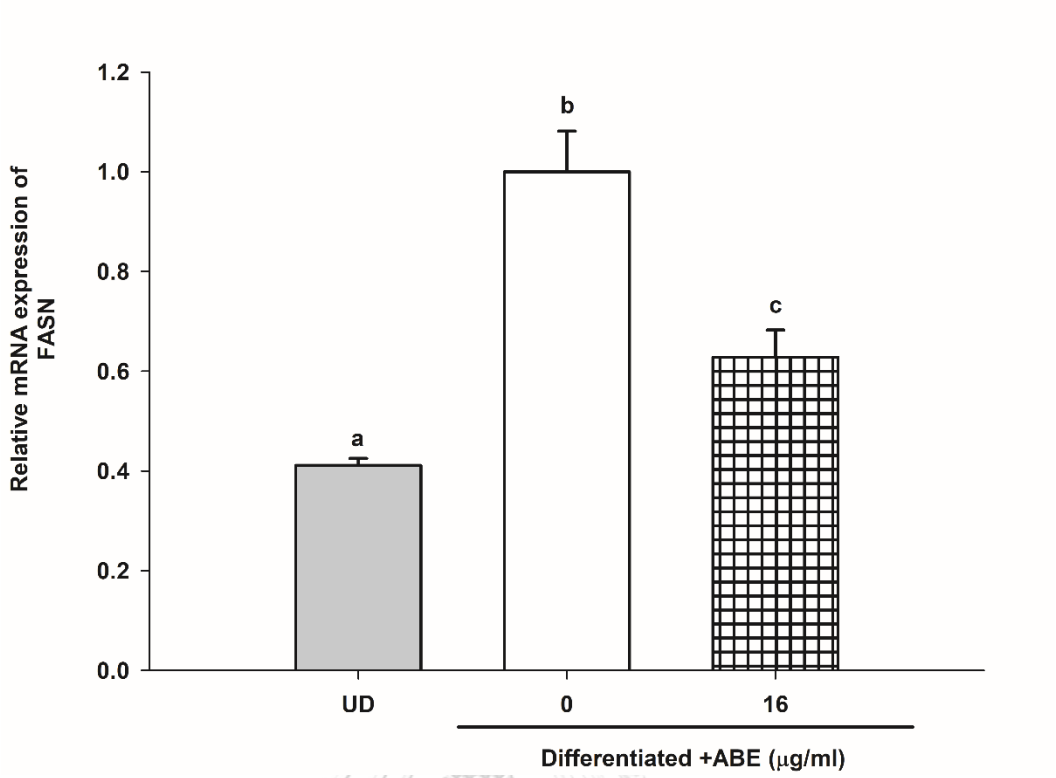


Figure 46 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on mRNA expression of fatty acid synthase (FASN) in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (16 μg/ml) after 10 days of differentiation period. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

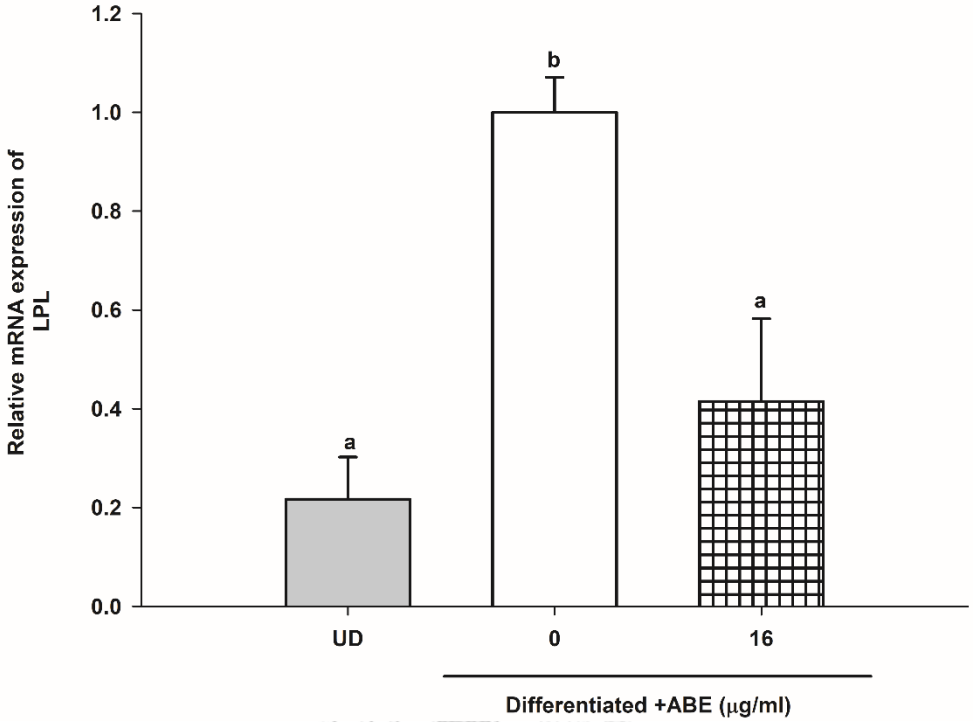


Figure 47 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on mRNA expression of lipoprotein lipase (LPL) in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (16 µg/ml) after 10 days of differentiation period. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

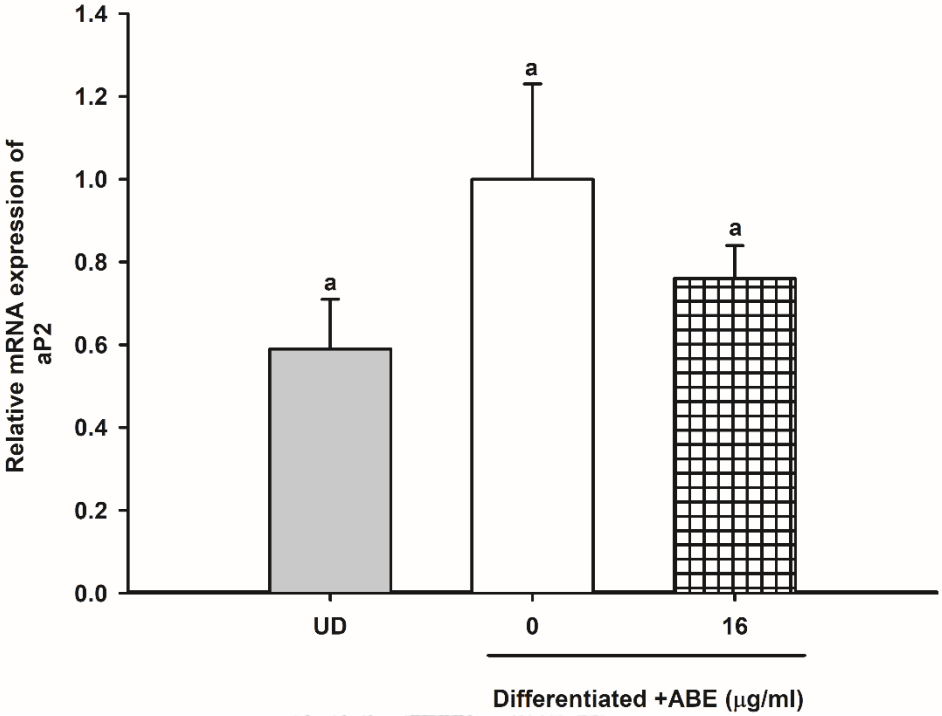


Figure 48 The effect of anthocyanins-rich fraction of *Antidesma bunius* fruit extracts (ABE) on mRNA expression of fatty acid-binding protein (aP2/FABP) in 3T3-L1 cells. 3T3-L1 cells were cultured in adipocyte differentiation cocktail media with ABE (16 µg/ml) after 10 days of differentiation period. Data are expressed as mean ± SEM (n=4). Results were statistically analyzed by one-way ANOVA, following Duncan's multiple range test. The different superscripted letters in the same group are significantly different ($p < 0.05$). UD: Undifferentiated cells.

CHAPTER V

DISCUSSION

5.1 The effect of Thai berry extracts on carbohydrate digestive enzymes and antioxidant activity

Phytochemicals, especially anthocyanins, have been interesting in preventing or delay the progression of diabetes and its complications (Castro-Acosta et al., 2016). A meta-analysis of prospective cohort studies described that dietary anthocyanins and berry fruits had been associated with the reduced risk of type 2 diabetes risk (Guo, Yang, Tan, Jiang, & Li, 2016). The present study showed that the anthocyanins-rich fraction of *Antidesma bunius* (ABE), *Lepisanthes rubiginosa* (LRE), and *Syzygium nervosum* (SNE) contained total phenolic contents were in the range between 237.90-300.91 mg GAE/ g extract. The anthocyanins contents of these extracts were ranging 31.66-39.93 mg C3G/ g extract. According to these findings, the fraction of Thai berry extract contained a higher phenolic and anthocyanin contents than previous studies of ABE with values of 3.11-119.37 mg GAE/ g extract and 69.12 mg/ 100 g extract, respectively (Islary, Sarmah, & Basumatary, 2017; Jorjong et al., 2015) and SNE with values of 5.36-61.09 mg GAE/ g extract and 0.50 mgC3G/ g extract (Poontawee, Natakankitkul, & Wongmekiat, 2016; Sukprasansap et al., 2017). Moreover, Thai berry extracts exhibited a higher phytochemical content than other berries such as Chilean berry, Portuguese wild blackberry, and blueberry with values of 5.11-97.47 mg GAE/ g extract and 0.53-1.54 mg C3G/g extract, respectively (Ramirez et al., 2015; Spínola, Pinto, Llorent-Martínez, Tomás, & Castilho, 2019;

Wang et al., 2012). According to a previous study, the used solid-phase extraction (SPE) in anthocyanin extraction from berries could remove sugar (94.4%) and acid (88.5%) in the fruit extract, resulting in the anthocyanin recovery of more than 90% (Denev et al., 2010). Therefore, this study's obtained data suggest that the SPE system may increase the contents of phenolic and anthocyanin in Thai berry extracts. This study found that Thai berry extracts contained anthocyanins, including cyanidin-3-glucoside and delphinidin-3-glucoside, especially in ABE and LRE. The present study is concomitant with previous studies that found that ABE's major anthocyanins were cyanidin-3-glucoside, following cyanidin-3-rutinoside and malvidin-3,5-diglucoside (Jorjong et al., 2015). Meanwhile, the anthocyanins in SNE included cyanidin-3-glucoside, cyanidin-5-glucoside, and cyanidin-3,5-diglucoside (Charoensin et al., 2012). Similarly, the anthocyanin profile of blueberry and blackcurrant was cyanidin and delphinidin (Hui et al., 2020).

Scientific evidence suggested that the inhibition of pancreatic α-amylase and intestinal α-glucosidase related to blood glucose management (Pałasz et al., 2019; Yan, Zhao, Yang, & Zhao, 2019). One of the well-established antidiabetic mechanisms of anthocyanin-rich berries is the inhibition of pancreatic α-amylase, intestinal α-glucosidase, and glucose absorption (Bell, Lamport, Butler, & Williams, 2017; Castro-Acosta et al., 2016; Guo et al., 2016; Törrönen et al., 2012). The present findings exhibited the inhibitory effects of Thai berry extracts, including ABE, LRE, SNE against intestinal α-glucosidase, including maltase and sucrase, *in vitro*. Specifically, ABE showed the most potent to inhibit intestinal maltase and sucrase. Meanwhile, Thai berry extracts exhibited slightly pancreatic α-amylase inhibition, except for LRE. The previous studies suggested that anthocyanins could inhibit α-

glucosidase activity. For example, the freeze-dried black currant extract containing anthocyanins, including 232 mg/kg cyanidin-3-glucoside (C3G) and 392 mg/kg delphinidin-3-glucoside (D3G), exhibited yeast α-glucosidase inhibition. In contrast, the green currant extract did not show an inhibitory effect on this enzyme (Barik et al., 2020). Moreover, the previous study reported that the individual C3G and D3G at the concentration of 66 μg/ml also inhibited the α-glucosidase activity (Barik et al., 2020). Additionally, C3G was able to inhibit intestinal sucrase and pancreatic α-amylase activities. It showed the synergistic effect with acarbose on intestinal maltase and sucrase and pancreatic α-amylase activities (Akkarachiyasit, Charoenlertkul, Yibchok-Anun, & Adisakwattana, 2010). Similarly, D3G (100μM) exhibited the inhibition of α-glucosidase and α-amylase by 44.5% and 24.2%, respectively (Mojica et al., 2017). Based on a previous study, the delphinidin-3-glucoside showed the most potency of α-glucosidase and α-amylase inhibition and was followed by cyanidin-3-glucoside, cyanidin-3-rutinoside, and malvidin-3-glucoside (Hui et al., 2020). A previous study reported that anthocyanins could inhibit the intestinal α-glucosidase by the competitive inhibition between the glycosyl groups of anthocyanins and the active site of enzymes (Boath, Stewart, & McDougall, 2012). The scientific evidence demonstrates that cyanidin and delphinidin's inhibitory effect on intestinal α-glucosidase may involve the hydrogen bond between hydroxyl groups of their structure and the polar groups on the active site of the enzyme (Akkarachiyasit et al., 2010). There also found that the number of hydroxyl groups in the B ring of anthocyanins associated with an increase of binding affinity to the enzyme (Hui et al., 2020; Zhang et al., 2019). Moreover, the inhibitory effect of C3G and D3G on α-glucosidase and α-amylase also depends on the binding affinity with the enzyme and

their chemical structure (Hui et al., 2020). According to the molecular docking study, the binding of D3G and α-glucosidase or α-amylase depends on the polar interaction, hydrogen bonding, and hydrophobic interaction in the enzyme's catalytic site (Mojica et al., 2017). It is noteworthy that the molecular docking predicted D3G could bind with amino acid residues (Gly402 and Val380) of α-glucosidase through hydrogen bonding. In the meantime, C3G showed the binding affinity with amino residues, including Glu231, Val335, Gly402, and Val380 (Hui et al., 2020). For α-amylase inhibition, D3G showed the binding affinity with the enzyme's amino residues, including His305, His299, Ser163, and Gln63, whereas the binding affinity of C3G and amino residues was His491 and Lys457 (Hui et al., 2020). Therefore, it is hypothesized that Thai berry extracts could inhibit α-glucosidase (maltase and sucrase) and α-amylase through enzymes' interaction, resulting from cyanidin-3-glucoside and delphinidin-3-glucoside as a predominant anthocyanin in extracts.

Chronic hyperglycemia increases the free radicals production leading to accelerating the progression of diabetes and its complications (Sancho & Pastore, 2012). Previous studies indicated that the diminished free radical generation could prevent oxidative stress related to the progression of diabetes and its complications (Sancho & Pastore, 2012; S. Y. Wang et al., 2012). In the current study, Thai berry extracts demonstrated the antioxidant activity indicated by DPPH scavenging activity, TEAC, and FRAP, similar to previous studies (Jorjong et al., 2015; Sukprasansap et al., 2017). Moreover, the scavenging activity of superoxide and hydroxyl radicals was found in Thai berry extract. Previous studies suggested that berries such as blueberry, blackberry exhibited antioxidant activity (Spínola, Pinto, et al., 2019; S. Y. Wang et al., 2012). In addition, the antioxidant activity of blueberry extract correlated to

anthocyanin in extract (S. Y. Wang et al., 2012). A previous study described that 10 μM of C3G or D3G scavenged DPPH radicals with the percentage of inhibition of 32% and 42%, respectively (Kähkönen & Heinonen, 2003). The scientific evidence demonstrates that the hydroxylation and methoxylation in the B ring of their structure impact the ability of C3G and D3G for DPPH radicals scavenging. The increased hydroxyl groups in the B ring could increase the radical scavenging activity indicating that delphinidin decreased free radicals greater than cyanidin (Rice-Evans, Miller, & Paganga, 1996). Additionally, the highest value of ferric reducing ion power among anthocyanins was found in cyanidin-3-glucoside and delphinidin-3-glucose, following petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside. The reducing capacity is influenced by the pyrogallol or catechol type on B rings of their structure (Jordheim, Aaby, Fossen, Skrede, & Andersen, 2007).

The anthocyanin-riched fraction of Thai berry extracts, including ABE, LRE, and SNE, were evaluated for their potential to prevent or delay the progression of diabetes and its complications by improving postprandial glucose level and decreasing oxidative stress *in vitro* study. According to our findings, these Thai berry extracts could inhibit intestinal α-glucosidase, including maltase and sucrase, and reduce the free radicals generation, as shown in Figure 49. These effects might result from cyanidin-3-glucoside and delphinidin-3-glucoside, which are a major anthocyanin in Thai berry extracts.

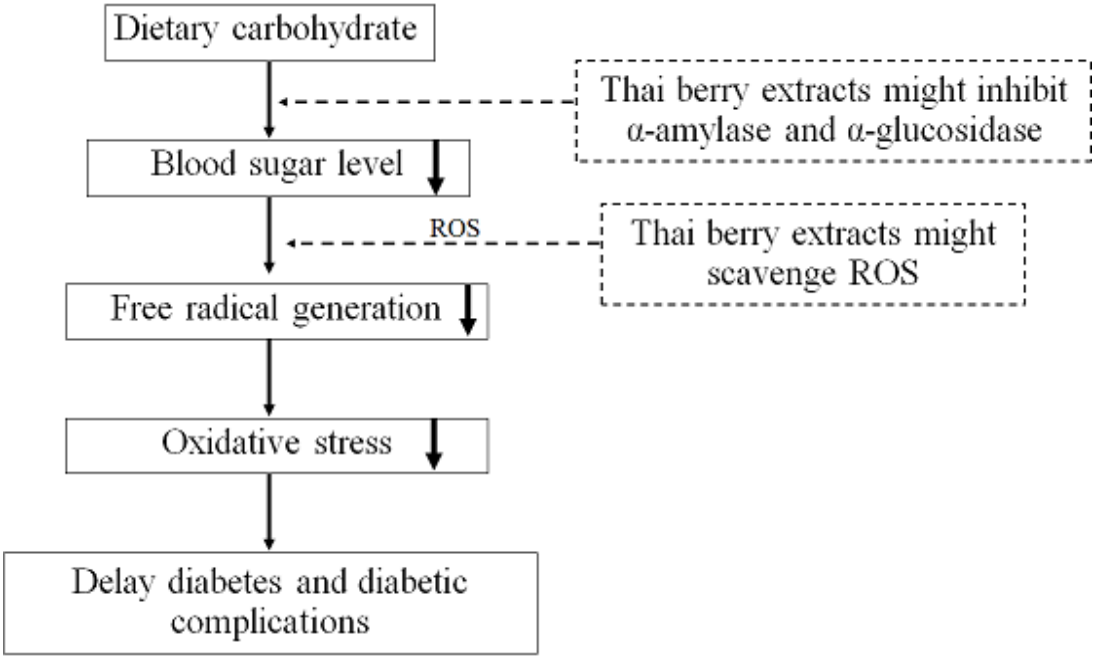


Figure 49 The proposed mechanism of the anthocyanin-rich fraction of Thai berry extract on the carbohydrate digestive enzyme inhibition and antioxidant activity.

5.2 The effect of ABE on protein glycation in BSA-induced by monosaccharides including fructose and glucose *in vitro*

Recently, berry fruits have been reported to prevent protein glycation (Ma et al., 2018). The anti-glycation of berries might relate to their phenolic compounds and antioxidant activity (C. S. Harris et al., 2014). The obtained data from LC-MS/MS revealed that ABE contained phenolic compounds including phenolic acids (quinic acid, gallic acid, and ellagic acid), flavonols (quercetin-3-galactoside, kaempferol-3-glucoside, and myricetin-3-galactoside), and anthocyanins (delphinidin-3-glucoside, cyanidin-3-sambubioside, and cyanidin-3-glucoside). These results are similar to previous studies that found a methanolic extract of ABE contained phenolic compound such as quercetin (4.06 mg/ 100 g DW), myricetin (3.16 mg/ 100 g DW), Kaempferol (0.16 mg/ 100 g DW), gallic acid (159.47 mg/ 100 g DW), cyanidin (58.56 mg/ 100 g DW), delphinidin (0.86 mg/ 100 g DW) (Jorjong et al., 2015).

Chronic hyperglycemia spontaneously modifies circulating and structural proteins through induction of protein glycation, leading to advanced glycation end productions (AGEs). Glycation causes protein modification and increases oxidative stress-induced protein damage, resulting in microvascular and macrovascular diabetic complications (Rhee & Kim, 2018). In general, AGEs can be categorized by their chemical structures into two main groups: fluorescent and cross-link AGEs (e.g., pentosidine, crossline, and methyl glyoxal-lysine dimer or MOLD) and non-fluorescent and non-crosslink AGEs (e.g., N^{ε}-(carboxymethyl lysine) or CML, N^{ε}-(carboxyethyl lysine) or CEL, and pyrraline) (Wu et al., 2011). The present study

demonstrated that fructose has a higher reactivity rate than glucose to form protein glycation, similar to previous studies. According to previous studies, the order of glycating activity was D-ribose > D-fructose > D-glucose (A. Meeprom, W. Sompong, C. B. Chan, & S. Adisakwattana, 2013; Thilavech et al., 2015). Monosaccharides-mediated protein glycation's reaction rate is related to their structures, indicating ketose sugar has more reactive than aldose sugar. Fructose has a more open-chain structure than glucose (Bunn & Higgins, 1981; Ramkissoon et al., 2016), leading to an increase in the higher reaction rate about 10 times than glucose (Suárez et al., 1989). In the present study, ABE (0.25 mg/ml) decreased the formation of fluorescent AGEs and the level of N^{ε}-CML in fructose and glucose-glycated BSA throughout the study period. Furthermore, ABE (0.25 mg/ml) exhibited a similar effect on reducing AGE formation as aminoguanidine (0.25 mg/ml) in the fructose system. Similar to a previous study, the anthocyanins-enriched extracts of berries including blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry exhibited a higher anti-glycation activity than anthocyanins-free of these plants (Ma et al., 2018). It is indicated that anthocyanins of these berry extracts were the main active anti-glycating agent to reduce the protein glycation process (Ma et al., 2018). The previous study demonstrated that cyanidin could reduce all phases of AGEs formations (Suantawee et al., 2015). The anthocyanin effect on anti-glycation may involve the anthocyanin bind with albumin at the hydrophobic cavity in site II (subdomain IIIA) that is the same binding site of aminoguanidine (Shi, Wang, Zhu, & Chen, 2014; Suantawee et al., 2015). According to a previous study, C3G prevented the lysine and arginine residues of the protein, leading to reduced fructosamine formation and BSA glycation (Prasanna & Jing, 2018). Interestingly, the molecular

docking indicated that C3G exhibited competitive inhibition on BSA glycation through the hydrogen bonding with Glu186, Arg427, Ser428, Lys431, Arg435, and Arg458. Moreover, C3G also reduced the BSA glycation by hydrophobic interaction with Leu189 and Ile455 (Prasanna & Jing, 2018).

The reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide are generated via the oxidation of Schiff bases or Amadori products during the early stage of the glycation process (Mossine et al., 1999; Smith & Thornalley, 1992). Moreover, the interaction of AGEs and its receptor also modulates ROS production through the MAPK pathway (Vlassara & Striker, 2013). The ROS oxidizes the thiol group of amino acid cysteine and methionine in protein molecules, resulting in the loss of thiol groups and enzyme inactivity (Acimovic, Stanimirović, & Mandic, 2009; Zeng & Davies, 2006). Besides, the oxidation of amino acids (Arg, Lys, and Thr) increases protein carbonyl content (Dalle-Donne, Giustarini, Colombo, Rossi, & Milzani, 2003). The increasing carbonyl content and decreasing have been used as a marker of protein oxidation (A. Meeprom et al., 2013). Our findings found that the fructose and glucose-mediated BSA glycation continuously increased protein carbonyl levels throughout the experimental period. Previous studies showed that edible plants containing phenolic compounds reduced the protein oxidation in glycated BSA. For example, *Mesona chinesis* extract (0.5-1 mg/ml) decreased the protein carbonyl content in fructose-mediated BSA glycation by 36.2%-46.7% (Adisakwattana, Thilavech, & Chusak, 2014). The flower petal extract of *Clitoria ternatea* containing anthocyanins and phenolic compounds prevented the formation of protein carbonyl by 8.23%-11.34% at the concentration of 0.25-1 mg/ml (Chayaratanasin et al., 2015). In addition, cyanidin (0.125-1 mM) could reduce the

protein carbonyl content in glucose-induced BSA glycation by 9.1-21.2% (Suantawee et al., 2015). The obtained data from the present study, ABE reduced the level of protein carbonyl content in glycated BSA throughout the experimental period. Several studies support that the free radical scavenging activity of flavonoids may be the mechanism for glycation inhibition and the reduction of protein carbonyl content (Jariyapamornkoon et al., 2013; Ramkissoon et al., 2016; Thilavech et al., 2015). Flavonoids could scavenge free radicals produced from the interaction between reducing sugars and protein during the Schiff bases oxidation, leading to blocking the formation of protein glycation and oxidation (Spínola, Llorent-Martínez, et al., 2019).

During the protein glycation, the monosaccharide-mediated BSA glycation induced the change of helical structure of BSA to the β-sheet structure, resulting in the formation of insoluble proteins, known as amyloids (Prasanna & Jing, 2018; Wei, Chen, Chen, Ge, & He, 2009). The previous study has reported that the protein glycation decreased the secondary α-helix structure of BSA and increased the β-sheet structure formation (Joglekar, Panaskar, Chougale, Kulkarni, & Arvindekar, 2013). The formation of the cross-β structure may involve two pathways, including (i) the covalent bonding between carbohydrate and amino acid residues (Lys and Arg) leading to unfolding protein, (ii) the intramolecular or intermolecular AGE-crosslinks resulting in the local or global unfolding of protein (Bouma et al., 2003). Consequently, the unfold polypeptides rearrange to form the amyloid fibrils (Bouma et al., 2003). Our findings demonstrated that fructose-induced the formation of the β-amyloid cross structure higher than glucose, whereas ABE could reduce the β-amyloid cross structure level. A previous study revealed that C3G could decrease amyloid fibril level during the protein glycation (Prasanna & Jing, 2018). According

to the molecular docking study, C3G entered the hydrophobic cavity in the subdomain IIA of BSA.

Moreover, C3G interacted with BSA through the Van der Waals force and hydrogen bonding, preventing the BSA secondary structure (Shi et al., 2014). In addition, the inhibitory effect of anthocyanin glycoside on amyloid formation is involved in the interaction between the aromatic ring of anthocyanins and BSA (Rivière et al., 2008). Therefore, it could be hypothesized that ABE may help preserve BSA structure, resulting in reduced fructose- and glucose-induced AGE and β-amyloid formation. The decreasing of the β-amyloid cross structure is helpful to prevent or delay the progression of diabetic complications.

The obtained data from these findings indicate the proposed mechanism of anthocyanin-rich fraction of *Antidesma bunius* (ABE) on monosaccharide-induced protein glycation in the BSA model in Figure 44.

1) ABE might interact with amino residues of BSA, resulting in the prevention of AGEs formation and protein aggregation.

2) ABE might scavenge ROS leading to the decreasing Amadori products and dicarbonyl compounds. Moreover, the antioxidant activity of ABE might reduce the protein carbonyl content and protein-cross β structure.

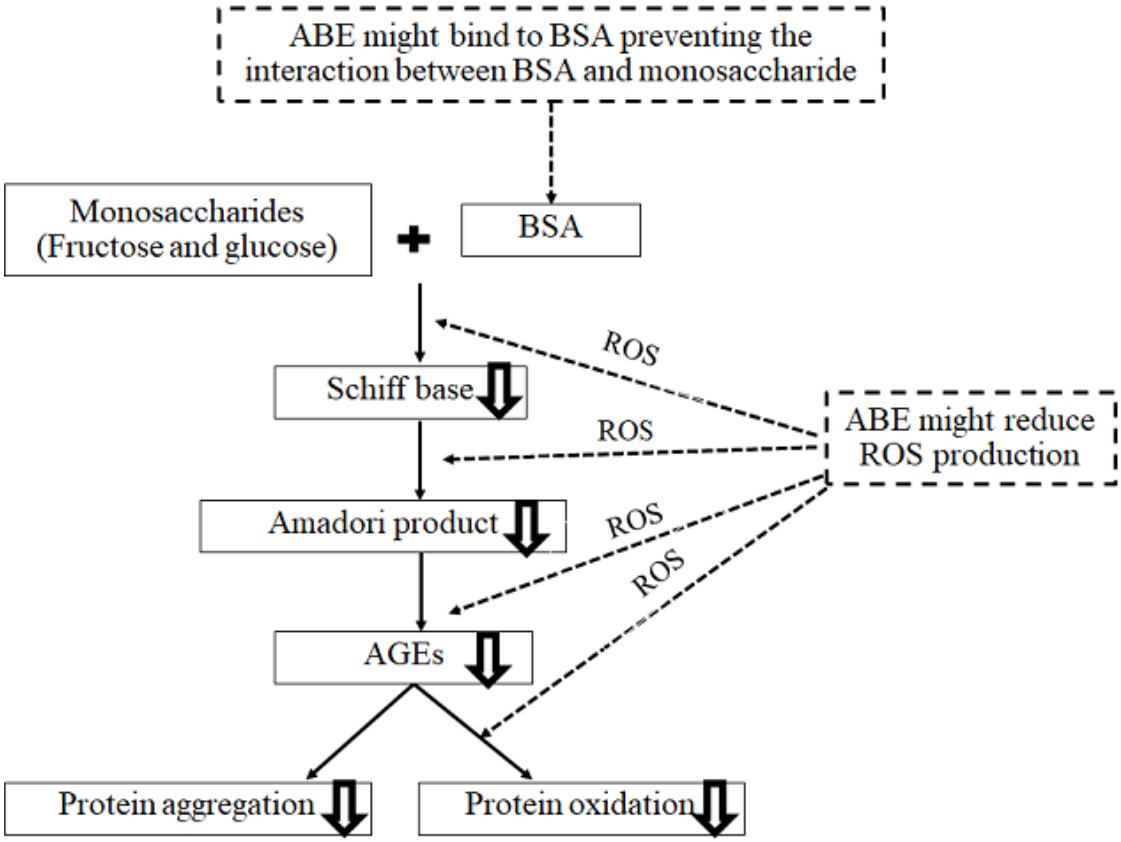


Figure 50 The proposed mechanism of the anthocyanin-rich fraction of *Antidesma bunius* extract (ABE) on protein glycation mediated by monosaccharides (fructose and glucose) in bovine serum albumin (BSA) model.

5.3 The effect of ABE on adipogenesis in 3T3-L1 preadipocytes *in vitro*

Obesity is a metabolic disorder and it can induce insulin resistance leading to the progression of type 2 diabetes (Matsukawa et al., 2015). Obesity is the lipid deposition in adipose tissue by adipogenesis, which involves the differentiation of preadipocytes (fibroblast-like shape) into fat storage cells (Jeong et al., 2013). Many therapeutic approaches have been used to treat obesity, including the balancing between energy intake and expenditure, inhibiting adipocyte differentiation and lipogenesis, the regulation of lipolysis, and the induction of adipocyte apoptosis (K. K. Li et al., 2016). Adipogenesis is regulated by PPARγ and C/EBPα that are the major adipogenic transcriptional factors. C/EBPα could induce PPARγ, which similar to PPARγ could induce C/EBPα, then increase their expression (Jeong et al., 2013). The expression of PPARγ and C/EBPα continuously increases from the preadipocytes to adipocytes during the adipocyte differentiation (White & Stephens, 2010). Our current study demonstrates that ABE (16 μg/ml) might decrease the expression of PPARγ and C/EBPα. According to previous studies, cranberry-containing anthocyanin reduced the gene expression of PPARγ and C/EBPα (Kowalska et al., 2014). Similarly, anthocyanins from grape skin (40μg/ml) decreased PPARγ and C/EBPα in both mRNA and protein expression (B. Lee, M. Lee, M. Lefevre, & H. R. Kim, 2014). Besides, it has been reported that anthocyanidin-enriched bilberry extracts decreased the PPARγ and C/EBPα mRNA levels. Moreover, it found that delphinidin (50 μM) exhibited the most potential in suppressing PPARγ and C/EBPα

expressions than others, including pelargonidin, cyanidin, peonidin, and malvidin (Rahman et al., 2016). The effects of C3G on adipogenesis found that C3G decreased lipid accumulation and reduced the PPARγ gene expression (Jang et al., 2015).

The expression of *PPARγ* and *C/EBPα* mediates the adipogenesis-related genes, including fatty acid-binding protein (*aP2*), fatty acid synthase (*FASN*), acetyl-CoA carboxylase (*ACC*), and lipoprotein lipase (*LPL*) (Li et al., 2016). *FASN* and *ACC* are important enzymes for *de novo* fatty acid synthesis. ACC catalyzes the conversion of acetyl-CoA into malonyl-CoA, which is a precursor for fatty acid (Li et al., 2016). *FASN* catalyzes the saturated long-chain fatty acids from the acetyl CoA and malonyl-CoA in the presence of NADPH (Fan, Wu, Tian, & Ma, 2013). *LPL* catalyzed the triglyceride hydrolysis in circulating lipoprotein to produce free fatty acids enter to adipocytes. (Goldberg, 1996). Moreover, *aP2* is a cytoplasmic fatty acid-binding protein involving fatty acid uptake (Aranaz et al., 2019; Jeong et al., 2013). As mention above, these genes expression led to triglyceride synthesis and lipid accumulation in adipocytes. The present findings exhibited that ABE (16 µg/ml) significantly decreased the expression of the lipogenic genes, including *ACC, FASN,* and *LPL*, and slightly reduced the expression of *aP2*. These findings are consistent with Oil Red O staining and triglyceride accumulation in adipocyte cells. Previous studies have been reported that anthocyanins-rich plants showed inhibitory effects on adipogenesis. *Cliteria ternetea* flower extract inhibited the late stage of adipocyte differentiation by suppressing lipogenic gene expression, including *FASN* and *ACC* (Chayaratanasin et al., 2019). The anthocyanin-enriched Rice berry rice extract exhibited the downregulation of *aP2, ACC,* and *LPL* m RNA expressions (Kongthitilerd et al., 2020).

Furthermore, the other phytochemicals have been reported for adipogenesis inhibition. Kaempferol reduced the fat accumulation by suppressing the protein expression of *PPARγ, C/EBPα*, *aP2*, and *FASN* during adipocyte differentiation in 3T3-L1 cells (Lee et al., 2015). The molecular docking demonstrated that kaempferol could bind with amino residue Cys285, Arg288, and Leu333 of the ligand-binding domain of *PPARγ* (Aranaz et al., 2019). The comparative inhibitory effect of phenolic acids on adipogenic gene expression demonstrated that ellagic acid, ferulic acid, and gallic acid inhibited the *FASN* gene expression, meanwhile p-coumaric acid reduce the *LPL* and *aP2* genes expression. Besides, these phenolic acids inhibited the gene expression of *PPARγ* (Aranaz et al., 2019). Myricetin exhibited decreasing lipid accumulation by reducing mRNA levels of adipogenic transcription factor (*PPARγ* and *C/EBPα*) and lipogenic genes, including *aP2* and *LPL* (Q. Wang, Wang, Yang, You, & Zhang, 2015). The purified extract of *Hibiscus sabdariffa* could increase the inhibitory effect of phenolics mixture on adipogenesis compared to the whole extract. Besides, the purified extract of *Hibiscus sabdariffa* could reduce triglyceride accumulation in mature adipocytes greater than the sum of its fraction, indicating the synergistic effect of the plant containing phenolics is an important factor to prevent adipogenesis (Herranz-López et al., 2012).

According to our findings, anthocyanin-riched ABE has assessed the potential of the anti-obesity *in vitro* model. The proposed mechanism of ABE for preventing obesity is shown in Figure 51.

1) ABE might inhibit adipocyte differentiation through the decreasing major transcription factors, including *PPARγ* and *C/EBPα*.

2) ABE might reduce fat storage in adipocytes by inhibiting lipogenic gene expression, including *ACC, FASN, LPL,* and *aP2*.

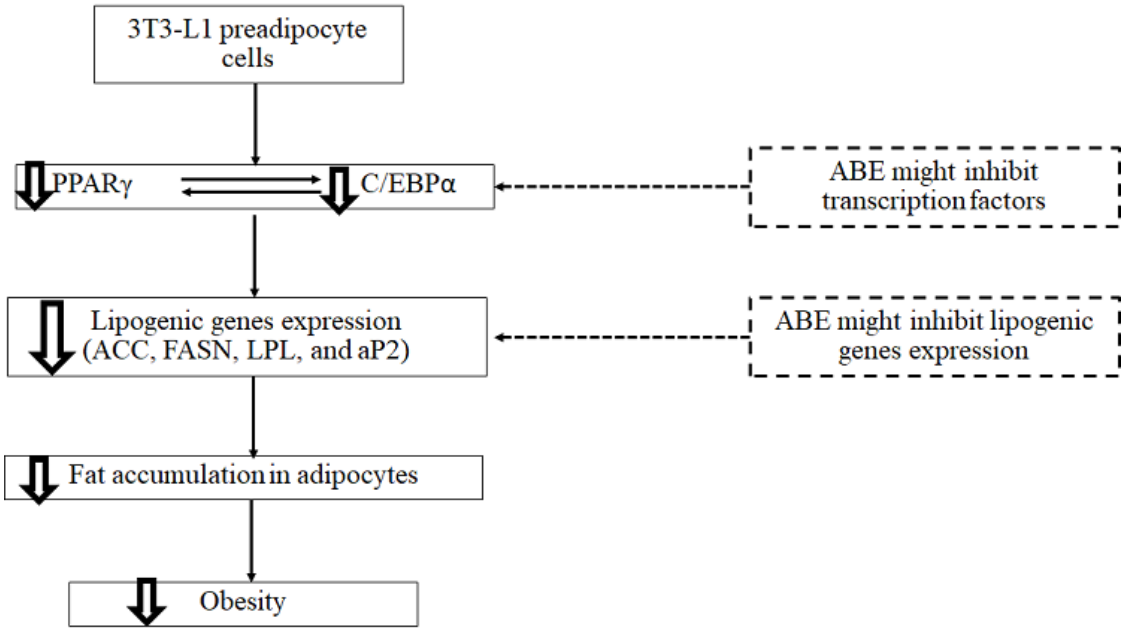


Figure 51 The proposed mechanism of the anthocyanin-rich fraction of *Antidesma bunius* extract (ABE) on 3T3-L1 preadipocyte cell differentiation.

CHAPTER VI

CONCLUSION

The present study indicates that the anthocyanins-rich fraction of *Antidesma bunius* (ABE), *Lepisanthes rubiginosa* (LRE), and *Syzygium nervosum* (SNE) have high contents of polyphenolic and anthocyanin. Specifically, all Thai berry extracts contain cyanidin-3-glucoside, whereas delphinidin-3-glucoside has been found in ABE and LRE. This study presents the effect of anthocyanin-rich fraction of ABE, LRE, and SNE on the carbohydrate digestive enzymes. ABE exhibits the most potent to inhibit intestinal maltase and sucrase among anthocyanin-rich fraction of Thai berry extracts. In the meantime, the most inhibition of pancreatic α-amylase was found in LRE. Besides, the anthocyanin-rich fraction of Thai berry extracts exhibit antioxidant activity. ABE can reduce the free radical generation higher than others through DPPH, TEAC, FRAP, and SRSA.

The proposed phenolic composition of ABE includes phenolic acids (quinic acid, gallic acid, 6-galloylglucose, 2,5-dihydroxybenzoic acid, and ellagic acid), flavonols (quercetin-3-O-arabinoglucoside, quercetin-3-galactoside, kaempferol-3-rhamnoside, kaempferol-3-glucoside, and myricetin-3-galactoside), and anthocyanins (delphinidin-3-glucoside, cyanidin-3-glucoside, and cyanidin-3-sambubioside). This study also reveals that ABE showed the anti-glycation through the reducing fluorescence and non-fluorescence AGEs in monosaccharides-mediated BSA glycation. ABE prevents protein oxidation by reducing protein carbonyl level as well as decreases the β-amyloid cross structure formation.

Interestingly, ABE prevents 3T3-L1 cell differentiation and lipid accumulation. The antiadipogenic of ABE involves the inhibition of major adipogenic transcription factors, including *PPARγ* and *C/EBPα*, resulting in the downregulation of lipogenic genes such as *ACC, FASN, LPL*.

Based on these findings, the anthocyanins-rich fraction of *Antidesma bunius* (ABE) may be a promising natural source for suppressing carbohydrate digestion, inhibiting monosaccharides-induced protein glycation, and preventing adipogenesis-related obesity.

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