EFFECT OF POMELO CRUDE EXTRACT ON LEARNING AND MEMORY THROUGH THE ACTIVATION OF PI3K/AKT SIGNALING PATHWAY IN THE BRAIN OF TYPE 2 DIABETIC RATS

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2020

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ชลธิชา วงค์ไชยา

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
วิทยาศาสตรมหาบัณฑิต สาขาวิชาชีวภาพการแพทย์
คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2563

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of MASTER OF SCIENCE

(Biomedical Sciences)

Faculty of Medicine, Srinakharinwirot University

2020

THE THESIS TITLED

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BY

CHONTICHA WONGCHAIYA

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

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Author	CHONTICHA WONGCHAIYA
Degree	MASTER OF SCIENCE
Academic Year	2020
Thesis Advisor	Assistant Professor Dr. Amporn Jariyapongskul

Type 2 diabetes (T2DM) induces defective in brain insulin signaling pathway, leading to impair cognitive function. Pomelo belongs to the genus Citrus and contains phyto*chemicals* such as *flavonoids* which are classified as a contributor in antioxidant and anti-inflammatory activities. The present study investigated the effect of Pomelo crude extract (PM) on cognitive impairment and related mechanism in type 2 diabetic rats. 6 weeks old male Sprague-Dawley rats (150-180 gram) were randomly divided into 3 groups: normal control (Control) and diabetes with (T2DM-PM) or without PM (T2DM) supplementation. Type2 diabetic rats were induced by feeding high fat diet (40.0% fat with 5,085 kcal/kg diet) for 12 weeks. Rats fed with high fat diet were injected intravenously with low dose streptozotocin (30 mg/kg/B.W.) once at 3rd weeks of diet. Daily gavage feeding of PM with 200 mg/kg BW in T2DM-PM-rats was performed for 12 weeks. The effect of PM on 1) blood glucose (BG), hemoglobin A1C (HbA1C) blood cholesterol (CHOL), triglyceride (TG), serum insulin (S. insulin), and calculated HOMA-IR index, 2) learning and memory performance using Morris Water Maze test, 3) expression of brain insulin signaling molecules, pIRS-1^{s312}, PI3K, pAkt, and pGSK-3 beta^{s9} 4) levels of insulin degradation enzyme (IDE), Amyloid beta$_{42}$, Tau protein phosphorylation(pTau^{s396}) 5) malondialdehyde (MDA), a lipid peroxidation end product were evaluated. The elevated BG, HbA1c, CHOL, TG were observed in T2DM-rats. The present results showed that insulin resistance, as evaluated by an increasing in S. insulin and HOMA-IR were developed in HFD-low STZ induced T2DM rat model. As compared to T2DM-rats, T2DM-PM rats demonstrated a significantly reduced in BG, CHOL, TG, S. insulin and HOMA-IR index. It was observed that T2DM-rats induced learning and memory deficits under MWM testing, which were significantly recovered by PM supplementation in T2DM-PM-rats. T2DM-rats exhibited defective in hippocampal insulin signaling molecules by increasing the expression of pIRS-1^{s312} and decreasing the expression of PI3K, pAkt and pGSK-3 beta^{s9}. In addition, it was found that levels of Amyloid beta$_{42}$, pTau and MDA were significantly increased, while levels of IDE were significantly decreased in the hippocampal tissues when compared with that of Control-rats. Interestingly, 12 weeks for PM supplementation could restored the alteration of insulin signaling molecules associated with decreasing the levels of Amyloid beta$_{42}$, pTau^{s396} and MDA. Meanwhile, IDE levels were increased in T2DM-PM-rats. In conclusion, long-term Pomelo crude extract supplementation in type 2 diabetic rats could recovered cognitive impairment by ameliorating impaired brain insulin signaling pathways, increasing IDE levels associated with decreased Amyloid beta$_{42}$ accumulation and tau protein phosphorylation. Moreover, PM supplementation demonstrated antioxidant effect which could help promote the cognitive function in T2DM-PM-rats.

Keyword : Pomelo crude extracts, Learning, Memory, Type 2 Diabetic rats

ACKNOWLEDGEMENTS

I would like to express major advisor, Asst. Prof Amporn Jariyapongskul for her valuable advice, helpful guidance and encouragement, for starting the time sitting lab, kindness and understanding in this study. All of this has enabled me to carry out of my study successfully.

I would like to thank Department of Physiology, Faculty of medicine, Srinakharinwirot University for providing me all instrument and facilities on my research.

I also sincerely thank to all lecturers and staff of my department for their valuable advice during study and special thank committee Assoc.Prof.Dr.Pornprom Yoysungnoen, Department of Physiology, Faculty of Medicine, Thammasat University and Dr. Ratchadaporn Pramong, Department of Anatomy, Faculty of Srinakharinwirot University for their support, helpfulness and guidance.

Finally, I would like to give all my heart to Miss Chonticha Areebambud and Mr. Nontapat Benjathum and my family for their helpfulness and guidance, love care, understand and many encouragements throughout my research. Without their support my success would never become true. And lastly, I would like to thank Faculty of Medicine Srinakharinwirot for support and my work with research budget.

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CHAPTER I
INTRODUCTION

Diabetes is a metabolic disorder that leads to health complications over a period of time, and causes morbidity and mortality. The world health organization (WHO) estimates that diabetic patients worldwide will increase to 200 million by the year 2025, and type 2 diabetes mellitus (T2DM) will account for 90% of all diabetes cases [(1)].

T2DM is the most common type of diabetes mellitus which is characterized by hyperglycemia and insulin resistance. The pancreatic β-cells of patients with this type of diabetes, unlike people with type 1 diabetes, can produce insulin, but the insulin signaling is impaired. This is called insulin resistance and is characterized by a decreased ability of tissues to respond to the action of insulin. To compensate for insulin resistance, the pancreatic β-cells secret more insulin that leads to hyperinsulinemia [(2)]. The major chronic complications associated with diabetes include retinopathy, neuropathy, nephropathy, coronary artery disease and cerebrovascular disease [(3-5)]. Recent studies have demonstrated a link between T2DM and Alzheimer disease (AD) [(6, 7)]. From a study of Mayo Clinic AD, Janson et al (2004) reported that T2DM was more prevalent in AD than in non-AD patients, with 81% of AD patients exhibiting T2DM [(8)]. The evidence supporting a link between T2DM and AD have also been found in animal models. For instance, Bruno et al. (2009) and Jolivalt et al (2010) have demonstrated impaired cognitive function in diabetic mice with over expression of amyloid precursor protein (APP), and beta amyloid (Aβ) peptide in the brain tissues, especially in the hippocampal area [(9, 10)]. Aβ peptide is the major component of extracellular senile plaques that play an important role in neurotoxicity through induction of oxidative stress [(11, 12)]. In addition, many studies have suggested that a contribution of Aβ peptide accumulation in the brain is one of the importance factor of cognitive impairment and dementia in both diabetic human and animal models [(13, 14)]. Several mechanisms have been hypothesized on the increasing impaired cognition and increasing risk of AD in T2DM, including hyperglycemia, insulin resistance, endothelial dysfunction, increased

oxidative stress, and inflammation [15-17]. Furthermore, current information from diabetic human and animal models indicates that insulin resistance and impaired insulin signaling pathway are the primary mechanisms underlying diabetic-induced cognitive impairment [18-20]. It is known that insulin is also synthesized in the central nervous system. In the brain, when insulin binds to insulin receptors (IRs), it activates insulin signaling pathways. Subsequently, insulin activates insulin receptor substrates (IRS), and then stimulates downstream proteins, phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) pathway with the downstream involvement of glycogen synthase kinase3 (GSK3). Importantly, brain insulin signaling has a crucial role for the regulation of neuronal growth, synaptic activity. and cognitive function [21]. Studies in diabetic animals have demonstrated impaired insulin signaling with increased activation of GSK3β, hyperphosphorylation of tau protein, increased accumulation of Aβ peptide in hippocampal tissues and associated with cognitive dysfunction [22, 23]. Schubert et al (2004) studied an insulin receptor knockout mice and found that these mice exhibited a loss of insulin-mediated activation of PI3K and reduced phosphorylation of Akt and GSK-3β, leading to an increase in phosphorylation of tau protein which is a major marker of neurodegenerative diseases and cognitive impairment. Furthermore, insulin is a common substrate for most of the beta amyloid degrading enzymes, such as insulin degrading enzyme (IDE). IDE is a major enzyme responsible for insulin and beta amyloid peptide degradation [24]. In T2DM, increased blood insulin promoted Aβ accumulation by decreasing IDE activity and increasing activation of glycogen synthase kinase-3β (GSK-3β) [25]. Thus, defects in brain insulin signaling transduction, decrease IDE levels associated with increase Aβ accumulation, and increase phosphorylation of tau protein are the most important mechanisms that contribute to cognitive impairment in type2 diabetes mellitus.

It is well established that chronic hyperglycemia, hyperinsulinemia, dyslipidemia, and other hallmarks of type2 diabetes accelerate glucotoxicity and lipotoxicity, leading to the activation of pro-inflammatory cytokine production and the formation of reactive oxygen species (ROS) [26]. Moreover, the metabolic alteration in

peripheral insulin resistance can affect the induction of brain oxidative stress. This evidence was confirmed by a recent study which demonstrated a positive correlation between brain oxidative damage and the HOMA-IR index an index for assessment insulin resistance [27]. This correlation implies the involvement of peripheral insulin resistance in the development of brain oxidative stress. In addition, several studies have demonstrated that enhanced production of ROS promotes the Aβ accumulation, leading to neuronal degeneration and cognitive impairment [28, 29].

Oral antidiabetic drugs such as thiazolidinedione and metformin have been shown to have beneficial effects in slowing the progression of cognitive impairment and dementia in both clinical and animal studies [30, 31]. However, these drugs are associated with several undesirable side effects, which are weight gain, fluid retention, and increased risk for heart failure. Herbal medicine and some plants have been used for a long time to manage disease, and to maintain health. Recently, extracts of various plant materials capable of decreasing blood sugar or increasing insulin sensitivity have been tested in animal models and their effects are confirmed. In recent years, the pomelo has received much attention because of its nutritional, antioxidant, antidiabetic, and antitumor properties [32, 33]. Pomelo (*Citrus grandis* (L.) Osbeck), known as grapefruit, is one of the most common fruits in Thailand with many different cultivars. Pomelo belongs to the genus *Citrus* of the family Rutaceae and is the largest citrus fruit. Citrus fruits are rich in various nutrients, such as vitamins A and C, folic acid, and dietary fiber [34, 35]. Furthermore, p*omelo is* source of bioactive compounds, such as flavonoids, coumarins, limonoids, and carotenoids [36, 37]. Among the flavonoids, pomelo presents considerable amounts of flavanones, flavones, flavonols, and anthocyanins [38]. The major flavonoids of pomelo are neohesperidin, hesperidin, naringenin, and naringin, which are present in high amounts in the fruit juice [39].

Recently, a number of studies on pomelo extract have reported the antioxidant properties through free radical-scavenging effects [40, 41]. However, the mechanisms of pomelo extract, especially on the brain insulin signaling pathway and cognitive function have not been clarified in high-fat diet combined with low dose

streptozotocin induced type 2 diabetic rat model. With the background knowledge above, therefore, the present study aimed to investigate the effect of pomelo crude extract on cognitive impairment and explore possible mechanism on brain insulin signaling through insulin-PI3K/Akt and GSK-3β pathway in high fat diet feeding combined with low dose of streptozotocin-induced type 2 diabetic rat.

Hypothesis

Long term oral supplementation of pomelo crude extract could improve cognitive impairment by ameliorating brain insulin resistance and regulating PI3K/Akt and GSK-3β activities in type 2 diabetic rats.

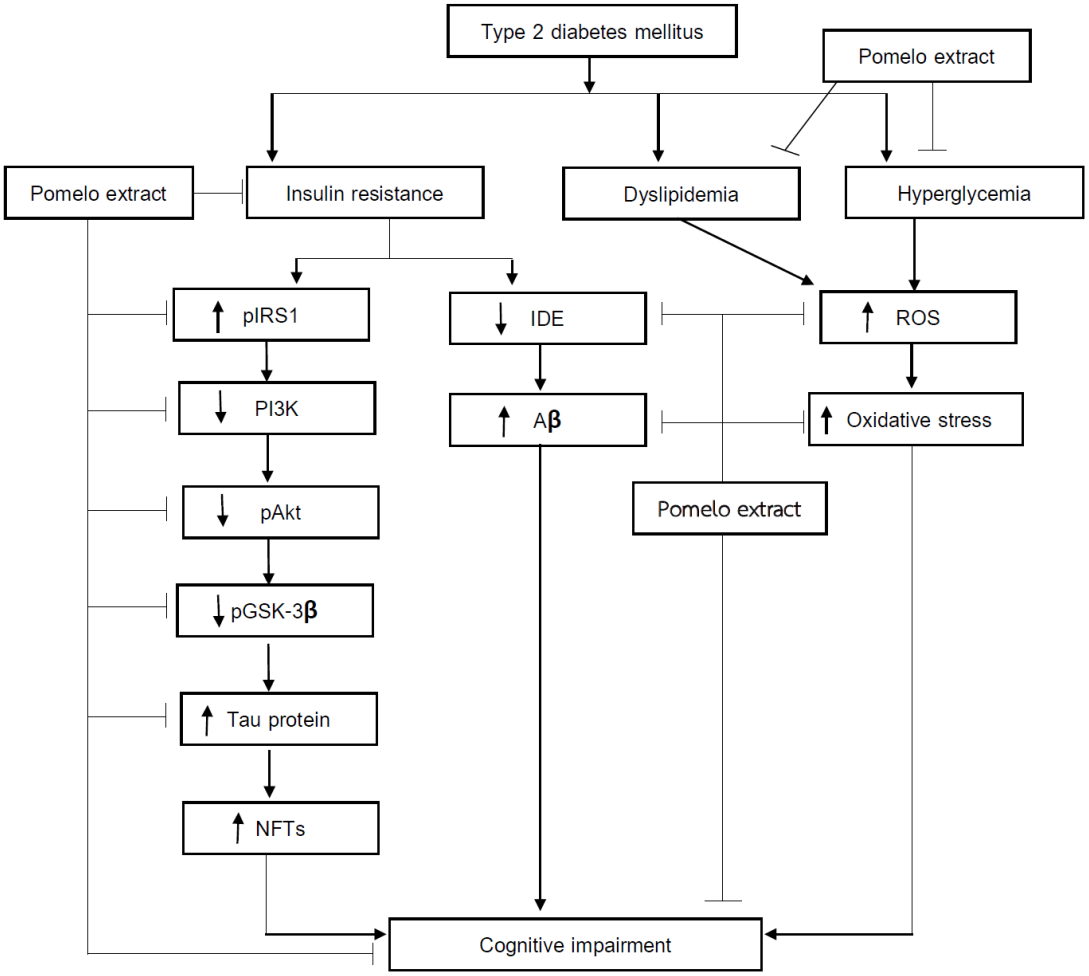
Objectives

To examine the effect of pomelo crude extract on cognitive function in type 2 diabetic rats

To examine the effect of pomelo crude extract on insulin signaling through PI3K/Akt and GSK-3β pathway in type 2 diabetic rats

To examine anti-oxidative effect of pomelo crude extract in type 2 diabetic rats

Conceptual framework



CHAPTER II
LITERATURE REVIEW

1. Diabetic mellitus (DM)

Diabetes mellitus (DM) is a group of metabolic diseases, characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Insulin is a peptide hormone produced by pancreatic beta-cells (β-cells). The function of insulin hormone is to regulate blood glucose levels. The insulin deficiency or loss of its function is a major cause of high blood glucose levels or hyperglycemia [42, 43].

The criteria for diagnosis of diabetes mellitus

1. Plasma glucose concentration more than 200 mg/dL (11.1 mmol/L)
2. Fasting plasma glucose (FBG) more than 126 mg/dL (7.0 mmol/L)
3. Oral glucose tolerance test (OGTT) at 2-hours post load glucose more than 200 mg/dL (11.1 mmol/L)
4. HbA1C more than 6.5% (48 mmol/mol)

1.1. Classification of diabetes mellitus

Type 1 diabetes mellitus (T1DM) is characterized by the

pancreatic β-cells destruction, usually leading to complete insulin deficiency. It has two forms;

-Immune-mediated diabetes mellitus is term insulin dependent results from cellular mediated autoimmune disruption of the pancreatic β-cells.

- Idiopathic diabetes mellitus is unknown ethilogies.

1.1.2. Type 2 diabetes mellitus (T2DM)

resulted from insufficient insulin function or reduced insulin sensitivity, reducing glucose transport into insulin dependent cells such as muscle and fat.

1.1.3. Other specific types

- Genetic defect of pancreatic β-cell
- Genetic defect of insulin action
- Disease of the exocrine pancreas
- Endocrinopathy

- Gestational diabetes mellitus
- Uncommon forms of immune mediated diabetic
- Other genetic syndrome

1.2. Type 1 diabetes mellitus (T1DM)

T1DM is also known as insulin-dependent diabetes mellitus (IDDM). The characteristic of T1DM is high levels blood glucose (hyperglycemia) because insulin deficits. The major cause of T1DM is β-cells dysfunction is an inability to produce insulin. This type is the most recent estimation in youth or child onset diabetes. T1DM is also associated with autoimmune disease that immune system cannot recognize β-cells. There are around 80% of patients with T1DM who have autoantibody against islet cells, insulin or secretory granule protein of islet cell, and the symptoms will be severe if autoantibodies are present to more than one β-cells antigens. The symptoms of T1DM are composed of hyperglycemia, polyphagia, polyuria, polydipsia and weight loss [44, 45].

1.3. Type 2 diabetes mellitus (T2DM)

T2DM is also known as non-insulin independent diabetes mellitus (NIDDM) that is the most common type of diabetes mellitus, about 90-95% of diabetic patients have T2DM. The characteristics of T2DM are insulin resistance and hyperglycemia [46]. In T2DM, the pancreatic β-cells can produce and secretory insulin at normal level but decrease in bioavailability or insulin signaling impairment, called insulin resistance. Insulin resistance is characterized by decreased ability of tissue to respond to the action of insulin. There are two metabolic defects that lead to type 2 diabetes, 1) a decreased ability of peripheral tissues in response to insulin (insulin resistance; IR), and 2) β-cell dysfunction which is manifested as inadequate insulin secretion in the face of insulin resistance and hyperglycemia [47, 48]. Figure 1 shows time causes for the development of hyperglycemia and dysfunction β-cell in T2DM that can be divided into three phases;

-Phase I is an early or pre-diabetic the insulin resistance and hyperinsulinemia are observed In this phase, IR leads to increase insulin release to try to decrease high blood glucose levels. Therefore, the fasting blood glucose level remains normal.

- **Phase II** is the period of β-cell dysfunction that reflects the inability of these cells to continue to produce enough insulin. As a result, blood insulin levels fall to the point where they can no longer for insulin resistance. However, the blood insulin levels are still high above normal. And hyperglycemia is observed.

- **Phase III** is the period that further decreases insulin production due to a severe B-cell dysfunction, resulting in decreased blood insulin levels (below than normal levels) and hyperglycemia [49].

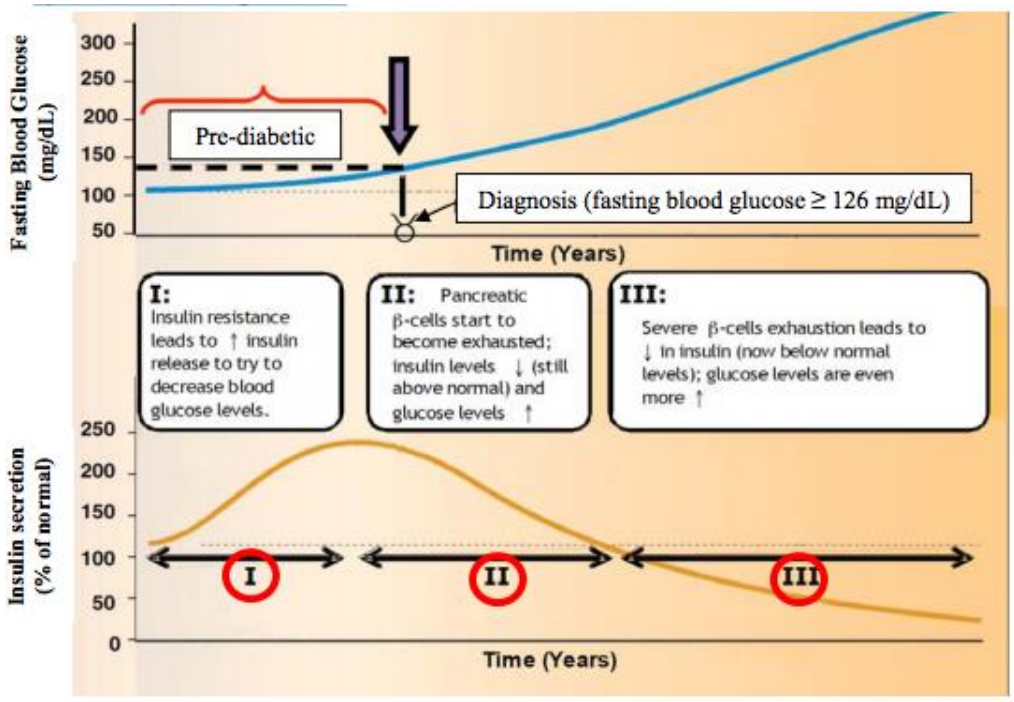


Figure 1 Phases of type 2 diabetes development

2. Insulin signaling pathway

2.1 Normal insulin signaling

Normally, insulin mediates its action through binding to insulin receptor. Insulin receptor (IR) consists of 2 alpha- and 2 beta-subunits that and link together by disulfide bonds [50]. Insulin signaling starts when insulin binds to its receptor (insulin receptor). Consequently, insulin receptor substrate (IRS) is phosphorylated in tyrosine, what leads to the downstream activation of PI3K and Akt. The Akt stimulates GLUT4 translocation to cell membranes (Figure 2) [51]. Through activation of these signaling

pathways, insulin acts as a powerful regulator of metabolic function. Insulin receptor-mediated activation of the mitogen-activated protein (MAP) kinase pathway implicated in cell growth, proliferation gene expression [52-54].

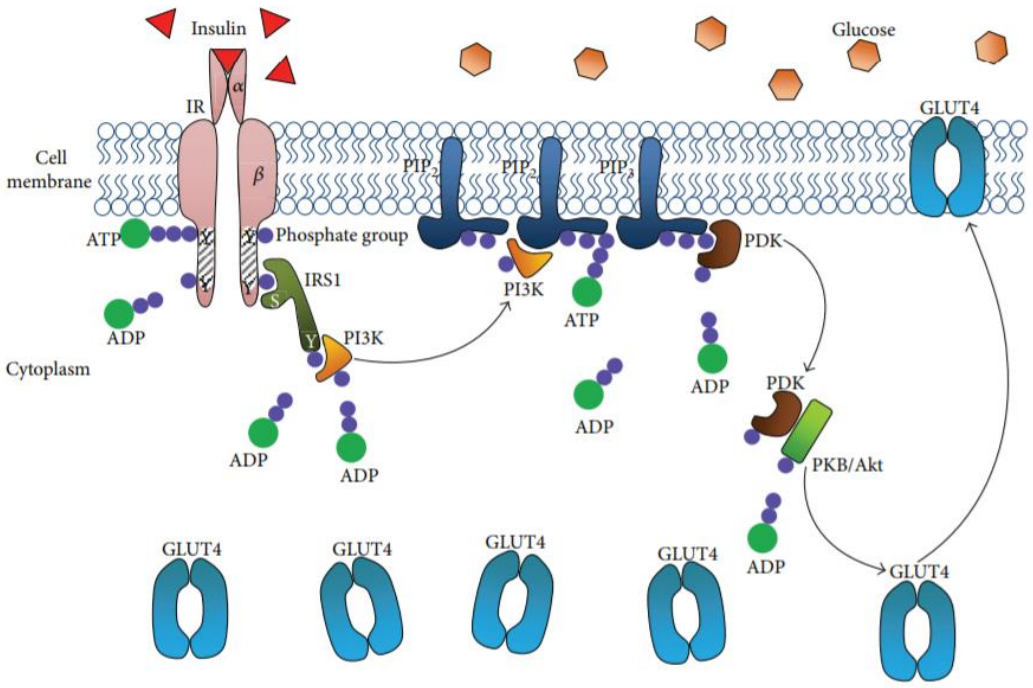


Figure 2 Insulin signaling pathway

2.2. Insulin resistance (IR)

Aging causes the peripheral tissues to become insensitive to the action of insulin. This causes an increase in blood glucose and insulin levels. It is not known what causes insulin resistance in elder population. However, it is believed that there are alterations in the signal transduction of peripheral tissues for example through a decrease of tyrosine phosphorylation in IRS proteins [55-57]. In addition IRS may fail to associate with PI3K to decrease the MAPK activity [58]. In an aging brain, the glucose metabolism in neurons decreases mainly, but not exclusively, because a decrease in insulin signal transduction of neurons [59].

3. Type 2 diabetes (T2DM) associated with cognitive impairment

It has been well established that T2DM is associated with cognitive impairment and increased risk of dementia or Alzheimer's disease [60]. Clinical evidences suggest that cognitive impairment is observed in not only in patients with diabetes but also in patients with early onset of diabetes. Biessels et al (2006) reports that 38% patients with diabetes exhibited ischaemic lesions and significantly higher cerebral atrophy as compared to the non-diabetic group, and only less than 5% had reported history of stroke in both diabetic and non-diabetic group [61]. Where as large body of evidences suggest that diabetes is associated with poor cognitive performance and increase risk of dementia the underlying cause remains unclear [62-64]. Interestingly, accumulating evidences suggest that both T2DM and Alzheimer's disease (AD) share similar behavioral, clinical and biochemical features [65]. Diabetes has been associated with memory and cognitive impairments; several lines of evidences suggest that diabetes impairs synaptic structure and function of hippocampus. Korf et al (2006) demonstrates that in older men with type 2 diabetes (average age of 81) has twice the increased risk of hippocampal atrophy, as compared to the age matched non diabetic subjects [66]. More recently, Gold and his colleagues (2007) reported that the cognitive impairment in T2DM patients may be more selective for hippocampal-related memory performance and that impairment is correlated with the degree of glycemic control [67]. These clinical data are supported by observations from animal models of T2DM. For instance, the *db/db* mouse exhibits deficits in the morris water maze test, a hippocampal-dependent spatial learning task [68]. The hippocampus is a major component of the brain of humans and other vertebrates. The hippocampus belongs to the limbic system and plays important roles in the incorporation of information from short-term to long-term memory, and in spatial memory that enables navigation. Several mechanisms have been hypothesized on the increased impaired cognition and increased risk of AD in T2DM, including hyperglycemia, insulin resistance, endothelial dysfunction, increased oxidative stress and inflammation [35, 36] . Moreover, current information from diabetic human and animal models indicated that hyperglycemia,

insulin resistance, impaired insulin signaling pathway and oxidative stress are the primary mechanisms underlying diabetic-induced cognitive impairment [37, 38].

3.1 Brain insulin signaling pathway

Figure 3 shows the brain insulin pathways. Insulin, which is secreted into the blood stream by pancreatic β-cells, is able to cross the blood-brain barrier (BBB) via a specific carrier. Once inside the central nervous system it binds to specific receptors, for example in the hippocampus. Insulin, then, produces tyrosine phosphorylation and activate similar signaling cascades as in the peripheral tissues. After autophosphorylation of the tyrosine receptor, PI3K/Akt and MAPK/ERK pathways are activated. In the hippocampus, disruption of the insulin signaling can occur at several points within the cascade (step1). There could be dysregulation of the specific transporter at the BBB (step 2), or changes in the phosphorylation state of IRS (step 3), or the Akt, a protein kinase, may fail to become phosphorylated (step 4). It could also happen that other downstream components may be affected. For example those involving the GSK3β (step 5). The enzyme GSK3β regulates the phosphorylation of the G-protein and the transcription factor FOXO (step 6) [69, 70].

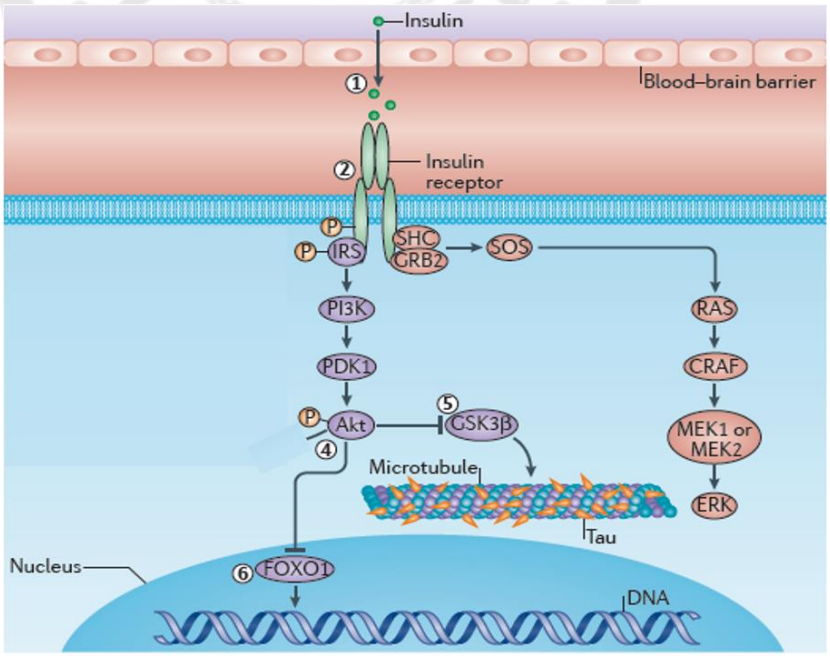


Figure 3 Brain signaling pathway

3.2. Insulin resistance induced beta-amyloid (Aβ) accumulation and cognitive impairment

The Aβ-peptide is generated from the amyloid precursor protein (APP). The APP is a transmembrane protein that is cleaved by secretase enzymes resulting in products that can go into amyloidogenic or non-amyloidogenic pathways [71]. If the APP is cleaved in the β-amyloid domain by the action of α-secretase, it enter the non-amyloidogenic pathway. On the other hand, if β- or γ-secretases cleave the APP, an insoluble Aβ-peptide is generated, which aggregates and accumulates (Figure 4) [72]. The high insulin levels in brain can cause high accumulation of Aβ by competitive binding with insulin degradation enzyme (IDE). Normally both insulin and Aβ are the target substances of IDE. In normal condition IDE can degrade the Aβ and protected Aβ accumulation in the brain but in insulin resistance stage, the high insulin levels lead to poor sensitivity of IDE to Aβ because of the competitive binding. Thus insulin resistance stage, the Aβ clearance by IDE was decreased and Aβ plaques lead to cognitive impairment [73-75].

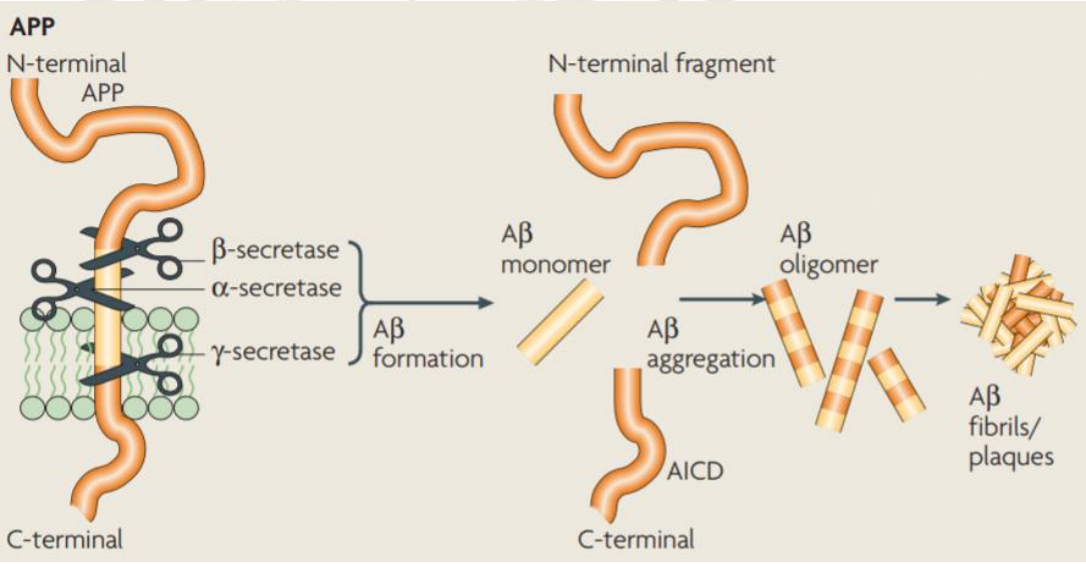


Figure 4 Amyloid beta processing

3.3. Insulin resistance induced Tau-protein phosphorylation

Tau-protein is a group of protein known as microtubules associated protein (MAPs). The function of Tau-protein is stabilized microtubules, axonal outgrowth and neuron cell trafficking. Normally, the structure of Tau-protein is an unfold protein and soluble. However, hyper-phosphorylation of Tau-protein (p-Tau) can change in structure into insoluble and accumulated in neuron cell known as neurofibrillary tangle (NFTs) which lead to neurodegenerative. Tau-protein can be phosphorylated by several enzymes and the most important is glycogen synthase kinase-3β (GSK3β). GSK3β was widely considered to be the primary kinase response to Tau-protein phosphorylation and turn it into NFTs. In normal condition, activation of GSK3β was controlled by PI3K/Akt signaling pathway. Activated Akt can phosphorylation GSK3β and turn it into the inactive form however, in insulin resistance condition PI3k/ Akt signaling pathway was inhibited, leading to high activation of GSK3β and hyperphosphorylation of Tau-protein (Figure 5) [76].

Both p-Tau and Aβ are activated the cognitive impairment via the oxidative stress by increasing reactive oxygen species (ROS) produced.

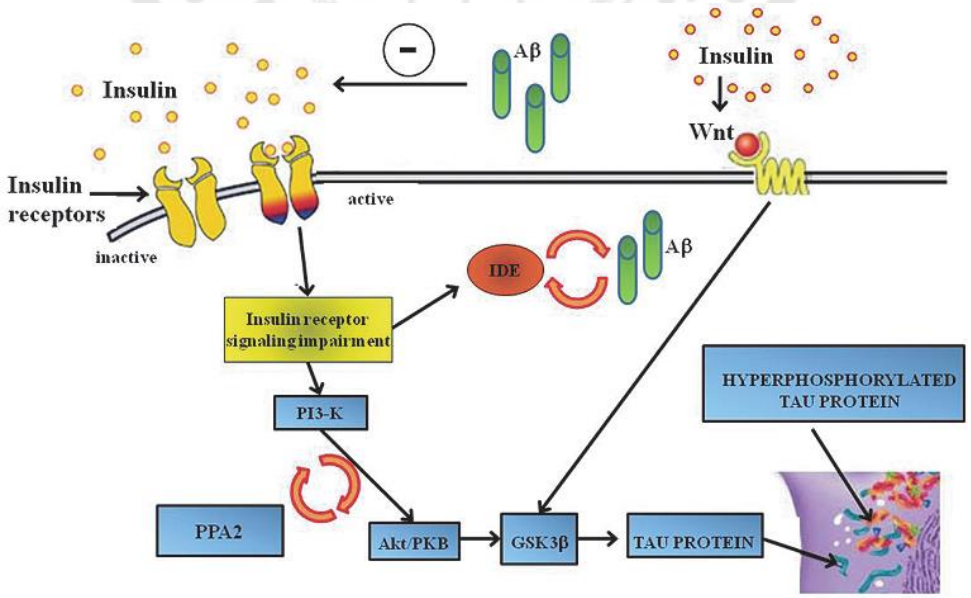


Figure 5 Insulin resistance signaling associate to AD

3.4. Hyperglycemia and oxidative stress induced cognitive impairment

Persistently high blood glucose impairs cognitive function and alters the structure of the hippocampus. A longitudinal study conducted for six years, reports that high glucose levels increases the risk of dementia in an elderly population [77]. It is possible to envision that high glucose levels may be toxic to the brain by several mechanisms. For example, osmotic changes may alter cell volume, or oxidative stress may cause protein damage. High blood glucose causes glycation of proteins leading to advanced glycation end products (AGEs) which are toxic to neurons. Consistent with this idea, a study on AD patients found that those with type 2 diabetes have higher AGEs in the brain together with microglial activation [78]. AGEs together with free radicals damage neurons by an oxidative mechanism [79]. In addition, in a diabetic state a decrease in oxidative capacity may also increase the oxidative stress. It has been postulated that oxidative stress via neuronal dysfunction leads to neuronal damage [80].

4. Brain and cognitive function

Cognitive function is process of acquiring knowledge and understanding through thought, experience, and the senses such as attention, the formation of knowledge, memory and working memory, judgment and evaluation, reasoning problem solving and decision making, comprehension and production of language. Cognitive processes use existing knowledge and generate new knowledge. Cognitive function part of the brain consists, the frontal lobe that links and integrates all components of behavior, the parietal lobe that is largely responsible for construction ability and language, and the temporal lobe that perceives and recognizes verbal material. The occipital lobe is primary visual cortex, which receives and interprets information from the retinas of the eyes. The hippocampus to be principally involved in storing long-term memories and in making those memories resistant to forgetting, though this is a matter of debate and an important role in spatial and non-spatial [81, 82].

5. Herbal medicine and diabetes mellitus

For type 2 diabetes mellitus treatment, oral hypoglycemia or lipid-lowering drugs and drug that improved insulin sensitivity are the convention therapy for T2DM. However, they are expensive and often associated with the side effects in long-term use such as hypoglycemia. At present, herbal medicines have increasingly gained as prevention and therapeutic for type 2 diabetes mellitus and its complications. In addition, the herbal medicines have shown the anti-diabetic, anti-inflammation, and anti-oxidant activities. The bioactivity phytochemicals of herbal medicine include flavonoid, phenolic and other compounds.

Pomelo (*Citrus grandis* L.) is the largest citrus fruit from the Rutaceae family, a natural citrus fruit and similar in appearance to a large grapefruit. Previous studies have shown that pomelo fruits are a rich source of vitamin C, flavonoids, and carotenoids, particularly in the peel and pulp [83]. The major flavonoids of pomelo are hesperidin, naringin, and another class of O-methylated aglycones of flavones such as nobiletin and tangeretin in which strongly associated with therapeutic properties including anti-oxidant, anti-atherogenic, anti-inflammatory, anti-microbial and anti-cancer effects [84-86].

Anti-diabetic property of pomelo extract

The pomelo extracts have ability to improve insulin secretion from pancreatic β-cells, insulin sensitivity, enhancing peripheral glucose uptake, muscle GLUT-4 translocation. Moreover, anti-hyperglycemic action may be through potentiating the pancreatic secretion of insulin from pancreatic β-cells and improved plasma lipid metabolism [87, 88].

Anti-oxidation property of pomelo extract

The pomelo antioxidant properties are free radical scavenging effects, reduces the reactive oxygen species (ROS) and inhibit lipid peroxidation in hydrogen peroxide (H_2O_2)-treated HepG2 cells [89].

Anti-inflammation property of pomelo crude extract

The pomelo flavonoids decreased the levels of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production, and inhibit transcription factor NF-**K**B activation in mice colitis [90].

6. Assessment of cognitive function in animal model

In order to obtain clinically relevant outcomes, it's necessary to support the histological, physiological, and behavioral outcomes of any tested cognitive impairment in animals. When using behavioral tests in animals, learning and memory are the most commonly used subjective endpoints [91].

6.1 Morris water maze (MWM) test

To test spatial learning in small rodents, the Morris water maze is used. It uses the assumption that an animal will try to escape from an obnoxious situation such as a large pool of water. The pool contains a small platform that be placed either under the water level, or slightly below the water. That platform would enable the animal to stand on it to evade the water. During a training period with the platform barely abode the water, the animal learns to use clues in the room to find the platform. During a trial the platform is lowered just below the water level. In order for the animal to escape from the pond, it must remember its location. The animal will remember the location of the platform by remembering the cues in the testing room, for example a window. This type of special memory depends on the proper function of the hippocampus. Thus, the ability to remember the platform can be altered by administration of certain drugs or by procedures that simulate disease [92, 93].

7. Type 2 diabetic animal model

Type 2 diabetes mellitus is characterized by insulin resistance and hyperglycemia. Several animal models of type 2 diabetes mellitus are obese, reflecting the human condition where obesity is closely linked to type 2 diabetes development. In present proposed, high-fat (HF) feeding combination with low dose injection of steptozotocin (STZ; 30 mg/kg body weight) will be performed as type 2 diabetic rat model. The HF feeding and STZ rat model was originally described in 2000 by Reed et al [94]. This model aims to combine inducing insulin resistance through high fat diet with

reducing pancreatic β-cell mass to mimic the natural history and metabolic characteristics of Type 2 diabetes mellitus. This model is known as the HF diet/STZ (HFD/STZ) rat and using Sprague Dawley or Wistar rats. The HFD/STZ rat model, the state of obesity, insulin resistance and/or glucose intolerance in prediabetes is simulated by a period of a HF diet. Besides the duration of the high fat feeding timeframe, the composition of the diet seems to greatly affect the weight gain and fat (free fatty acid; FFA) distribution. The final event involved in the development of type 2 diabetes mellitus is pancreatic β-cells failure or death. STZ has been used in both type 1 and type 2 diabetes animal models. It is toxic to cells causing damage to the DNA. STZ is similar to glucose to be transported into the cell glucose transporter Protein 2 (GLUT2). STZ is not recognized by the other glucose transporters. This explain it's relative toxicity to pancreatic β-cell, because these cells have high level of GLUT2. Therefore, STZ induced hyperglycemia mainly by it's direct cytotoxic effect on the pancreatic β-cell [95].

CHAPTER III

MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Chemical	Source
1. Streptozocin (STZ; $C_8H_{15}N_3O_7$)	Sigma-Aldrich (St. Loius, MO, USA)
2. Glucostrip Accu-Check® performa	Roche (Boehringer Mannheiem, Germany)
3. Cholesterol and triglyceride strip Accutrend® GCT meter	Roche (Boehringer Mannheiem, Germany)
4. Insulin Enzyme-linked immunosorbent assay (ELISA) kit	Millipore (St.Charles, MO, USA)
5. Anti- PI3K antibody	Cell signaling, Technology (Beverly,MA, USA)
6. Anti-pTau (Ser^{396}) antibody	Cell signaling, Technology (Beverly,MA, USA)
7. Anti-Akt antibody	Cell signaling, Technology (Beverly,MA, USA)
8. Anti-pAkt antibody	Cell signaling, Technology (Beverly,MA, USA)
9. Anti- GSK3β antibody	Cell signaling, Technology (Beverly,MA, USA)
10. Anti- pGSK3β antibody	Cell signaling, technology (Beverly,MA, USA)
11. Anti- β-actin antibody	Millipore (St.Charles, MO, USA)
12. Anti-mouse IgG HRP- linked antibody	Sigma-Aldrich (St. Loius, MO, USA)
13. Anti-rabbit IgG HRP- linked antibody	Cell signaling, Technology (Beverly,MA, USA)
14. Thiobarbituric acid reactive substraces (TBARs) assay kit	Cayman Chemical (Ann Arbor, MI)
15. Glutathione assay kit	Cayman Chemical (Ann Arbor, MI)

16. Amyloid-beta (Aβ) ELISA assay kit	Invitrogen, U.S.A
17. 30% acrylamide/bis solution	Bio-Rad Laboratories, CA
18. Sodium dodecyl surface (SDS)	Millipore (St.Charles, MO, USA)
19. 10% ammonium persulfate	Millipore (St.Charles, MO, USA)
20. Tetramethylethylenediamine (*TEMED*)	Millipore (St.Charles, MO, USA)
21. Polyvinylidenedifluoride (PVDF) membranes	Millipore (St.Charles, MO, USA)
22. Skim milk	Millipore (St.Charles, MO, USA)

3.1.2. Animals

Male Sprague Dawley rats, weighting 150-180 g were purchased from Nomura Siam International Co., Ltd. (Thailand). Throughout the experiment, the rats were housed, and maintain under temperature 22±2 °C, and 12 hours light-drank cycle. The rats were allowed to acclimatize for one week before the experiment. The experiment was in accordance with the Nation Institutes of Guidelines for the use of laboratory animal and was approved by the Ethics committee of Faculty of Medicine, Srinakharinwirot University (SWU-A-008-2562)

3.1.3. Animal diet formula

- Normal diet consist of 4.5% fat and 24.0% protein with a total calorific value of 3,040 kcal/kg diet (Nation Laboratory Animal Center, Salaya campus, Mahidol University).

- High fat (HF) diet consist of 40.0% fat, 35.0% carbohydrate, and 25.0% protein with total calorific value of 5,085 kcal/kg diet. (Plus Value Enterprise Co., Ltd, Thailand).

3.1.4. Pomelo juice powder preparation

The pink pomelo " Thong Dee" (*Critrus grandis* (L.) Osbeck) were purchased from Nakhon Si Thammarat Province. Fruits were peeled and flesh samples were juiced and frozen at -30 °C until use. The frozen pomelo juice was dissolved with

water drinking. The juice was spray to powder with spray dry. The pomelo powder or crude extract was frozen at -20 °C until use. 3.86 kg of pomelo crude extract was originated from 100 kg of pomelo juice.

- **Determination of total phenolic content**

The total phenolic content of pomelo crude extracts was measured using the Folin-Ciocalteu assay [96]. 0.5 ml of the diluted extract was mixed with 7.5 ml of distilled water and 0.20ml of Folin-Ciocalteu reagent. After 10 min, 1 ml of 20% sodium carbonate was added. The tubes were kept for 20 min at 40 ºC before the absorbance was measured at 755 nm with a UV-Vis spectrophotometer (UV-1601, Shimadzu Corporation, Japan). The total phenolic contents were expressed as a gallic acid equivalent in microgram per gram of pomelo crude extracts.

- **Determination of total Flavonoid content**

The colorimetric assay adapted from Ramful et al. was used for the determination of total flavonoids present in the extracts [97] 0.5 ml of diluted extract was mixed with 150 µl of 5% aqueous $NaNO_2$ (0.15 ml). After 6 min, 150 µl of 10% aqueous $AlCl_3$ (0.15 ml) was added. After 6 min, 2 ml of 4% NaOH was added. The absorbance of the whole mixture was read at 510 nm with a spectrophotometer. The total flavonoid contents were expressed as a catechin equivalent of microgram per gram of pomelo crude extracts.

- **HPLC analysis**

The pomelo crude extracts were analysed by high performance liquid chromatography (HPLC) with column's size 250 mm*4.6 mm, 5 µm and with mobile phase (Podigy 5U ODS3 100R). Quantitative analysis of naringin was performed. The naringin concentrations was expressed as microgram per gram of pomelo crude extracts.

Table 1 Composition of bioactive compounds of pomelo powder

Percent yield	Unit
Total phenolic (μgGAE/g)	3389.73 ± 360.98
Total flavonoids (μgGAE/g)	192.2 ± 10.8
Naringin (μg/g)	416.70 ± 21.82

3.2. Methods

3.2.1. Induction of experimental type 2 diabetic rat model

Type 2 diabetic rat model was induced as described following. Rats were daily feed with high-fat diet with a total calorific value of 5,085 kcal/kg diet. After 3 weeks of dietary manipulation, rats were injected intravenously (I.V.) with streptozocin (STZ) 30 mg/kg body weight in 0.9% sodium chloride solution (0.9% NaCl) [98]. The normal control rat was received normal diet for 3 weeks followed by 0.9% NaCl (0.5 mL) I.V.injection. Fasting blood glucose (FBG) was examined 1 week after STZ or 0.9% NaCl injection. The rats with a FBG level more than 250 mg/dL were defined hyperglycemia and included into the research project.

3.2.2. Experimental designed

Twenty four rats will be randomly divided into 3 groups of 8 each. Normal control group (CON) will be received normal diet for 12 weeks. Type 2 diabetic group (DM2) will be continuously fed with high-fat diet for 12 weeks after STZ injection. Type 2 diabetic rat treated with pomelo juice (DM2-PM) will be fed with high-fat diet continuously and received gavage feeding of pomelo juice (200 mg/kg B.W./day) for 12 weeks after STZ injection.

Pomelo juice powder (200 mg/kg B.W.)[99,100] will be dissolved with drinking water daily in the volume of 0.5 mL per rat. The supplementation will be started from 7th day of STZ injection.

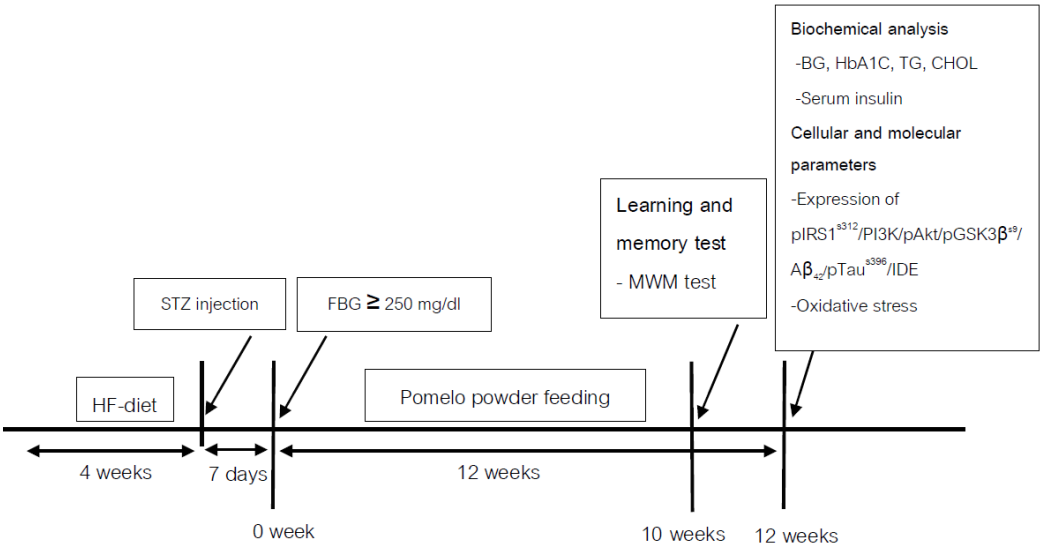
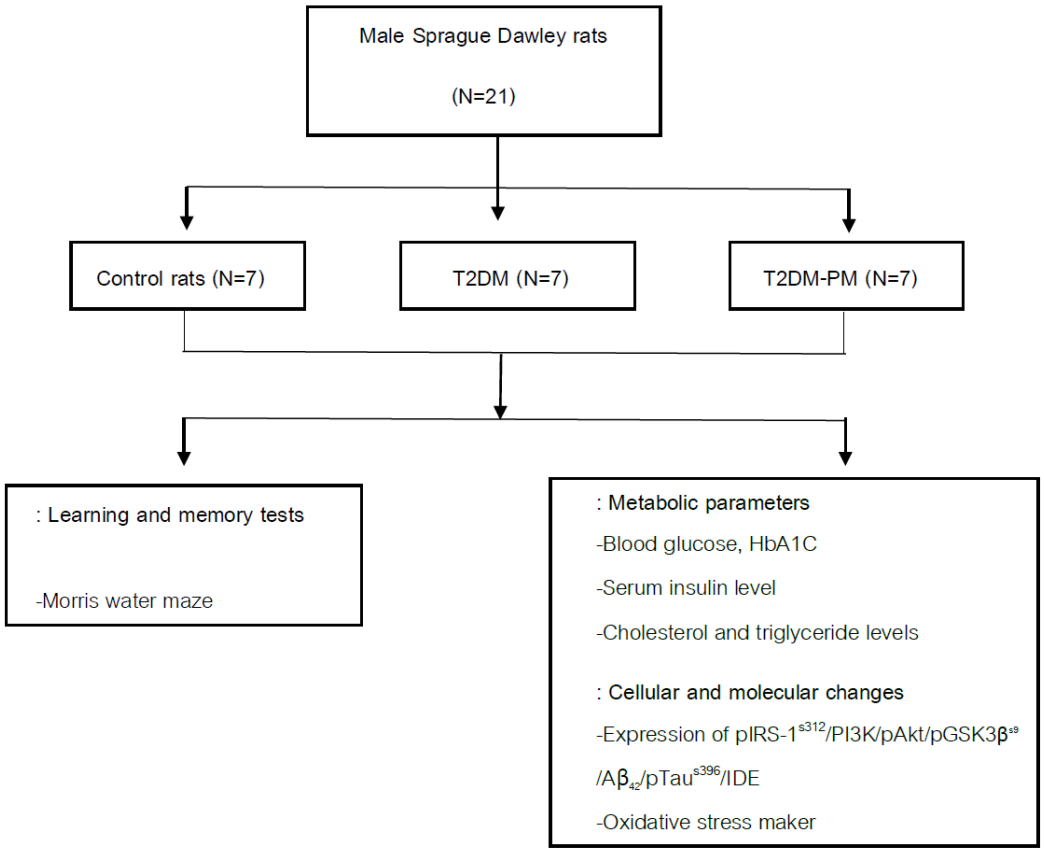


Figure 6 Time table experimental design



3.2.3. Biochemical analysis

Fasting blood glucose blood cholesterol and triglyceride levels were measured every 2 weeks by Gluco-Cho-Tri (GCT) meter (Acc-Check® performa Roche, Boehringer Mannheiem, Germany). Plasma HbA1c was determined by turbidimetric immunoinhibition method (Professional Laboratory Management Crop.Co.,Ltd.Thailand).

3.2.4 Determination of serum insulin level by EILISA

Serum insulin level was evaluated by sandwich ELISA technique, following manufacturer's protocol. Briefly, 10 µL volumes of serum was added into microwells precoated with insulin specific antibody, then the plate was sealed with cover strip and incubated at 37 °C for 2 hours. The liquid of each well was discarded without any wash. Next,100 µL of biotin-conjugated antibody specific for insulin was added to the wells and incubated at 37 °C for 1 hour, followed by 5 wash. The color was developed by adding 90 µL of TMB substrate to each well and leaving the plate at 37 °C for 15-30 minutes in dark. To stop the enzymatic reaction 50 µL of stop solution was added to a wells and mixed. The absorbance was measured at 450 nm, and 570 nm was used to subtract from the reading at 450 nm with multi-detection microplate reader (BioTex® Instruments, Inc., USA). All standards and sample were assayed in duplicate. Insulin levels were determined from standard curve.

- **Homeostatic model assessment of insulin resistance (HOMA-IR)**

The blood glucose (BG) and serum insulin levels were used to calculate the homeostatic model assessment of insulin resistance (HOMA-IR), according to the following formula:

$$HOMA-IR = \frac{Glucose \times Insulin}{22.5}$$

Where IR is the insulin resistance in international dosage units (U) per liter and glucose is given in mmol/L. The HOMA-IR is a procedure to determine insulin resistance designed by Matthews *et al.* in 1985 [101].

3.2.5 Assessment of cognitive (learning and memory) function

To assess spatial and non-spatial memory capacity of the rats, Morris water maze (MWM) was performed, respectively on the 10th week after STZ injection or pomelo juice supplementation.

3.2.5.1 Morris water maze (MWM) test

In order to assess spatial learning and memory the Morris water maze (MWM) test was performed in this study [92]. The MWM test consisted of a pool, with a hidden platform submerged just below the water surface. The circular pool (214 cm in diameter and high 50 cm) and a platform (15 cm in diameter and high 80 cm) were prepared. The pool was divided equally into 4 quadrants, water was filled into the pool until the platform submerge 1-1.5 inch the water. Non-Toxic white dry added to make water opaque rendering platform invisible. The temperature of water was maintained at 25±0.5 °C. The hidden platform was located in the middle of a certain quadrant, and reminded unchanged throughout study. The MWM test was performed in a quiet and light-controlled room. Before start the MWM test, rat was acclimatized to the environment of the test room for one day. Each rat's swimming behavior was recorded by a digital video camera throughout the study.

There are 2 phases in MWM test, the first phase, rat was learn to escape from the water by locating a hidden flatform (escape latency time). The second phase, platform was removed out of the pool for assessing memory (probe trial time). The MWM test was carried out for 6 consecutive days, the five days for learning test (escape latency time) and the last day for memory test (probe trial time).

- Learning test (escape latency time)

The escepe latency time is the time that rat required to reach the hidden platform. Each rats were received 5 consecutive daily training trilas, with 4 intervals for one trial. Each interval have maximal time of 120 seconds (sec). For each interval, the rat was placed into the water at randomly start quadrant, by turning the rat's head facing the pool wall. The rat was swam to find the platform until it climb onto the platform, allowed to stay there for 20 sec. If the rat fails to reach the hidden platform within the maximal time of 120 sec, it was conducted to climb onto the platform and

allowed to stay there for 20 sec. The resting peroid for starting next interval is 30 mins. The time to reach the hidden platform was measured as escepe latency time in seconds.

- Memory test (probe trial time)

After 5 days of learning test, the memory test was performed on day 6. The hidden platform was removed out of the pool, and rat was placed into the pool from the starting location at the quadrant opposite to the former platform quadrant. Number of times of crossing the former platform and the time spent in the former platform was recorded for 60 seconds.

3.3. Determination of the molecular parameters

3.3.1. Brain tissue preparation for Western blot and EILISA

At the end of each experiment, rat was sacrificed with high dose of sodium pentobarbitial (100 mg/kg B.W.). After, rat was perfused transcardially with cold 0.1 M phosphase-buffered saline (PBS, pH 7.4) to wash out the remined blood, and the brain was removed. The hippocampal tissues were rapidly separated from the whole brain and homoginized in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich St. Loius, MO, USA) phos protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 12,000 rmp for 10 minutes at 4 °C and the supernatant collected. The protein concentration in supernatant was determined by Bradford protein assay (Bio-Rad, CA, USA). These protein samples were stored at -80 °C untill use for western blot and EILISA assay.

3.3.2 Determination of pIRS-1^{s312} levels by ELISA assay

The pIRS-1^{s312} was determined using the ELISA assay kits (ThermoFisher, U.S.A). determined by EILIZA assay. A monoclonal antibody specific for pIRS-1^{s312} and per-coated on the microplate. Standard, control and samples were added the appropriate to wells with pIRS-1^{s312} detected specific to pIRS-1^{s312}. Next, pIRS-1^{s312} conjugated to anti-rabbit IgG Horseradish Peroxidase (HPR) solution added into each wells and incubated. After, TMB substrate solution added each well, only those wells that contain pIRS-1^{312} the substrate solution begins to turn to blue. The enzymes reaction

yield a blue product which turns to yellow when adding the stop solution. The color change was measured spectrophotometrically at an absorbance at 460 nm. The concentration of pIRS-1^{s312} in the samples were tested determined by comparing the O.D. of samples to the standard curve.

3.3.3 Determination of pTau PI3K and Akt protien expression by western blot analysis

The protien sample of hippocampal tissue was diluted to 5 µg/uL for 10% sodium dodecyl surface gel eletrophorosis (SDS-PAGE). The SDS-PAGE loading gel consists of 30% acrylamide/bis solution (37.5:1), 1.5 M tris-HCL (pH 8.8), 10% sodium dodecyl surface (SDS), DI water, 10% ammonium persulfate and tetramethylethylenediamine (*TEMED*). Twenty five micrograms of protein sample was loaded and runs at 30 mA for 45-60 minutes. The gel was removed from the electrophoresis apparatus, incubated with tranfer in solution buffer for 30 minutes and transferred into polyvinylidenedifluoride (PVDF) membranes (Millipore). The non-specific binding was blocked by 5% skim milk blocking grade (Millipore St.Charles, MO, USA) with 0.1% tween in tris-buffer saline (TBST) for 1 hour at room temperature. The membrane was inclubated with primary antibodies agents pTau^{S396} (1:1,000), pGSK3β^{s9}(1:1,000), pAkt (1:5,000) and PI3K (1:1,000) in 5% BSA and in 1% skim milk at 4 °C, overnight (18 hrs.) with gentle shake. The membrane was washed by TBST 3 times each time for 5 minutes. After washing, secondary antibody. (1:5,000) was prepared in 5% bovine serum albumin (BSA) and inclubated for 1 hour at room temperature. β-actin (1:40,000) was used as loading control. After 3 washed with TBST, specific bands of immunoreactive protien was visualized after membrane inclubation with enchanced chemiluminescense (ECL) for 5 minutes by imaging flim and the density of protien bands were calculated using the Graph pad program

3.2.4 Determination of $A\beta_{42}$ levels by ELISA assay

The levels of $A\beta_{42}$ in hippocampal homogenate was determined using the ELISA assay kits (Invitrogen, U.S.A). This assay employs the quantitative sandwich enzme immunoassay technique. A monoclonal antibody specific for $A\beta_{42}$ has been pre-

coated on a microplate. Standards, control and samples were added to the wells and $A\beta_{42}$ present is bound by the immobilized antibody. After washing the unbound substances, an enzyme linked polyclonal antibody specific for $A\beta_{42}$ was added to each wells. Following a wash to remove any unbound antibody-reagent, a substrate solution was added to the wells. The enzyme reaction yields a blue product that turns yellow when add the stop solution. The intensity of the color was measured is in propotion to the amount of $A\beta_{42}$ bound in the initial step. The optical density was measured within 30 minutes using a multi-detection microplate reader (BioTex® Instruments, Inc., USA) at 450 and 570 nm. The absorbance reading at 570 nm was used to substract from the reading at 450 nm with multi-detection microplate reader (BioTex® Instruments, Inc., USA). All standards, control and samples were tested in duplicate. Rat $A\beta_{42}$ levels were determined from $A\beta_{42}$ standard curve.

3.2.5 Determination of insulin degrading enzymes (IDE) level

The insulin degrading enzymes (IDE) is a zinc-binding protease known to cleave short polypeptides with variable sequences. The IDE was measured by ELISA assay kit. The microtiter plate provided in this kit has been pre-coated with an antibody specific to IDE. Standards or samples were added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to IDE. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution was added, only those wells that contain IDE, biotin-conjugated antibody and enzyme-conjugated Avidin was exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of IDE in the samples were tested, then determined by comparing the O.D. of the samples to the standard curve.

3.2.6. Determination of lipid peroxidation by thiobabituric acid reactive substrance (TBARs) assay

Lipid peroxidation was determined by the TBARs assay. This assay measures the level of malonaldehyde (MDA), an end product of lipid peroxidation that

serves as a biomaker of oxidative stress in cell and tissues. MDA forms a complex with thiobarbituric acid (TBA) in a 1:2 ratio. The MDA-TBA complex was measured colorimetrically using a spectrophotometer at 540 nm. The TBARs levels were determined from a MDA standars curve. Rat MDA was measured using TBARs assay kit (Cayman chemical, USA) following the manufacturer's protocal. 100 µL of standard and hippocampal homogenate was added into each vial. Then, 100 µL of sodium dodecyl sulfate (SDS) solution was added into each vials and mixed. After adding 4 mL of color reagent forcefully down side of each vial, the vials were mixed and boiled at 100 °C for one hour. The vials were then placed on ice bath for 10 minutes to stop reaction and centrifuged at 1,600 g, 4 °C for 10 minutes. The vials may appear clear or cloudy and were incubated at room temperature for 30 mintues. Finally, 150 µL of solution in each vial was loaded into 96-well plated (duplicate) and the absorbance at 540 nm of each well's contents were measured using multi-detection microplate reader (BioTex® Instruments, Inc., USA). Between mixing step, each vial was mixed using vortex mixture. The MDA concentration in each sample was calculated by using the following formular.

$$\text{MDA } (\mu M) = \frac{\text{(Corrected absorbance)-(y-interept)}}{\text{Slope}}$$

3.4. Statistical analysis

All data were presented as means±standard error means (mean±SEM) and using the Statistical Package for the Social Sciences (IBM SPSS) 23.0 software (IBM Corporation, USA). Data was analyzed by One Way Analysis of Variance (ANOVA) with multiple comparisons using Tukey post hoc test (IBM SPSS). A value of $p \leq 0.05$ was considered statistically significant.

CHAPTER IV
RESULTS

There are four parts of the result were described according to the experimental designs as follow:

Part I demonstrated the effect of pomelo crude extract (PM) on metabolic parameters.

Part II demonstrated the effect of PM on cognitive function.

Part III demonstrated the possible mechanisms underlying the effect of PM in associate with cognitive function and brain insulin signaling pathway.

Part IV demonstrated the effect of PM on ROS (lipid peroxidation).

Part I demonstrated the effect of pomelo crude extract (PM) on metabolic parameters.

1.1 Fasting blood glucose (FBG), plasma HbA1C blood cholesterol and triglyceride serum insulin and HOMA-IR

To examine cognitive impairment induced by type 2 diabetic animal model, Sprague Dawley rats feeding with 40% high fat diet (HFD) for 12 weeks and low dose of STZ was injected once at 3rd weeks after HFD feeding. During the course of the experiment, the control rats maintain a normal FBG level, while all the T2DM rats exhibited hyperglycemia after STZ injection. Interestingly, 2 weeks after supplementation with PM in T2DM rats, a decreasing trend in FBG level was exhibited but not statistical significant. After 4 weeks supplemented with PM, the FBG level significantly decreased when compared with that of the T2DM rats and the levels were remained stable after 6 weeks of supplementation until the end of the experiment (Figure 7). To further estimate the mean blood glucose concentration during PM supplementation, the plasma levels of HbA1C were measured. Plasma HbA1C levels were significantly increased in T2DM rats compared with that of the Control-rats but significantly reduced in T2DM-PM rats compared with that of the T2DM-rats (Figure 8).

Moreover, the blood triglyceride levels of T2DM-rats were significantly higher than that of the Control-rats after 3 weeks of HFD feeding (week 0 in the Figure 10). The blood triglyceride level in T2DM-PM-rats was significantly decreased after supplementation in with PM for 2 weeks and remained normal as control rats until the end of the experiment (Figure 10). Apart from triglyceride-lowering effect of PM, the present result was shown that blood cholesterol levels in T2DM-PM –rats were not stable but had a tendency to decrease and significantly decreased after 8 and 12 weeks supplemented with PM compared with that of T2DM-rats.

To evaluate the effect of PM on insulin secretion, serum insulin level was analyzed by using ELISA analysis. As shown in figure 9, serum insulin levels were significantly increased ($p < 0.001$) in T2DM-rats compared with CON-rats. Moreover, serum insulin were reduced in T2DM-PM rats compared with that of T2DM-rats. Regarding serum insulin, we observed that its levels were 16 fold and 2 fold higher in T2DM rats than in Control-rats and T2DM-PM-rats, respectively. Importantly, this result

suggested that long term oral feeding of PM significantly reduced hyperinsulinemia in type 2 diabetes.

Taken together, these metabolic results suggested that long term supplementation of PM was able to counteract hyperglycemia, hyperlipidemia and hyperinsulinemia.

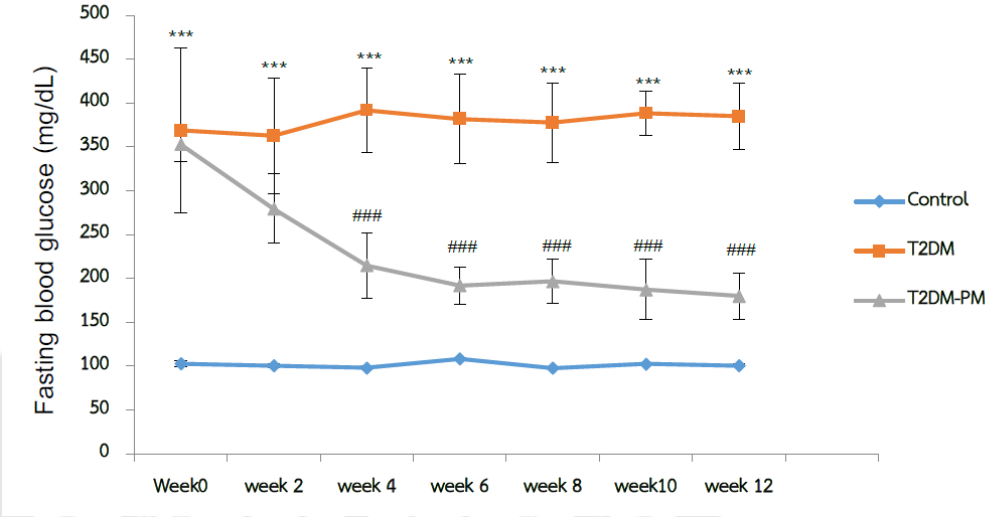


Figure 7 Effect of Pomelo crude extract supplementation for 12 weeks on FBG level

Data are means ± SEM (N =7)

*** indicates significantly different as compared to Control (p<0.001)

indicates significantly different as compared to T2DM (p<0.001)

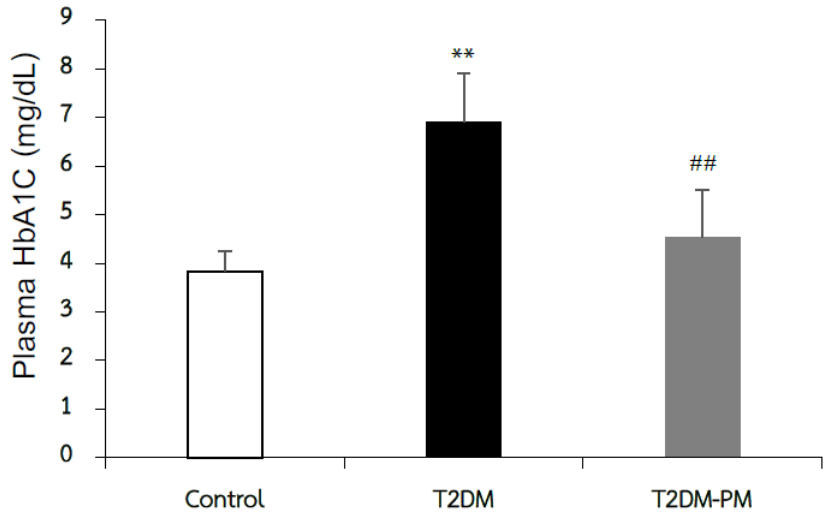


Figure 8 Effect of Pomelo crude extract supplementation for 12 weeks on glycosylated hemoglobin (HbA1C)

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

indicates significantly different as compared to T2DM (p <0.01)

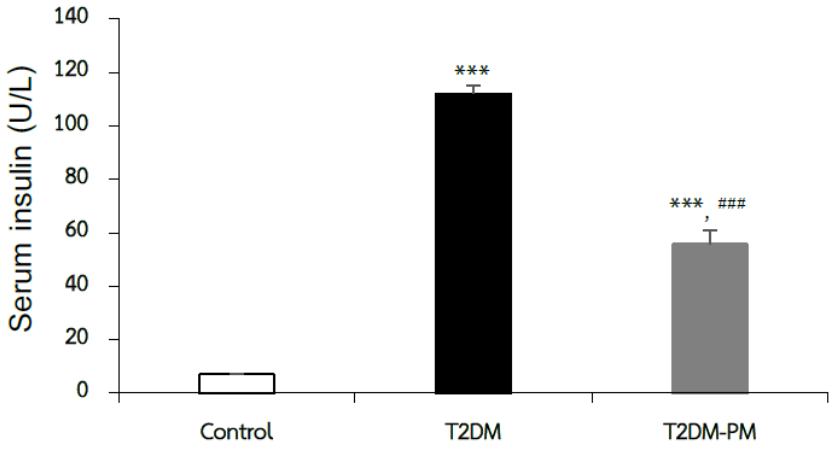


Figure 9 Effect of Pomelo crude extract supplementation for 12 weeks on Serum insulin

Data are means ± SEM (N=7)

*** indicate significantly different as compared to Control (p<0.001)

indicate significantly different as compared to T2DM (p<0.001)

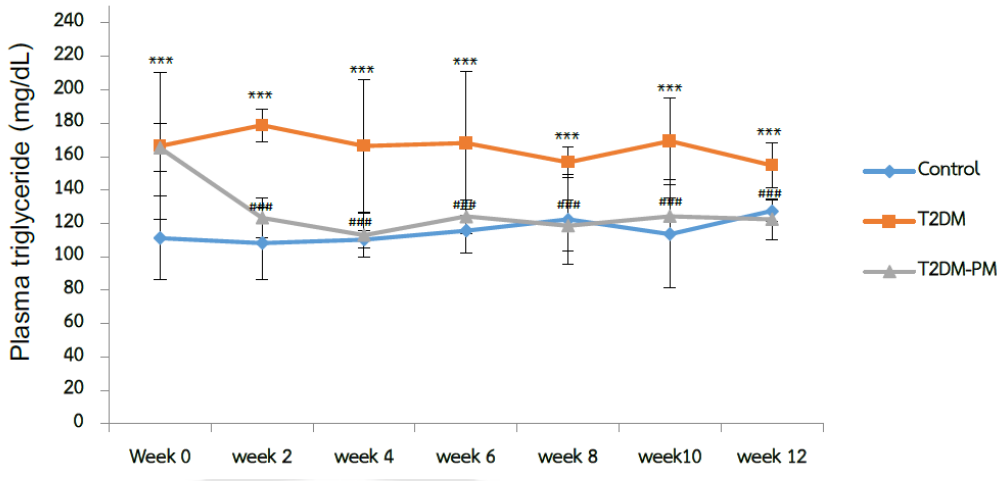


Figure 10 Effect of Pomelo crude extract supplementation for 12 weeks on plasma triglyceride level

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

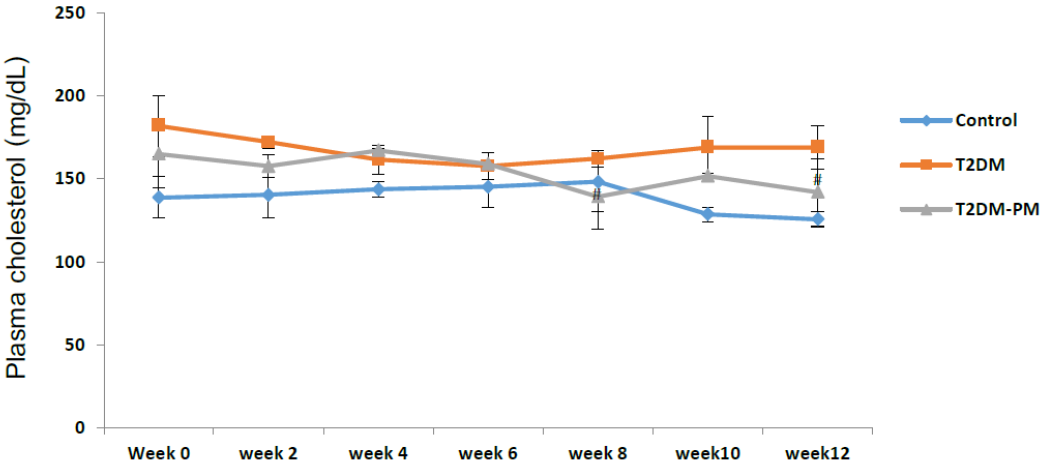


Figure 11 Effect of Pomelo crude extract supplementation for 12 weeks on plasma cholesterol level

Data are means ± SEM (N=7)

# indicates significantly different as compared to T2DM (p <0.05)

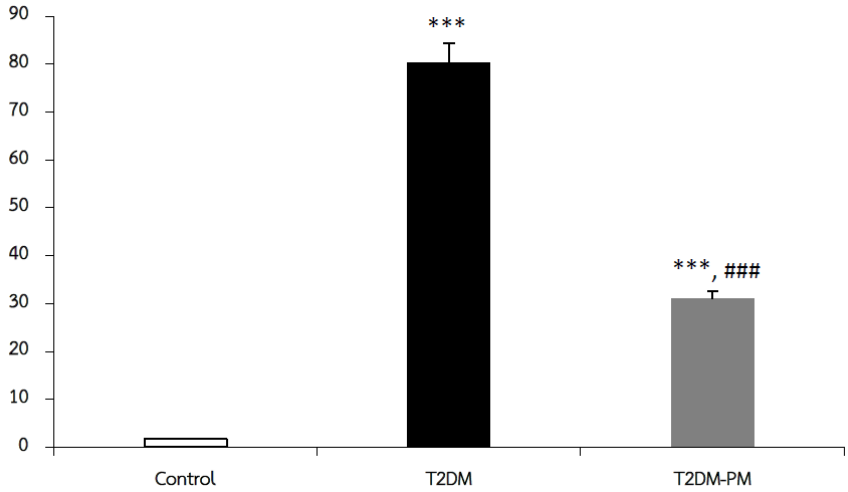


Figure 12 Effect of Pomelo crude extract supplementation for 12 weeks on HOMA-IR

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

Part II The effect of Pomelo crude extract (PM) on cognitive function

It is well recognized that type 2 diabetes is a major risk factor for learning and memory impairment. Therefore in this study, the Morris water maze (MWM) test was performed to evaluate whether long term PM supplementation could restored or improved learning and memory function (Figure 12). In MWM test, spatial learning was evaluated by using the time to find a hidden platform. This time is presented by escape latency time. As shown in Figure 12, T2DM-rats showed a significantly longer escape latency time than that of Control rats (p <0.01) on every test day. In addition, diabetic rats supplemented with PM (T2DM-PM) exhibited shorter the escape latency time over the 5 days of testing period compared with that of T2DM-rats (Figure 12). Importantly, escape latency time of T2DM-PM on day 2 to day 5 of testing period were not different from Control-rats. To evaluated spatial memory, probe trial test was performed on the 6th day of the MWM test. As shown in Figure 13, time spent in the platform area of T2DM-rats was significantly reduced when compared with that of Control-rats. Importantly,

when compared with T2DM-rats, the time spent in the platform area of T2DM-PM rats significantly higher (p <0.001).

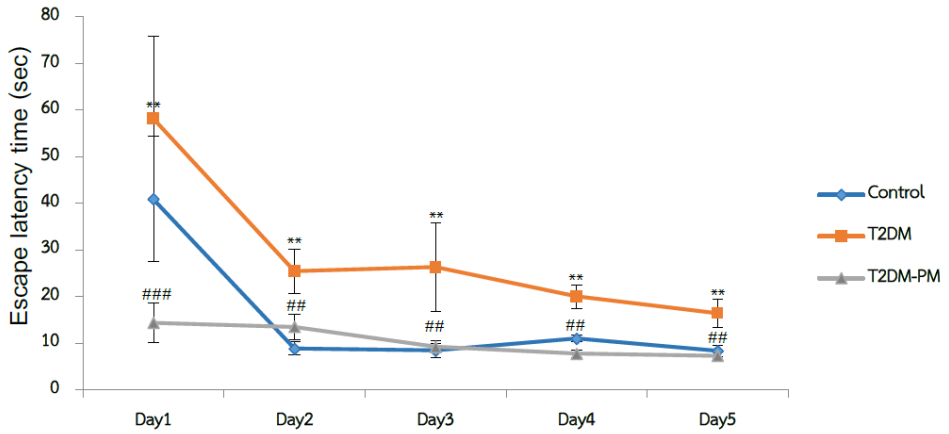


Figure 13 Effect of Pomelo crude extract supplementation for 12 weeks on the learning performance

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

##,### indicates significantly different as compared to T2DM (p<0.01,p<0.001, respectively)

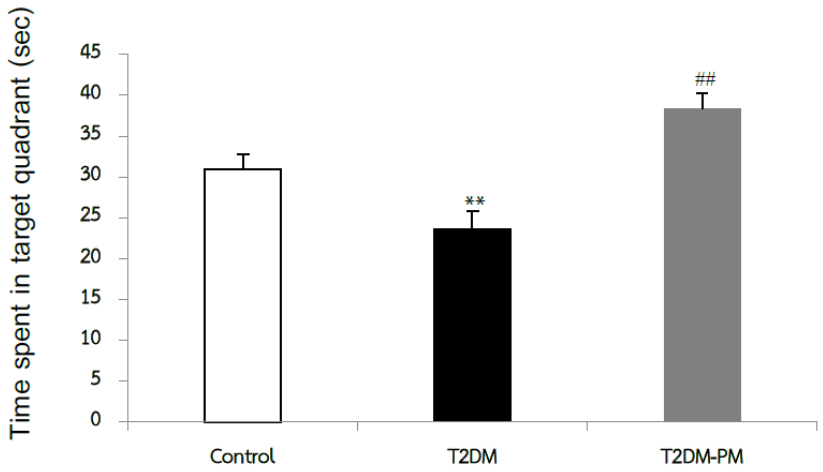


Figure 14 Effect of Pomelo crude extract supplementation for 12 weeks on memory performance

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

indicates significantly different as compared to T2DM (p <0.01)

Part III Pomelo crude extract (PM) ameliorates type2 diabetes-induced brain insulin resistance

3.1 The effect of PM on brain insulin signaling transduction Tau protein phosphorylation

To determine whether brain insulin resistance was induced by HFD-low STZ-induced T2DM, both ELISA and Western blot assay were performed in hippocampus.

The level of pIRS-1_{s312} in T2DM-rats was significantly increased (p <0.001) when compared with that of Control-rats. PM supplementation in T2DM-PM-rats significantly decreased the level of pIRS-1_{s312} (p <0.001) when compared to that of T2DM-rats (Figure 14). This result suggested that PM supplementation improved the type 2 diabetes-induced impairment of upstream insulin signaling. Additional, the present study further explored effects of PM on alterations of PI3K, Akt and GSK-3β^{s9} in the hippocampus. Therefore, The expression of PI3K, pAkt, and pGSK-3β^{s9} were analyzed. The expression of PI3K and pAkt were decreased compared with that of

Control-rats. Supplemented T2DM rats with PM can attenuate the decreasing of PI3K and pAkt expression. On the other hand, the expression of phosphorylated GSK-3β^{s9} significantly decreased in T2DM rats, and after supplementation with PM in T2DM-PM-rats the expressed can be reversed (Figures 15).

To evaluate the effect of PM on Tau protein phosphorylation, which was regulated by the insulin signaling pathway in type 2 diabetic rat's hippocampus. The expression of pTau^{s396} was analyzed. The expression of pTau^{s396} in T2DM- rats was significantly increased (p <0. 001) when compared to that of Control-rats. Supplementation with PM in T2DM-PM-rats significantly (p <0.01) decreased expression of pTau^{s396} when compared with that of T2DM- rats (Figure 16).

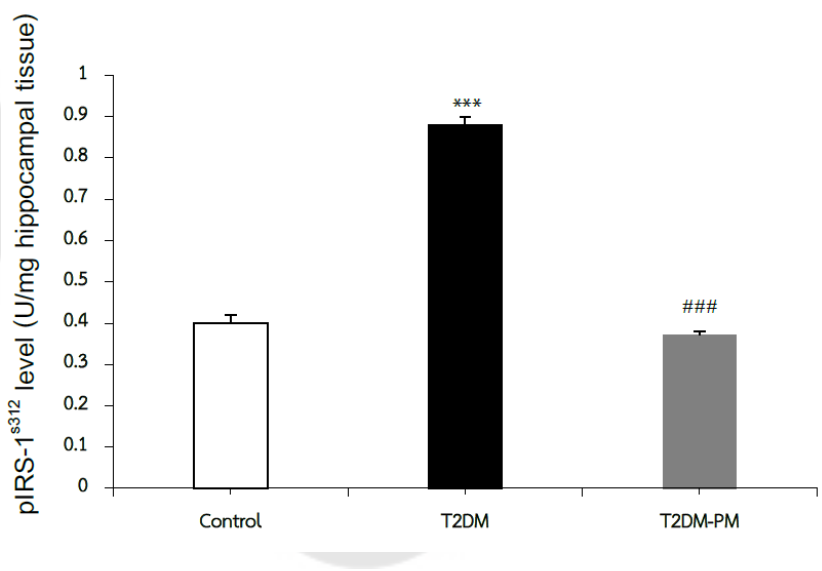


Figure 15 Effect of Pomelo crude extract supplementation for 12 weeks on the pIRS-1^{s312} expression in hippocampus

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

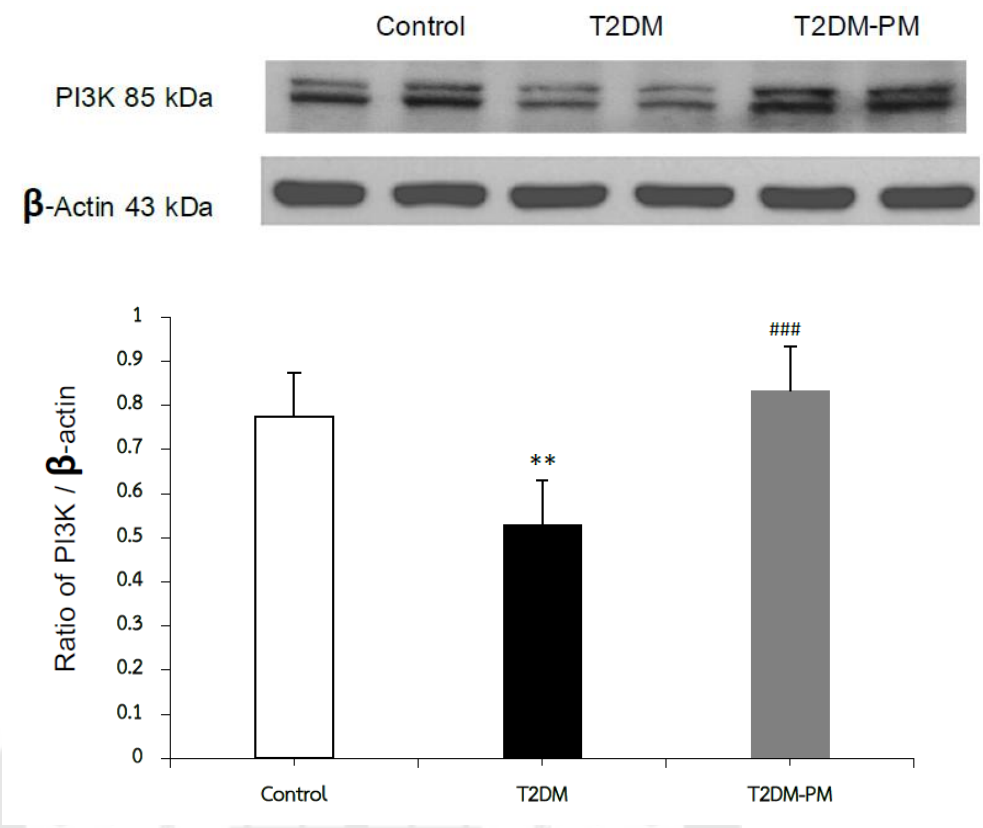


Figure 16 Effect of Pomelo crude extract supplementation for 12 weeks on the insulin signaling of PI3K

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

indicates significantly different as compared to T2DM (p <0.001)

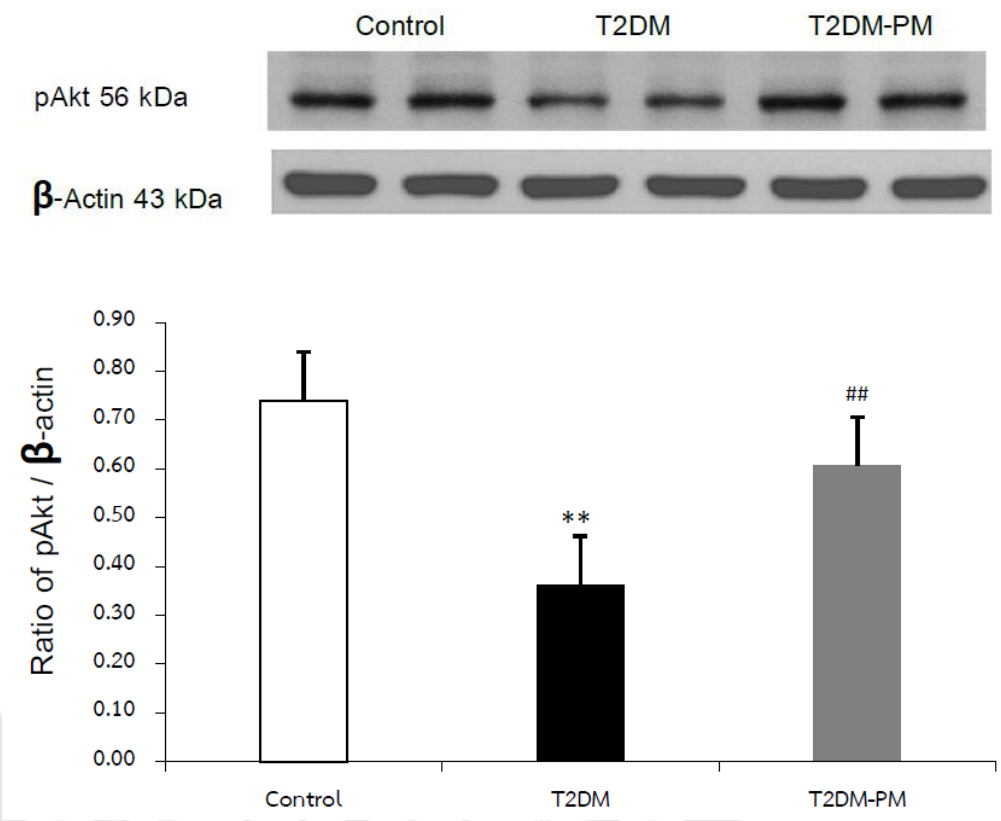


Figure 17 Effect of Pomelo crude extract supplementation for 12 weeks on the insulin signaling of pAkt

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

indicates significantly different as compared to T2DM (p <0.01)

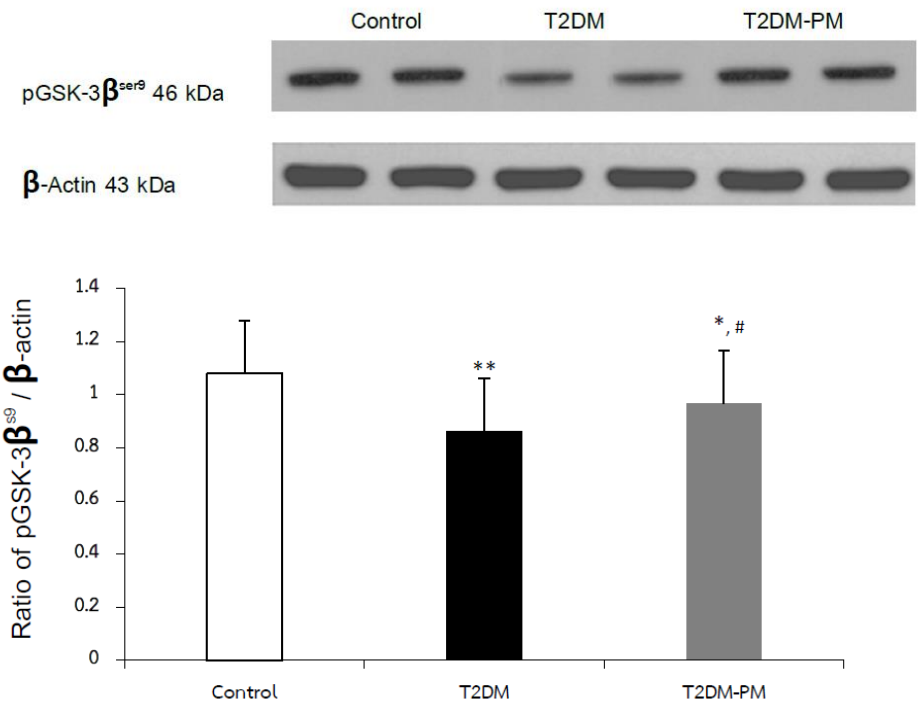


Figure 18 Effect of Pomelo crude extract supplementation for 12 weeks on the insulin signaling of pGSK-3β^{s9}

Data are means ± SEM (N=7)

*,** indicates significantly different as compared to Control (p <0.05, 0.01, respectively)

# indicates significantly different as compared to T2DM (p <0.05)

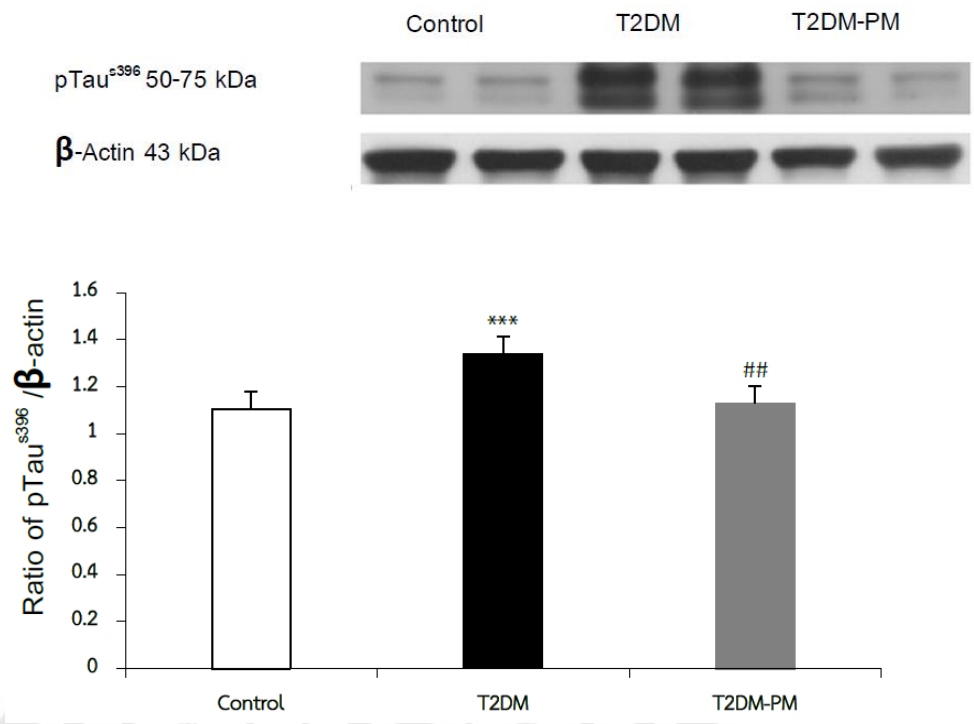


Figure 19 Effect of Pomelo crude extract supplementation for 12 weeks on the pTau^{s396} expression

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.01)

3.2 The effect of PM on the levels of insulin-degrading enzymes (IDE) and Beta-amyloid ($A\beta_{42}$)

IDE acts as a regulator for $A\beta_{42}$ degradation. In the present results, in T2DM-rats, the IDE level was significantly decreased ($p<0.001$) compared with that of Control-rats (Figure 17). In addition, the $A\beta_{42}$ content was significantly elevated ($p<0.001$) when compared with that of Control-rats (Figure 18). Importantly supplementation of PM in T2DM-PM-rats significantly increased the IDE level ($p<0.001$) (Figure 17) and significantly reduced ($p<0.001$) the $A\beta_{42}$ content when compared with that of T2DM-rats (Figure18).

These results indicated the PM supplementation in type 2 diabetic rats improved the IDE function and could clearance the accumulation of the $A\beta_{42}$ in hippocampus tissues.

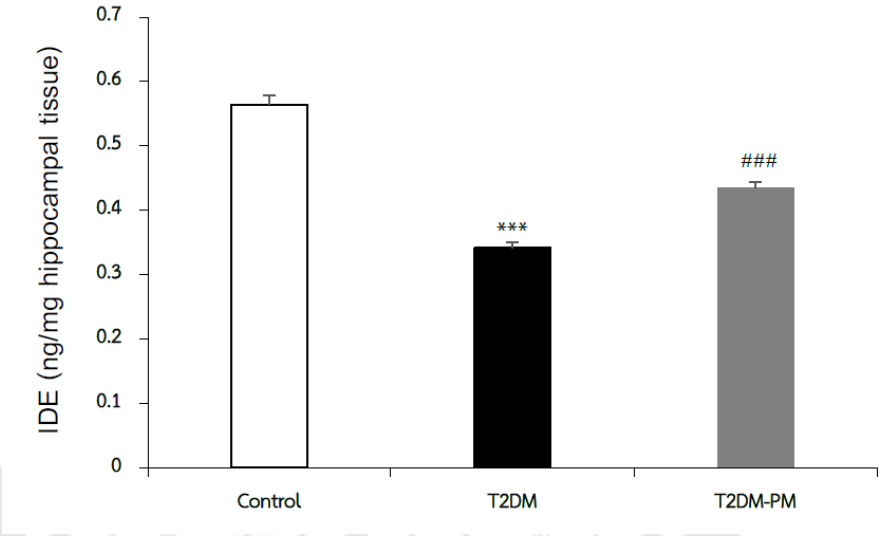


Figure 20 Effect of Pomelo crude extract supplementation for 12 weeks on the IDE levels in hippocampus tissues

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

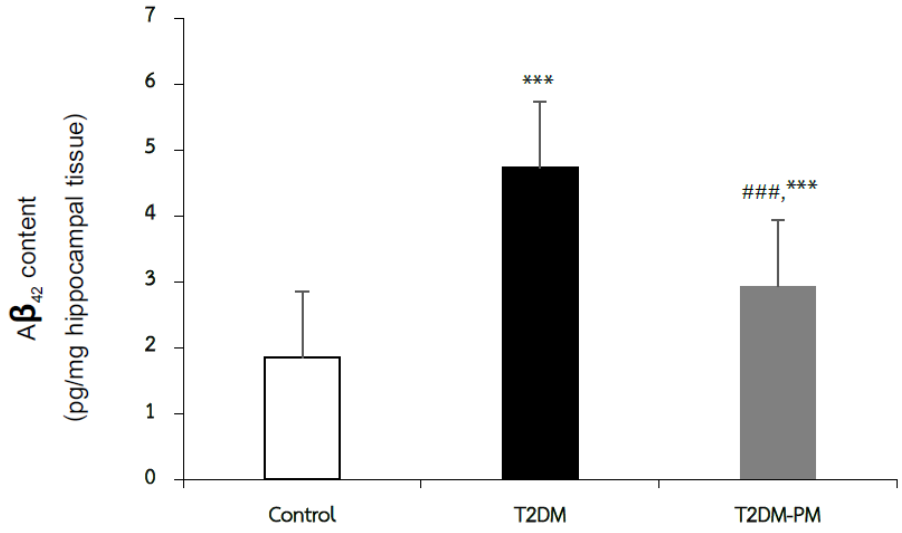


Figure 21 Effect of Pomelo crude extract supplementation for 12 weeks on the $A\beta_{42}$ accumulation in hippocampus

Data are means ± SEM (N=7)

,* indicates significantly different as compared to Control (p <0.01 and 0.001, respectively)

indicates significantly different as compared to T2DM (p <0.001)

Part IV The effect of Pomelo crude extract (PM) on lipid peroxidation

In the present study, malondialdehyde (MDA), an end product of lipid peroxidation was used as an indicator of oxygen free radical. The hippocampal MDA level was significantly higher in T2DM-rats than that of the Control-rats. Interestingly, PM supplementation in the T2DM-PM-rats was able to reduced hippocampal MDA level by 56.4% as percent changes from the levels of T2DM-rats (Figure 19).

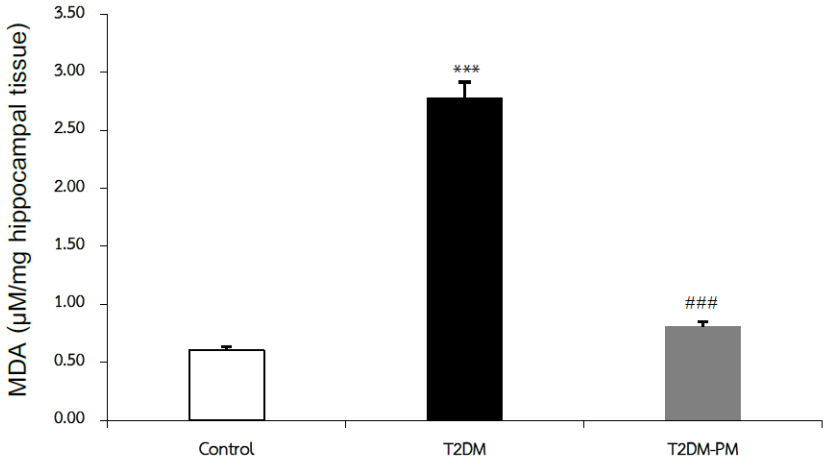


Figure 22 Effect of Pomelo crude extract supplementation for 12 weeks on malondialdehyde in the hippocampus

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

CHAPTER V
DISCUSSION

The present study hypothesized that long term oral supplementation of pomelo crude extract (PM) improves cognitive impairment by recovering the defective insulin signaling, including phosphorylation of PI3K/Akt/GSK3β^{s9} in high fat feeding combined with low dose streptozotocin (HFD-STZ) induced type 2 diabetic rats (T2DM). The results in this study demonstrate that HFD-STZ treatment induced hyperglycemia, dyslipidemia, peripheral and brain insulin resistance, and impaired cognitive function. The present study has shown that 12 weeks supplementation with PM improved cognitive function, decreased blood glucose, cholesterol, triglyceride, restored peripheral insulin sensitivity. The recovered defective insulin signaling in hippocampus involved activation of phosphorylation of IRS-1^{s312}, PI3K/Akt/ and inactivation of GSK-3β^{s9}, and a decrease in p-tau^{s396} expression and Aβ_{42} accumulation. In addition, supplementation with PM significantly increased IDE and decreased lipid peroxidation end products levels in DM2-PM rats.

Pomelo crude extract supplementation attenuates the metabolic disorders in HFD-STZ induced DM2 rats

The present results are consistent with previous studies that a combination between high fat feeding and low dose STZ injection induced hyperinsulinemia, hyperglycemia, and dyslipidemia [102-104]. The HFD-STZ induced type2 diabetic animal model, feeding high fat diet stimulated insulin resistance and injected low dose STZ caused mild pancreatic β-cells dysfunction [105]. In the present study, T2DM-rats showed metabolic disorders, including markedly elevated fasting blood glucose, HbA1C, blood cholesterol, triglyceride, serum insulin levels, and HOMA-IR index with glucose intolerance. HOMA-IR index is an index to quantify insulin resistance [106]. Wang et al (2007) demonstrated that feeding high fat/high energy diet in rats for 4 weeks induced hyperinsulinemia and mild hyperglycemia. They suggested that the mechanism of developing hyperinsulinemia in early stage of DM2 was due to a compensatory

mechanism of pancreatic β-cells in order to maintain the normal blood glucose levels [107]. In the present study, low dose of STZ (35 mg/kg B.W.) was injected which partially damaged pancreatic β-cells, and there were some surviving β-cells left. In the present results, DM2 rats showed metabolic disorders, including markedly elevated fasting blood glucose, HbA1C, blood cholesterol, triglyceride, serum insulin levels, and HOMA-IR index with glucose intolerance. HOMA-IR index is an index to quantify insulin resistance [108]. From these results of metabolic disorders, it is suggested that hyperglycemia, dyslipidemia and insulin resistance have been developed in HFD-STZ rat model that was used in this study. The HFD-STZ rat model is appropriate because it showed similar features as those seen in type2 diabetic human [109]. As shown in Figure 7–10 supplementation with PM for 12 weeks in T2DM-PM rats decreased FBG, HbA1C, blood cholesterol, triglyceride, serum insulin levels, and HOMA-IR index. Compared with the T2DM-rats, the PM supplemented in T2DM-PM-rats presented with not only a lower insulin level but also improved insulin sensitivity. These findings indicated that PM has a potential to reduce T2DM-induced hyperglycemia, hyperlipidemia and hyperinsulinemia and to improve insulin sensitivity by repairing damaged pancreatic β-cells functions and maintaining the surviving β-cells function. Apart from anti-hyperglycemia and insulin sensitizing effects, PM could decrease blood cholesterol and triglyceride, the fasting blood cholesterol, and triglyceride in T2DM-PM-rats were decreased 13.93% and 26.06%, respectively from the values before PM supplementation. Pomelo is the largest citrus fruit which rich in various nutrients and is source of bioactive compounds, as flavonoids. The major flavonoids of pomelo are neohesperidin, narigenin, and naringin [110, 111]. These flavonoids are found to exhibit hypoglycemic, antioxidant and anti-inflammatory effects [112, 113]. Previous studies have shown that pomelo extract has great effect to improve insulin secretion, insulin sensitivity and enhancing peripheral glucose uptake [38, 114]. In the present study, from HPLC analysis, the one of flavonoid from pomelo crude extract is naringin. Previous studies have shown that naringin decreased fasting blood glucose and lipid levels in animal models of diabetes [115, 116]. A study by Jung et al demonstrated that in type 2 diabetic mice, naringin reduced hyperlipidemia

and hyperglycemia by partly regulating the fatty acid and cholesterol metabolism and affecting the gene expression of glucose-regulating enzymes [117]. *In vitro* and *in vivo* studies suggest that citrus flavonoids improve insulin sensitivity and insulin action [118, 119]. A study in male Wistar rats with a high fat diet feeding developed hyperinsulinemia, hyperglycemia and insulin resistance. All of these metabolic disturbances were significantly recovered by oral supplemented with naringin [120]. Therefore, from the previous finding together with present results it is suggested that PM has the potential to restore disorders of metabolic parameters in HFD-STZ induced type2 diabetic rats as indicating by the improved hyperglycemia, hyperlipidemia and peripheral insulin resistance through partly from the action of citrus flavonoid, naringin.

Pomelo crude extract restore cognitive impairment in HFD-STZ induced type2 diabetic rats

In type2 diabetes, alterations in glucose homeostasis, insulin function and insulin secretion increase the risk of cognitive impairment as well as dementia and Alzheimer disease [6, 121]. Moreover, peripheral insulin resistance could exacerbate insulin resistance in the brain by reducing brain insulin uptake [122, 123]. In normal physiology, insulin plays important roles for various neuronal functions which are involved in the regulation of learning and memory performance [124, 125]. Insulin is transported into the brain by binding to a specific receptor at blood brain barrier (BBB). Although, an acute increase in serum insulin levels is associated with an increase in CSF and intracellular insulin levels [126, 127]. Chronic hyperinsulinemia impairs BBB function and downregulates insulin receptor at BBB reducing insulin transport to the brain which accelerates the process of cognitive deficit and neurodegeneration [128, 129].

Numerous *in vivo* studies in type2 diabetic animal models have shown that impairment in cognitive function is linked to a defective in insulin signaling [130, 131]. Insulin is an important factor that regulates blood glucose levels and uptake of glucose to peripheral tissues by activating through PI3K/Akt signaling pathway [132]. In the brain, insulin- PI3K/Akt signaling regulates synaptic activities, learning and memory functions [133]. It was shown that hyperinsulinemia and down regulation of insulin signaling in the

brain of type2 diabetic animals was associated with neurodegenerative disorders, together with the activation of GSK-3β [134, 135]. In normal tissues including brain, PI3K-Akt signaling controls GSK-3β by phosphorylating at serine 9 residue inhibiting GSK-3β activity [74]. GSK-3β is one of the most important tau kinases [136]. Brain insulin resistance in type2 diabetes is characterized by reduction in brain insulin signaling increasing GSK-3β activity [75, 135]. Furthermore, increasing GSK-3β activity increased abnormal tau phosphorylation which promoted aggregation and formation of neurofibrillary tangles and accelerates Aβ production [137]. In addition, increasing an Aβ production is mediated by decreasing level of IDE. IDE or insulin is a thiol zinc-metaloendopeptidase [138]. Previous studies have shown that IDE expression levels in the cortex and hippocampus in STZ-induced diabetic rats [21, 139] were reduced. Moreover, the current study demonstrated that in the STZ rats' brain, decreasing in IDE expression was correlated with increased accumulation of Aβ [140].

Clinical and animal studies reported that high fat diet induced peripheral and brain insulin resistance leading to impairment of cognitive function [141, 142]. Thirumangalakudi et al (2008) reported that mice fed with high fat, high cholesterol diet for 8 weeks exhibited memory deficit associated with a significant increase in Aβ deposition, increase neuroinflammatory changes and increase oxidative stress [143]. Based on these studies, brain insulin resistance is an important factor that causes the cognitive impairment in HFD-STZ induced type2 diabetic animal model. Correlated with previous studies, in the present study, HFD-STZ rats demonstrated significant learning and memory impairment associated with brain insulin resistance as presented by increased level of phosphorylated form of IRS-1 and decreased expression of downstream target PI3K, phosphorylated form of Akt and GSK-3β in the hippocampus. In addition, the present results also showed increasing in phosphorylation of tau proteins and accumulation of Aβ in hippocampus of T2DM-rats. Furthermore, the decreased expression of IDE and increased lipid peroxidation end product; malondialdehyde were found in T2DM-rats. In the present study, learning and memory performance was evaluated by the Morris water maze (MWM) test which is a test of spatial learning and

memory. As shown in Figure 12 and Figure 13, results from MWM test showed that T2DM-rats demonstrated impaired learning and memory performance by increasing escape latency time and deceasing time spent in the platform area. Interestingly, supplemented with PM in T2DM-PM-rats improved learning and memory performance as demonstrated by decreasing escape latency time and deceasing time spent in platform area which suggested that PM can improve cognitive impairment. The positive effect of PM on cognitive function was associated with reduction in $A\beta_{42}$ levels and expression of p-tau in the hippocampus.

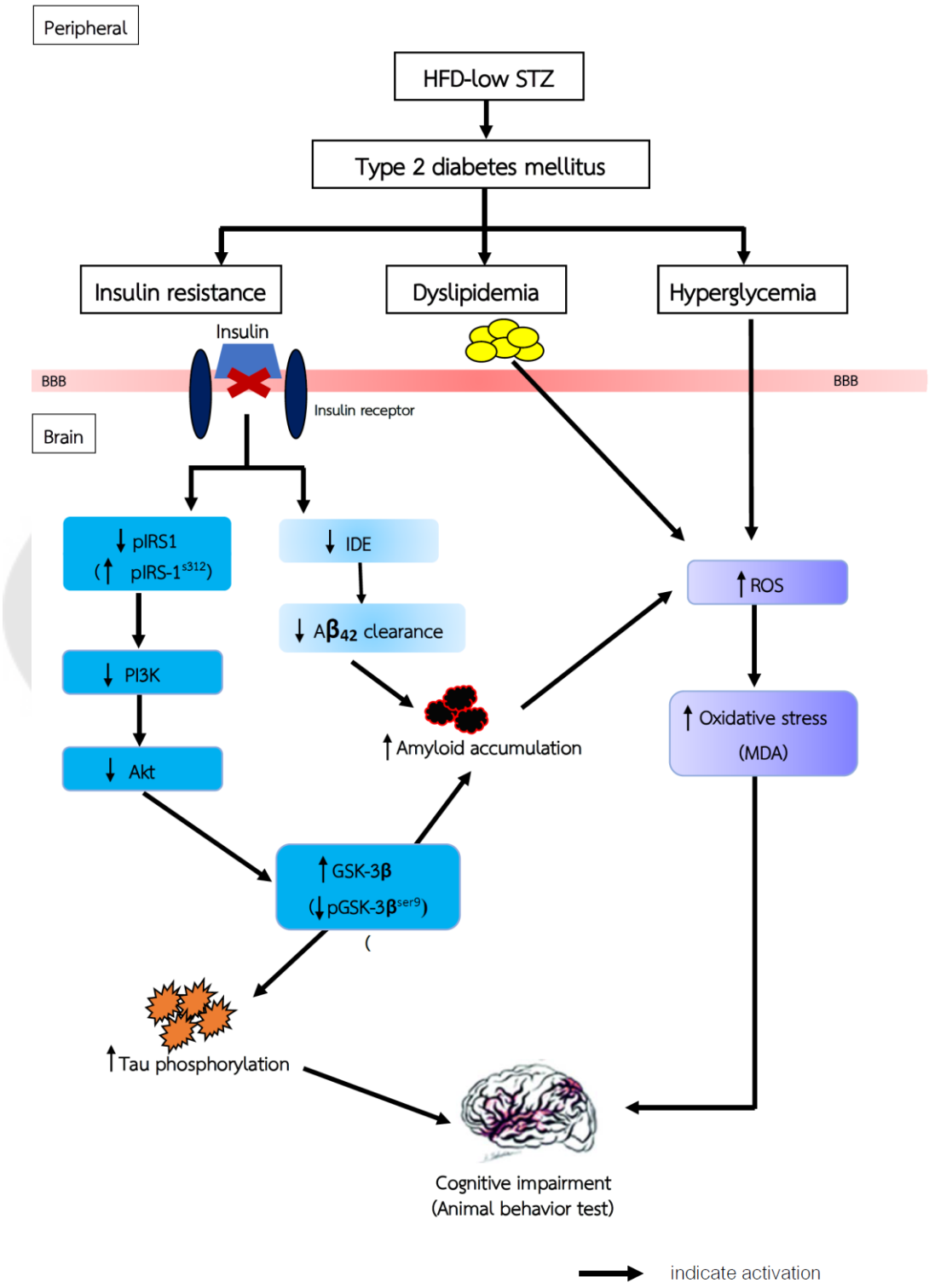
In the present study, the major biochemical compositions of pomelo crude extract were phenolic and flavonoid, naringin. The present results found the naringin in the amount of 416.70 ± 21.82 µg / g of pomelo crude extract. Several studies have shown the effects of flavonoids for health and diseases which include antioxidant, anti-tumor, anti-hyperlipidemia, anti-hyperglycemia, anti-inflammation, and neuroprotective effects [23, 144]. Anand et al (2014) found that treating to Alzheimer's disease rat model with 50 mg / kg of naringin for 21 days significantly improved cognitive impairment [145, 146]. They suggested that the positive effect of naringin on cognitive impairment was due to the reduction of oxidative stress and pro-inflammatory cytokines [147]. Recently, Wang et al (2015) demonstrated that naringin improves cognitive deficits in 20 weeks high fat feeding-induced obese mice by enhancing in neuronal insulin signaling and a reduction mitochondrial damages through the activation of AMPK [148]. Correlate with Wan et al.'s study, the expression of p-IRS-1, p-Akt and p-GSK-3β in the present study were recovered to near normal levels in the hippocampus of T2DM-PM-rats. It is indicated that PM has a great effect to recover brain insulin resistance of DM2-PM rats through p-IRS-1, p-Akt and GSK-3β pathway. Following the activation of brain insulin signaling, supplementation with PM significantly reduced Aβ and p-tau expression and improved learning and memory performance in T2DM-PM-rats. Moreover, IDE levels in hippocampus were higher in T2DM-PM-rats than those of control rats. IDE has been found to degrade both insulin [63] and Aβ proteins [149]. A recent study by Ho et al (2004) demonstrated that high fat feeding in mice promoted

insulin resistance, Aβ generation and decreased IDE activities [150]. The present study confirmed the occurrence of decreased IDE and increased Aβ productions in the brain of T2DM-rats and these two abnormal changes were recovered by supplementation with PM. Therefore, it is suggested that PM supplementation could decrease $A\beta_{42}$ productions in hippocampus by increasing IDE levels and inhibiting the GSK-$3\beta^{s9}$ activity.

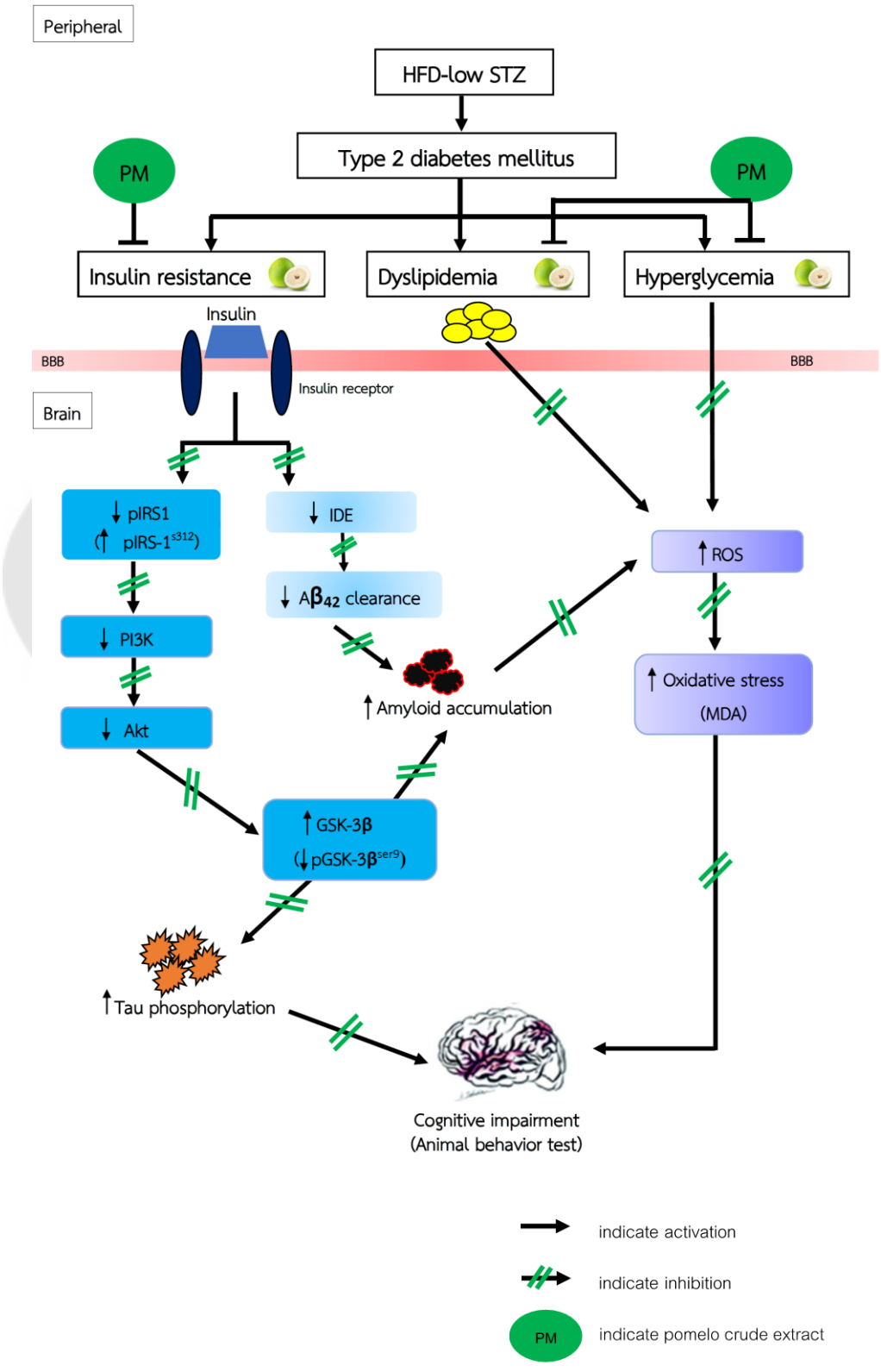
One possible mechanism by which diabetes could increase the incidence of cognitive impairment as well as dementia is oxidative stress. In type2 diabetes, hyperglycemia causes an increase in production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and oxidative stress markers, such as lipid peroxidation, associated with a decrease in antioxidant. This imbalance between ROS and antioxidant has been suggested to be responsible for the induction of cognitive impairment in diabetes mellitus [7, 27]. Oxidative stress is the major factor responsible for the disturbance in glucose and lipid metabolism, cellular damages as well as neuronal degeneration [151]. It has been shown that increased oxidative stress leads to loss of energy production of cells and tissues and has negative effect to the insulin secretion and sensitivity [152, 153]. It is well recognized that in type2 diabetes, metabolic alterations (such as enhanced plasma fatty acids, hyperglycemia, hyperinsulinemia and dyslipidemia) in peripheral insulin resistance are source of brain oxidative stress. In the brain, oxidative stress induced by increase ROS productions disrupt synaptic function and leads to cognitive impairment [154]. The enhanced diabetes-induced ROS was indirectly demonstrated by using lipid peroxidation end products, malondialdehyde (MDA) level as an indicator [155, 156]. In the present study, significantly higher malondialdehyde (MDA) levels were observed in the hippocampus of T2DM-rats. Importantly, the enhanced MDA was reduced by supplementation with PM in T2DM-PM-rats. It has been reported that naringin possess strong antioxidant, antihypertension, antidyslipidemia, and neuroprotective effects [157, 158] . In addition, to the antioxidant properties, naringin also inhibit hydrogen peroxide-induced lipid peroxidation [159]. Taken together with previous studies and the present finding, it is suggested that one

mechanism by which PM, which is rich in flavonoid; naringin, could improve cognitive function is through its antioxidant effect.

The mechanism of type2 diabetic rats induced cognitive impairment



The proposed mechanism for the effects of pomelo crude extract on type2 diabetic rats induced cognitive impairment



CHAPTER VI
CONCLUSION

The present findings indicate that high fat feeding combined with low dose STZ injection-induced type2 diabetic rats which is characterized by hyperglycemia, dyslipidemia, peripheral and brain insulin resistance. These metabolic disturbances lead disrupt learning and memory. Most important, this study has shown that 12 weeks supplementation with pomelo crude extract improves cognitive function together with decreased blood glucose, cholesterol, triglyceride and restored peripheral insulin sensitivity, and recovered defective insulin signaling in hippocampus. This recovery includes activation of phosphorylation of IRS-1^{s312}, PI3K/Akt and inactivation of GSK-3β^{s9}, and decreasing in ptau^{s396} expression and $A\beta_{42}$ accumulation. Moreover, the reduction in $A\beta$ accumulation may be involved in the mechanisms by which PM inactivates GSK-3β^{s9} activity and increases IDE level, leading to a reduced $A\beta_{42}$ production and increased $A\beta_{42}$ clearance, respectively. In addition, the present findings demonstrate the antioxidant effect of PM which is an important mechanism to revert cognitive impairment in type2 diabetes mellitus.

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APPENDIX

Table 2 Effect of Pomelo crude extract supplementation for 12 weeks on FBG level

	Fasting blood glucose (mg/dl)					
	week 2	week 4	week 6	week 8	week10	week12
Control	100.5±2.32	98±1.67	108.4±0.49	97.6±1.47	102.5±1.83	100.5±2.05
TDM2	362.6±48.12***	391.57±50.89***	381.71±45.15***	377.57±25.41***	388.29±37.81***	384.8±17.71***
T2DM-PM	354.6±37.14	214.75±20.84###	191.75±25.13###	196.5±34.24###	187±26.55###	170.8±20.29###

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control ($p < 0.001$)

indicates significantly different as compared to T2DM ($p < 0.001$)

Table 3 Effect of Pomelo crude extract supplementation for 12 weeks on metabolic parameter

	Metabolic parameters	
	HbA1C	S.insulin
Control	3.84±0.42	7.037±0.1482
T2DM	6.9±1.02**	111.9±3.338***
T2DM-PM	4.54 ±0.98##	55.85±5.136###

Data are means ± SEM (N=7)

,* indicates significantly different as compared to Control (p <0.01 and 0.001,respectively)

##, ### indicates significantly different as compared to T2DM (p <0.01 and 0.001, respectively)

Table 4 Effect of Pomelo crude extract supplementation for 12 weeks on plasma triglyceride level

	Plasma triglyceride (mg/dl)						
	Week 0	week 2	week 4	week 6	week 8	week10	week12
Control	111.2±12	108.18±13.79	110.27±7.8	115.56±12.01	122.27±17.77	113.55±4.51	127.33±4.73
DM2	166.29±18.42***	178.57±3.77***	166.33±8.54***	168±8.36***	156.57±5.01***	169.14±18.78***	154.8±12.82***
T2DM-PM	165.12±20.39###	123.33±7.2###	112.88±1.38###	124.05±2.27###	118.62±19.99###	124.25±1.55###	122.42±20.39###

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

Table 5 Effect of Pomelo crude extract supplementation for 12 weeks on plasma cholesterol level

	Plasma cholesterol (mg/dl)						
	Week 0	week 2	week 4	week 6	week 8	week10	week12
Control	138.67±12	140.45±13.79	143.64±7.8	145.18±12.9	148.27±17.77	128.67±4.51	125.67±4.73
TDM2	182±18.42	172.2±3.77	161.71±8.54	157.71±8.36	162.14±5.01	169±18.78	169±12.82
T2DM-PM	176±14.24	157.66±7.2	167.12±1.38	159±2.27	139.25±19.99#	151.75±1.55	142±20.3#

Data are means ± SEM (N=7)

# indicates significantly different as compared to T2DM (p <0.05)

Table 6 Effect of Pomelo crude extract supplementation for 12 weeks on HOMA-IR

	HOMA-IR
Control	7.0 ± 0.40
T2DM	81.49 ± 0.60***
T2DM-PM	50.29 ± 3.8*** ,###

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

Table 7 Effect of Pomelo crude extract supplementation for 12 weeks on the learning performance

	Escape latency time (Sec.)				
	Day1	Day2	Day3	Day4	Day5
Control	40.92±13.42	8.83±1.41	8.42±1.49	11±0.74	8.33±1.28
T2DM	58.21±17.58**	25.42±4.79**	26.3±9.56**	20±2.54**	16.42±2.98**
T2DM-PM	14.39±4.22###	13.5±2.72##	9.22±1.21##	7.72±0.79##	7.28±0.68##

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

##, ### indicates significantly different as compared to T2DM (p <0. 01 and 0.001, respectively)

Table 8 Effect of Pomelo crude extract supplementation for 12 weeks on memory performance

	Time spent in target quadrant (sec.)
Control	31.186±1.86
T2DM	23.57±2.24**
T2DM-PM	38.33 ± 1.96##

Data are means ± SEM (N=7)

** indicates significantly different as compared to Control (p <0.01)

indicates significantly different as compared to T2DM (p <0.01)

Table 9 Effect of Pomelo crude extract supplementation for 12 weeks on the pIRS-1^{312} levels in hippocampus tissues

	pIRS-1^{312} expression
Control	0.4±0.02
T2DM	0.88±0.02**
T2DM-PM	0.37±0.01###

Data are means ± SEM (N=7)

** indicates significantly different as compared to control (p <0.01)

indicates significantly different as compared to T2DM (p <0.001)

Table 10 Effect of Pomelo crude extract supplementation for 12 weeks on the IDE levels in hippocampus tissues

	IDE level
Control	0.56±0.01
T2DM	0.34±0.008***
T2DM-PM	0.43±0.009###

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control (p <0.001)

indicates significantly different as compared to T2DM (p <0.001)

Table 11 Effect of Pomelo crude extract supplementation for 12 weeks on the $A\beta_{42}$ accumulation in hippocampus

	$A\beta_{42}$ level
Control	1.84±0.21
T2DM	4.72±0.26***
T2DM-PM	2.93±0.14**,###

Data are means ± SEM (N=7)

,* indicates significantly different as compared to control (p <0,01 and 0.001 ,respectively)

indicates significantly different as compared to T2DM (p <0.001)

Table 12 Effect of Pomelo crude extract supplementation for 12 weeks on malondialdehyde in the hippocampus

	MDA level
Control	0.60 ± 0.52
T2DM	2.77 ± 0.11***
T2DM-PM	0.80 ± 0.40###

Data are means ± SEM (N=7)

*** indicates significantly different as compared to Control ($p < 0.001$)

indicates significantly different as compared to T2DM ($p < 0.001$)

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