ANTIGLYCATION PROPERTIES OF *MORINGA OLEIFERA* LEAF EXTRACT *IN VITRO*

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Food and Nutrition
Department of Nutrition and Dietetics
Faculty of Allied Health Sciences
Chulalongkorn University
Academic Year 2014

ฤทธิ์ของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง

นางสาวพรพิมล นันท์ธนะวานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาอาหารและโภชนาการ ภาควิชาโภชนาการและการกำหนดอาหาร
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	ANTIGLYCATION PROPERTIES OF *MORINGA OLEIFERA* LEAF EXTRACT *IN VITRO*
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Field of Study	Food and Nutrition
Thesis Advisor	Sathaporn Ngamukote, Ph.D.

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พรพิมล นันท์ธนะวานิช : ฤทธิ์ของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง (ANTIGLYCATION PROPERTIES OF *MORINGA OLEIFERA* LEAF EXTRACT *IN VITRO*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: สถาพร งามอุโฆษ, 173 หน้า.

ภาวะระดับน้ำตาลในเลือดสูงเรื้อรัง เป็นสาเหตุของการเกิดกระบวนการไกลเคชั่นโดยไม่อาศัยเอนไซม์ จากการจับกันระหว่างน้ำตาลรีดิวซ์ซิ่ง และหมู่อะมิโนของโปรตีน เกิดเป็นแอดวานซ์ไกลเคชั่น เอ็น โปรดักส์ (advanced glycation end products) ผลงานวิจัยในอดีตยืนยันแน่ชัดว่าผลิตภัณฑ์นี้เป็นปัจจัยสำคัญที่ส่งผลต่อการเกิดภาวะแทรกซ้อนในหลอดเลือดขนาดเล็ก และหลอดเลือดขนาดใหญ่ในผู้ป่วยเบาหวาน มะรุม (*Moringa oleifera*) เป็นหนึ่งในพืชสมุนไพรที่เป็นที่รู้จักและนิยมใช้อย่างแพร่หลายในประเทศไทย ซึ่งหลักฐานการศึกษาทางวิทยาศาสตร์พบว่า มะรุมมีฤทธิ์ในการป้องกันหรือรักษาโรคเบาหวานได้หลากหลายกลไก อย่างไรก็ตามยังไม่มีการศึกษาใดศึกษาฤทธิ์ของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นโดยการบ่มโปรตีนอัลบูมินจากวัว (Bovine serum albumin) ในฟอสเฟตบัฟเฟอร์ซาไลน์ ร่วมกับน้ำตาลกลูโคส (0.5 M) ฟรุกโตส (0.5 M) หรือสารเมทิลไกลออกกซอล (1 mM) ที่อุณหภูมิ 37°C กับสารสกัดใบมะรุมที่ความเข้มข้น 0.5-2.0 มิลลิกรัมต่อมิลลิลิตร จากผลการทดลองพบว่าสารสกัดใบมะรุมมีสารสำคัญจำพวกโพลีฟีนอลและฟลาโวนอยด์ อาทิ เช่น เฟอรูลิก แอซิด (225.04 ไมโครกรัม) รูทิน (0.09 ไมโครกรัม) เคอซิทิน (0.41ไมโครกรัม) และแคมเฟอรอล (0.15 ไมโครกรัม) เมื่อศึกษาความสามารถในการยับยั้งการเกิดไกลเคชั่น พบว่า สารสกัดใบมะรุมสามารถยับยั้งการเกิดโปรตีนไกลเคชั่น โดยลดผลิตภัณฑ์ที่เป็นฟลูออเรสเซนต์และไม่ใช่ฟลูออเรสเซนต์ (เอนแอปซีลอน-คาร์บอกซีเมทิลไลซีน) (N^{ε}-(carboxymethyl) lysine (CML)) อย่างมีนัยสำคัญตามความเข้มข้นที่เพิ่มขึ้น ร่วมกับการลดลงของระดับฟรุกโตซามีน (fructosamine level) อีกทั้งสารสกัดจากใบมะรุมสามารถยับยั้งการเกิดการตกตะกอนของโปรตีนโดยลดระดับของการสร้างโครงสร้างเบต้า อะไมลอยด์ (β-amyloid structure) นอกจากนั้นยังสามารถป้องกันการเกิดโปรตีนออกซิเดชัน โดยลดระดับของการเกิดโปรตีนคาร์บอนิล (protein carbonyl) รวมทั้งป้องกันการลดลงของหมู่ไทออล (thiol group) จากผลการทดลองสามารถสรุปได้ว่า สารสกัดจากใบมะรุมอาจนำไปประยุกต์ใช้เป็นสมุนไพรทางเลือกเพื่อป้องกันการเกิดภาวะแทรกซ้อนอันเนื่องมาจากกระบวนการไกลเคชั่นในผู้ป่วยเบาหวาน

ภาควิชา โภชนาการและการกำหนดอาหาร ลายมือชื่อนิสิต .....

สาขาวิชา อาหารและโภชนาการ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5576854037 : MAJOR FOOD AND NUTRITION

KEYWORDS: ANTI-GLYCATION, MORINGA OLEIFERA, MEDICINAL PLANT, 173 COMPOUNDS

PORNPIMON NUNTHANAWANICH: ANTIGLYCATION PROPERTIES OF *MORINGA OLEIFERA* LEAF EXTRACT *IN VITRO*. ADVISOR: SATHAPORN NGAMUKOTE, Ph.D., pp.

Chronic hyperglycemia causes non-enzymatic glycation between reducing sugars and amino groups of proteins, resulting in production of advanced glycation end products (AGEs). Strong evidences reveal that AGEs are important factors responsible for both microvascular and macrovascular diabetic complications. *Moringa oleifera*, one of the most medicinal plants that are commonly used in Thailand, has been shown to have the favorable effects in the prevention or treatment of diabetes through various mechanisms of action. However, anti-glycation property of *Moringa oleifera* leaf extract *in vitro* has not been investigated. Therefore, the aim of this study was to examine the ability of *Moringa oleifera* aqueous leaf extract (MOE) on protein glycation by incubated bovine serum albumin (BSA) in 0.1 M phosphate buffer saline (pH 7.4) with 0.5 M glucose, 0.5 M fructose or 1 mM methylglyoxal with or without MOE (0.5-2.0 mg/mL) at 37ºC. It was found that MOE contained polyphenol and flavonoids including ferulic acid (225.54 µg), rutin (0.09 µg), quercetin (0.41 µg), and keamferol (0.15 µg). Inhibitory effects of MOE on protein glycation was demonstrated by a significant dose-dependent reduction in the formation of fluorescence and non-fluorescence AGEs (N^{e}-(carboxymethyl) lysine (CML)), with concomitant a marked decrease in fructosamine level. In addition, MOE also inhibited the cross-linking of protein by reducing β-amyloid structure formation ($P<0.05$). Moreover, MOE prevented the oxidation of protein manifested by reducing protein carbonyl and increasing protein thiol in a dose-dependent manner ($P<0.05$). In conclusion, our findings indicate the possibility of using MOE as the therapeutic agent for preventing glycation-related diabetic complications.

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

Academic Year: 2014

ACKNOWLEDGEMENTS

First of foremost, I would like to express my deepest appreciation to my thesis advisor, Dr. Sathaporn Ngamukote for her patience, continued support and encouragement. She advised me not only how to succeed in the research career path but also how to succeed in everyday life.

I wish to express my sincere gratitude to Associate Professor Dr. Sirichai Adisakwattana and Dr. Kittana Mäykenen for the useful comments, the opportunity and all experiences in the laboratory to and also everyone in Adisakwattana research group.

I would like to thank my thesis committee members for the suggestions, comments, and their kindness during oral defense. Additionally, I would also like to thank faculty members in Faculty of Allied Health Sciences, Chulalongkorn University for their devoted instruction and warm welcome to be one of their students.

My sincere thanks also go to Thailand Research Fund for the financial support and The Halal Sciences Center, Chulalongkorn University for instrumental support of this research.

Last but not least, and most importantly, words cannot explain how thankful I am to be a part of the family. I would like to thank Flight Sergeant First Class Lamom and Mrs. Phorntawal Nunthanawanich, as well as my brother Mr. Auttapon Nunthanawanich for their supporting, encouraging, and standing beside me all the time I was dispirited. Moreover, I would like to express my special thanks to my Mekwichai and Nunthanawanich family especially my uncle Dr. Jirasith Mekwichai, M.D. who is the precedent in academic field and inspired me to be a master degree student.

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CHAPTER I

INTRODUCTION

1.1 Background and significance of this study

Diabetes mellitus (DM) is one of the top ten causes of death worldwide and the number of people with diabetes is expected to increase from 171 million in 2000 to 366 million in 2030 (Association, 2013). Diabetes mellitus is a chronic non-communicable disease caused by deficiency or diminished effectiveness of insulin secretion in pancreatic beta cells or both resulting in high blood glucose level (hyperglycemia) (Association, 2013). Hyperglycemia has been shown to play pivotal roles in diabetes-associated vascular diseases both microvascular complications, including retinopathy, nephropathy, and neuropathy and macrovascular complications such as coronary artery diseases, atherosclerosis, and peripheral vascular diseases (Ceriello, 1999). These deleterious complications are the leading cause of mortality and morbidity in this population. Although multiple metabolic pathways have been proposed to explain the adverse effects of hyperglycemia induced vascular complications, one of the most importance metabolic pathways is non-enzymatic glycation of proteins, lipids or nucleic acids which leads to the formation of advanced glycation end products (AGEs) (Vanessa Fiorentino, Prioletta, Zuo, & Folli, 2013).

Advanced glycation end products (AGEs) are a complex of heterogeneous group of molecules that are formed from non-enzymatic glycation of carbonyl group

of a reducing sugar with an amino group of proteins, lipids, or nucleic acids (Peyroux & Sternberg, 2006). The presence of AGEs are closely related to hyperglycemia and are regarded a major component responsible for diabetes-related complications (Peyroux & Sternberg, 2006). Glucose and fructose are the most reactive reducing sugars that react spontaneously with amino groups of proteins to AGEs. Many studies have tended to focused on using glucose and fructose to react with protein causing glycation in vitro. Many studies have reported that fructose accelerates glycation reaction faster than glucose (Semchyshyn, Miedzobrodzki, Bayliak, Lozinska, & Homza, 2014). Moreover, fructose can be formed through the polyol pathway, conversion of glucose to fructose with the formation of sorbitol as an intermediate product (Hamada, Araki, Horiuchi, & Hotta, 1996). Recently, a number of studies have also focused on methylglyoxal (MG), a highly reactive dicarbonyl compound, inducing AGEs formation because it is an intermediate product of glucose autoxidation, lipid peroxidation and also glycation reaction. Moreover, strong evidences showed that an elevated MG levels and an increased AGEs formation in plasma of patient with diabetes are correlated with the development of diabetic complications (Paul Thornalley, Langborg, & Minhas, 1999).

Many studies have shown that AGEs can generate reactive oxygen species (ROS), leading to increase oxidative stress (Tan, Forbes, & Cooper, 2007). Moreover, the accumulation of AGEs in various types of tissues in the body causes tissues damages and diabetic complications through both modify protein conformation (covalent cross-link formation) and interact with advanced glycation end products receptor (cell surface receptor-mediated pathways) (Rojas, Mercadal, Figueroa, & Morales, 2008) .

Thus, anti-glycating agents of natural or synthetic origin may be useful to delay or prevent diabetes-related complications and AGES-related diseases (Brownlee, Vlassara, Kooney, Ulrich, & Cerami, 1986). To ameliorate the level of AGE formation is using drug therapy. Aminoguanidine (AG) is one kind of therapeutic agents for the prevention of AGEs formation due to its ability to cleave AGEs-induced chemical cross-links (Brownlee et al., 1986). However, some studies have been shown side effects of AG including flu-like symptoms, a rare vascularitis, gastrointestinal disturbances and anemia. Therefore, much attention has focused on medicinal plants with anti-glycation property.

Moringa oleifera (Ma-rum) is the medicinal plant commonly found and used in tropical countries such as India, Afghanistan, as well as Thailand. It has been shown various pharmacological effects (Adisakwattana & Chanathong, 2011; Jaiswal, Kumar Rai, Kumar, Mehta, & Watal, 2009; Verma, Vijayakumar, Mathela, & Rao, 2009b). Many studies have reported the flavonoid contents such as keamferol, quercetin, ferulic acid, gallic acid, rutin, caffeic acid as well as other phenolic compounds in multi-part of Moringa tree. Additionally, antioxidant properties in previous studies have also found in Moringa oleifera aqueous leave extract by scavenging DPPH, superoxide radical, and also hydroxyl radical (Rout & Banerjee, 2007; Siddhuraju & Becker, 2003). Moreover, anti-diabetic properties of *Moringa oleifera* aqueous leave extract have also found both in vitro and in vivo studies (Siddhuraju & Becker, 2003; Vinson & Howard III, 1996) . The possible mechanisms involving in anti-diabetic action may be due to inhibition of alpha-glucosidase activity (Adisakwattana & Chanathong, 2011).

However, the anti-glycation of *Moringa oleifera* leaf extract has not been elucidated. To the best of our knowledge, this research is the first investigation on the mechanism of *Moringa oleifera* leaf extract against protein glycation.

Objectives of this study

1. To identify the phenolic constituents of MOE using High-Performance Liquid Chromatography.
2. To investigate the inhibitory effect of MOE on glucose, fructose, and methylglyoxal - induced protein glycation in vitro.

Hypotheses of this study

1. The MOE may be containing polyphenolic compound especially in flavonoid substituents.
2. The MOE can inhibit glucose, fructose, and methylglyoxal-induced protein glycation in in vitro.

Expected benefit of this study

Nowadays, Thai people are really concern about the degenerative disease such as DM because the trend is growing up rapidly and the relation with another disease is also dangerous. Thus, alternative medicines are also interesting to use as a therapeutic agent to prevent the DM complications because the drug are also terminated as well as side effect containing. Natural compounds are also remarkable in this moment. *Moringa Oleifera* leaf extract is one kind of the popular medicinal plant in Thailand because it is easy to find in every provincial part and relate to previous pharmacological properties such as antioxidant, anti-diabetic, hypolipidaemic effect and anti-glycation properties representing in this present study. It will provide additional data of medicinal herb regarding anti-glycation mechanisms that may provide a novel therapeutic strategy for the prevention of diabetic complications and can be used as the functional product.

CHAPTER II

LITERATURE REVIEWS

2.1 Definition and classification of Diabetes Mellitus (DM)

Diabetes mellitus (DM) is a group of metabolic diseases which is characterized by hyperglycemia resulting from defect in insulin secretion, insulin action, or both. Chronic hyperglycemia is clearly demonstrated as a major cause for diabetic complications both in macrovascular and microvascular diseases (Ceriello, 1999; Kaneko et al., 2005; Vanessa Fiorentino et al., 2013). According to the American Diabetes Association in 2013; DM is classified into 4 clinical types (Association, 2013).

❖ **Type 1 DM** is commonly occurs in childhood and adolescence which defined as the cellular-mediated autoimmune destruction of pancreatic β-cell leading to absolute insulin deficiency. Thus, the DM patients must require an insulin replacement therapy.

❖ **Type 2 DM** is characterized by insulin resistance which depends on the function of insulin including insulin secretion and its sensitivity. This type of diabetes mostly develops in the adult which is caused by family history, obesity, increasing age, non-physical activity. The combination of diet/lifestyle changes and medical therapies are commonly used for the treatment.

❖ **Gestational DM** is usually occurring in the second or third trimester of pregnancy. Underlying the mechanism, free fatty acid accumulation during pregnancy leading to secret the insulin abnormality which is the cause of GDM. As the ongoing epidemic of obesity and diabetes has led to more type II diabetes in women of

childbearing age, the number of pregnant women with undiagnosed type 2 diabetes has increased.

❖ **Other specific types of DM** include virus infection, genetic defect of beta-cell or insulin action, exocrine pancreas disease and also drug-or-chemical –induced diabetes

2.2 Diabetic complications

High level of glucose in the blood plasma, known as hyperglycemia, is the primary cause of many complications in diabetic patient because of the spontaneous reaction between glucose and the protein in endothelial wall which can develop the diabetic progression is advance glycation end product (AGE) (Vanessa Fiorentino et al., 2013). AGE production attached to the ligand of AGE receptor (RAGE) which induced oxidative stress by increased the ROS productivity in mitochondria. From that session, ROS also activated the PPAR generation that also related to rise the glycolytic intermediate compound thereby influx the polyol pathway, activate the protein kinase (PKC) level by increasing triacylglycerol (DAG) which effect to lower the level of endothelial nitric oxide synthetase (eNOS) by causing endothelial dysfunction due to blood flow abnormality (Tan et al., 2007; Vanessa Fiorentino et al., 2013). As well as increasing NADPH oxidases can enhance the level of ROS effecting to cause multiple effects such as lipid peroxidation, protein nitration, DNA damage and advanced glycation endproducts (AGEs) activation. Moreover, ROS can directly damage the tissue in human body by increasing PKC which effect to change

protein expression (Kaneko et al., 2005). Thus, this present study will focus on AGEs formation involving in diabetic complications.

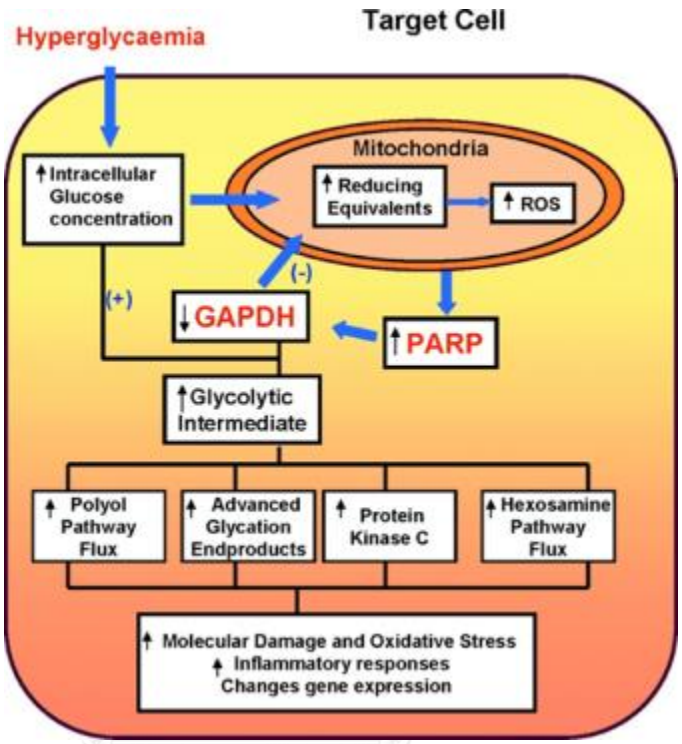


Figure 1. The major cause of diabetic complications resulting from hyperglycemia (Cil et al., 2012).

❖ **Macrovascular diseases**

Macrovascular diseases are the leading cause of death in the world (Organization). The pathogenesis of macrovascular complications is various determinants such as age, tobacco use, alcohol consumption, dietary behavior, hypertension, dyslipidemia including long term of high glucose-containing in the

blood plasma. Hyperglycemia obstructed the eNOS responsibility by increasing the ROS production (Ceriello, 1999).

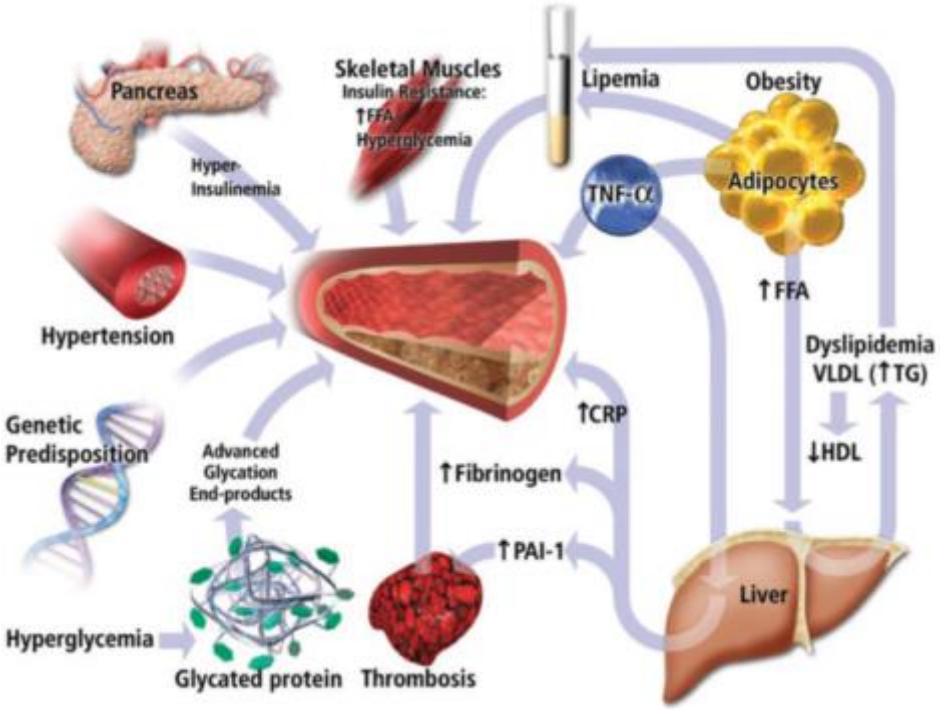


Figure 2. The multiple mechanisms related to macrovascular disease (Sudic et al., 2006).

❖ **Cardiovascular disease**

There is several risk factors unionize on the arterial wall to stimulate atherosclerosis in diabetic patients. Hyperglycemia is mainly cause to step up the progression of chronic inflammation by several pathways (Aronson, 2008). Chronic inflammation and the arterial wall injury is response to produce foam cell accumulated in the vascular system though LDL oxidation (Yoshida & Kisugi, 2010). In diabetic patients, insulin resistance is the stage that pancreases produce more insulin as known as hyperinsulinemia which is the risk factor of arteriopathy. In

addition, the elevation of free fatty acid is mostly found in diabetic obese patient that related to promote liver fat accumulation. Pro-inflammatory cytokine such as TNF-α is also released from adipocyte to activate atherosclerosis (Bruunsgaard, Skinhøj, Pedersen, Schroll, & Pedersen, 2000). Moreover, the binding between sugar containing in the blood and protein in arterial wall is cause AGE accumulation which stimulate protein modification such as collagen and endothelium thickness (Bruunsgaard et al., 2000).

❖ **Cerebrovascular disease**

DM plays a major role to cause cerebrovascular disease by increasing oxidize LDL formation throughout in the blood vessel which narrowed the carotid artery that located in head and neck area (Yan, Ramasamy, & Schmidt, 2008). Cerebrovascular disease was divided into 2 classifications. First of all, Transient Ischemic Attack (TIAs) was a transient part of blood supplying to the brain disorder because of neurological dysfunction which occurs mostly in diabetic patients (Palumbo, Elveback, & Whisnant, 1977). Second, stroke was a neurological deficit of cerebrovascular cause that persists beyond 24 hours or is interrupted by death within 24 hours which is the immediately and emergency response in human body. Dietary control, blood pressure manipulation and also tobacco recantation were generally suggested to follow before and after the disease progression (Palumbo et al., 1977).

❖ **Peripheral artery disease**

Additionally, peripheral artery disease or PAD was the dysfunction of blood supply to lower limb in the body. The obstruction of plaque is narrowed the arterial wall which blocked the blood flowing to peripheral organ such as feet and legs that means to express as muscle cramping, numbness, pain. That symptom could be associated to get the wound and also noticeable to change in color (blue or pale) in diabetic patients (Ouriel, 2001) .

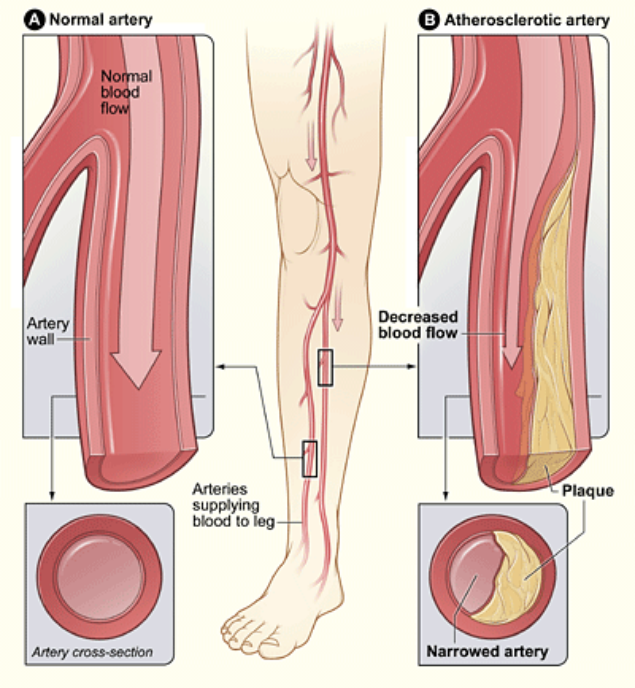


Figure 3. (A) Normal blood flow in leg arteries

(B) Narrowed blood flow because of plaque in the atherosclerotic arteries.

❖ **Microvascular diseases**

❖ **Retinopathy**

Due to long term of hyperglycemia in diabetic patients, the high level of glucose induces the flux of sugar though polyol pathway which can activate sorbitol formation that related to osmotic pressure in the eyes. From that reason, the basement membrane has been thickening and loss of function (Aiello et al., 1994). Moreover, AGE can also cause diabetic retinopathy as well because of ROS production during the process and non-enzymatic reaction between protein and glucose in the pericyte of the eyes (Brownlee, 2001). The adverse consequence of diabetic retinopathy developed the incidence case of blindness for 12% in the adults (20-64 years) (Jeganathan, Wang, & Wong, 2008).

❖ **Neuropathy**

In term of neuropathy is commonly known as the symptom and/or sign of peripheral nerve dysfunction in chronic diabetic patients (Ewing & Clarke, 1982). The symptom of this disease is included insensitivity to expose the pain/sharp pain/ or temperature, prickling sensation as well as loss of balance control. Uncontrolled glycemic level directly effect to the accumulation of polyol which undergo to form oxidative stress by dicarbonyl compound. On the other hand, PKC was increased from the DAG accumulation-derived hyperglycemia status that is one of caused associated to decreased eNOS level which effect to cause blood flow abnormalities (I.-K. Lee, Koya, Ishi, Kanoh, & King, 1999). Chiefly, mention to peripheral organ that

mostly affected by nerve dysfunction is foot. First step to treat that disorder is maintaining the glucose level in the blood system to prevent other nerve damage. Amputation is the last priority to save diabetic patients life from its complication (Pecoraro, Reiber, & Burgess, 1990).

❖ **Nephropathy**

Kidney is the key organ to filtrate some waste product though circulatory system. Long duration of hyperglycemia certainly affect to overmuch function. Osmotic pressure raised the level up via glucose reabsorption. Protein starts to leak in the urine as commonly known as micro albuminuria (Mogensen, 1987). The screening is attained 24-hour of urinary albumin excretion by defined in the range of 30-299 mg (Gall, Hougaard, Borch-Johnsen, & Parving, 1997). For long term of uncontrolled this situation, it obviously to cause proteinuria and uremia in the last stage of kidney failure (Mogensen, 1987). It also related to the increasing of blood pressure and damaging the kidney glomeruli. AGE also produced though AGE-induced programmed mesangial cell apoptosis and change extracellular matrix protein to patronize glomerular overfunction. Moreover, AGE productions also excessively stimulate insulin-like growth factor 1 and modify form of growth factor-β to encourage glomerular fibrosis which resulting in reduced surface area for filtration (Eddy, 2000).

2.3 Advanced Glycation End Products

❖ The production of AGE reaction

The end product (AGE) or Millard reaction occur both inside (endogenous) an outside (exogenous) of the human body. Regarding with exogenous glycation, cooking at high temperature such as frying, baking etc. can induce AGE formation (Siddhuraju & Becker, 2003). In tem of endogenous, the reaction happens when the functional group of protein (amino acid) binding with the carboxylic group of the reducing sugar resulting in forming an unstable Schiff's base and rearranges to form a stable ketoamine, called Amadori product (Maillard, 1912). That product can then be oxidized and generated dicarbonyl compound which can further react with protein to form AGE. Several studies have been shown the association between AGE and degenerative diseases such as cardiovascular complication of DM, neurodegenerative diseases, and also connective tissue disorders (Wu, Huang, Lin, & Yen, 2011).

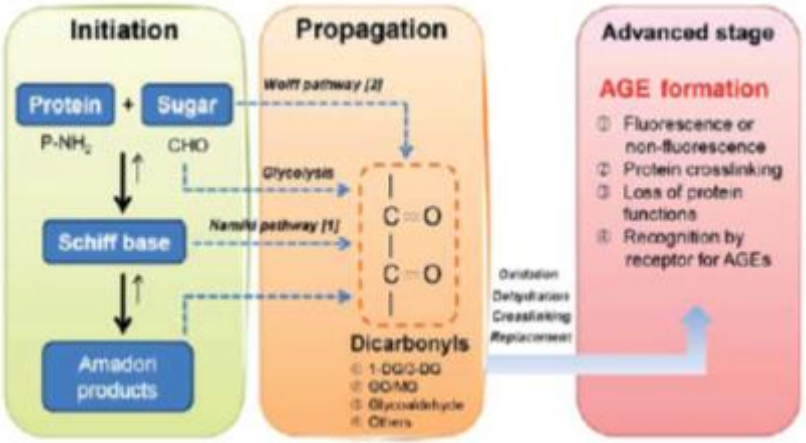


Figure 4. Pathway of AGE formation.

❖ **Schiff base**

Schiff base is the first product representing when amino acid triggering with the aldehyde group of reducing sugar which forming double bond between nitrogen atom and carbon atom that is the non-stable molecule owing to reversible product all time of reaction. So, the Schiff base can rearrange to produce Amadori product which is more stable (Yim, Yim, Lee, Kang, & Chock, 2001).

❖ **Amadori product**

Amadori product is one of product in the initiation stage or first stage of AGE reaction which undergoes from the Schiff base that transform to oxidize with another protein called as protein oxidation which supersede in term of dicarbonyl compounds such as glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) (Peyroux & Sternberg, 2006). The bonding occur between ketone group of the glucose and the amine group of the protein known as fructosamine which is the indicator to determine the level of HbA_1C in diabetic patients.

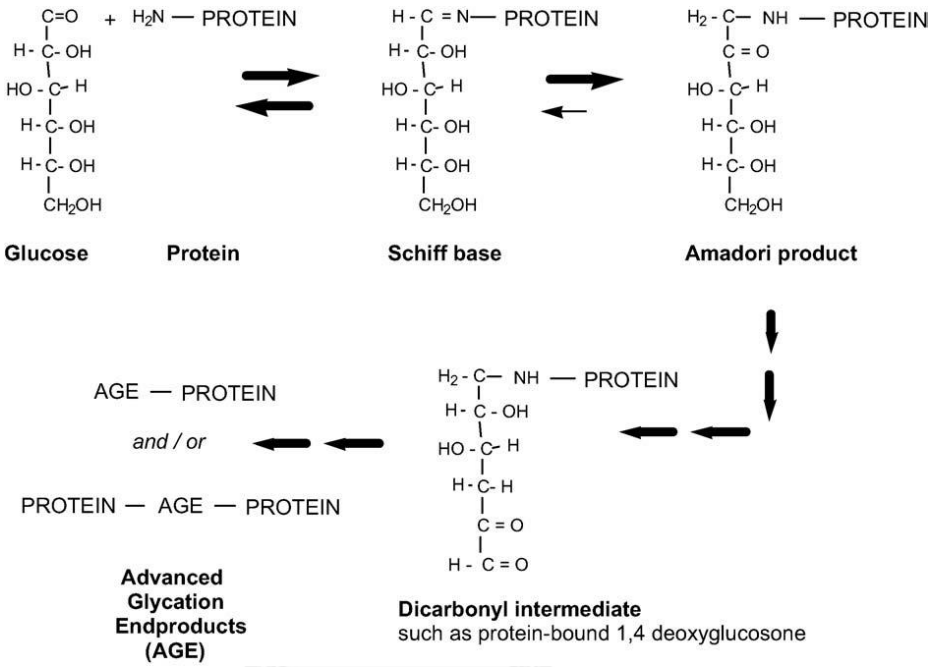


Figure 5. Pathway of AGE reaction resulting from glucose (Peyroux & Sternberg, 2006).

The end products of AGE occur in the last stage of the reaction which characterize by 2 major types of chemical structure. There are fluorescence and non-fluorescence AGE.

❖ **Fluorescence AGE**

Fluorescence AGE presents in many chemical structures as shown in figure5, pentosidine is the most abundant compound which can detect by spectrofluorometer. In diabetic patient, the number of pentosidine is growing up followed by the level of hyperglycemia. The accumulation of pentosidine cannot detect when the level of glucose less than 50mM in hyperglycemia status, the pentosidine However, it is only one percent or less of the total crosslink structures formed under physiological conditions (Sugiyama et al., 1998).

❖ **Non - fluorescence AGE**

N^{ε}—carboxymethyl lysine (CML) is the major product in AGE reaction. CML is one of indicator to progress the diabetic complications. Previous study has been exhibited that the accumulation of CML in diabetic patient developed the progression of chronic kidney failure (Baynes & Thorpe, 1999). That is the serious topic to focus on the compounds which can inhibit the formation of AGE production.

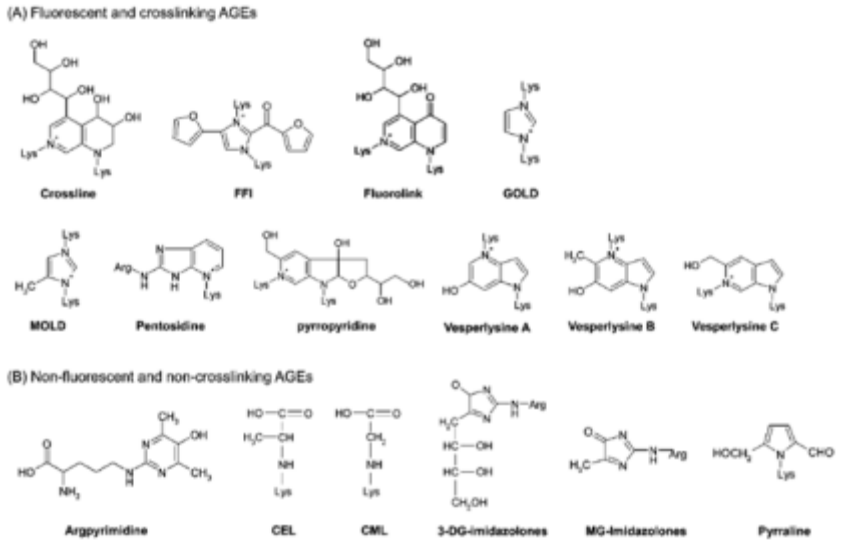


Figure 6. (A) Fluorescence AGE

(B) non-fluorescence AGE chemical structures (Wu et al., 2011).

❖ **Protein oxidation**

Protein oxidation was contributed while a lysine side chain of the protein attached to aldehyde group of the reducing sugar (Wolff, Jiang, & Hunt, 1991). The structure of protein was modified when the glucose concentration still up to 25mM in the BSA model which mimic as a hyperglycemia status in human body. Thiol group is the sulfhydryl-containing organic compound (C-SH or R-SH) which react as antioxidant in circulatory system (Lean et al., 2003). In contrast to carbonyl accumulation, it has been shown the relation to link with diabetic pathological complications (Baynes & Thorpe, 1999).

❖ **Protein aggregation**

β - amyloid cross structure was the indicator of protein aggregation which modified by pH changing, temperature changing, metal ion exposing, and also glycating reaction (ref). Focused on glycation process, the proportion of protein aggregation was quite much in diabetic patients for 2-3 folds when compared to healthy people, eventually it much larger proportion (90%) in the diabetic without uncontrolled glycemic diet. AGE receptor was such a primary transporter for amyloid protein which binds to AGE and develop the neurodegenerative progression. Alzheimer's disease developed from the aggregation of protein in the blood supplying to the brain which mostly occurred in aged-diabetic patients because the progression took a long time to expression (Irvine, El-Agnaf, Shankar, & Walsh, 2008).

Thus, AGE formation is the crucial mechanism which important pathological consequence in diabetic patient because of their pathway following;

1. The increased of intracellular AGE production is certainly affected to the protein and decreased the vascular cell function that is the majority of the microvascular and neurodegenerative diseases.

2. The increased of extracellular AGE production can interact with matrix-matrix, matrix-cell, cell-cell to rise the ROS accumulation which linked to the presented of diabetic vessels especially in basement membrane component in glomerular and retina endothelial cell. Thus, AGEs production induced abnormalities of the vascular cell by increasing fluid across carotid artery, decreasing vascular adhesion molecules, increasing permeability of basement membrane.

3. The increased of AGE accumulation altered AGE receptor that encourage to change in gene expression by increased adhesion of inflammatory cells, thrombomodulin, tissue factor, and vascular cell adhesion molecule-1 (VACM-1). Moreover, ROS also generated during the reaction, it directly effect to the signaling of the gene via increasing NF-KB which response to injure the gene.

2.4 Glycating agent

❖ Glucose

Glucose is one kind of reducing sugar that reacts non-enzymatically with amino residues of protein resulting in AGE formation under the physiological of hyperglycemic condition. The previous study has been shown that the glucose oxidation release ROS as a key role of AGE progression. During that reaction, α - oxoaldehyde, 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO) are the intermediate compound to activate the AGE accumulation. 3-DG is the major component to rearrange Amadori product by fructose-3-phosphate in polyol pathway to form immidazolone, pyrrarine, and CML (Paul Thornalley et al., 1999).

Moreover, MGO reacts with lysine to form methylglyoxal-lysine-dimer (MOLD) as well as carboxyethyl-lysine (CEL). In term of GO, it produce the various types of AGE production such as pentosidine, glyoxal-lysine-dimer (GOLD), CML, and another non-fluorescence AGE (Ahmed, 2005). The production of ROS in human cell are related to activate aldose reductase, protein kinase C (PKC), nuclear factor- κB (NF-κB) and also induce diacylglycerol to develop the pathologies of diabetic degenerative diseases as shown in figure 6.

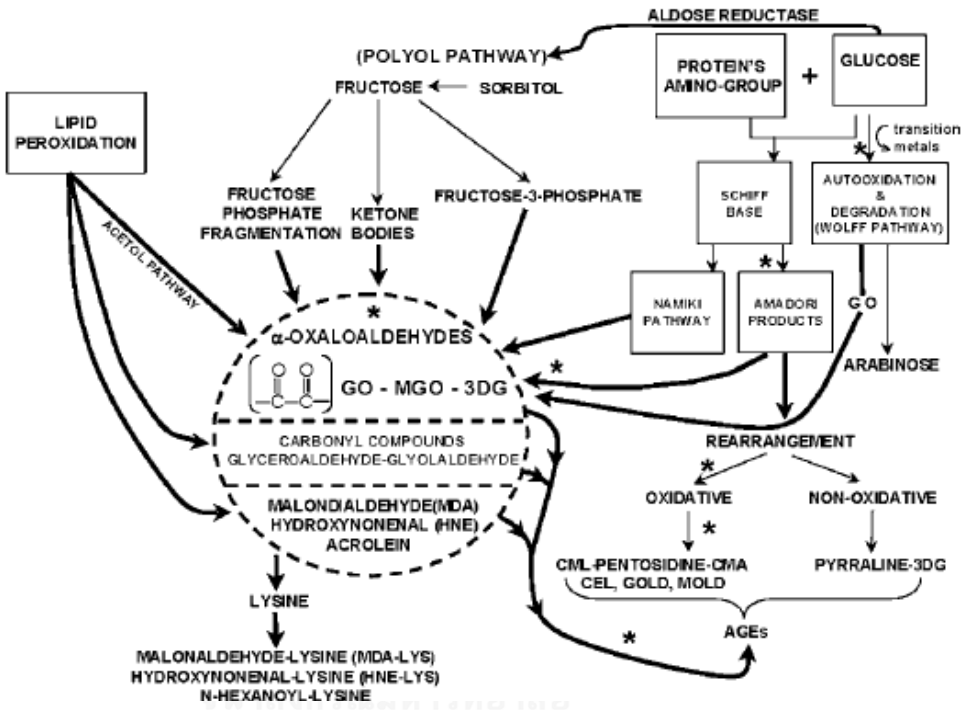


Figure 7. The association between glucose and dicarbonyl compound linked to AGE formation.

❖ **Fructose**

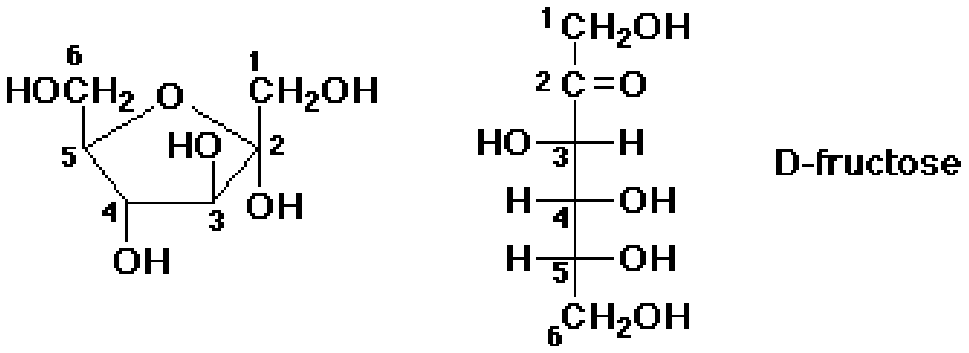


Figure 8. The chemical structure of fructose

Several studies has been shown the information about glycating agent, fructose; one kind of reducing sugar, metabolize by sorbitol-aldose reductase pathway or primarily known as polyol metabolic pathway (A. Y. Lee & Chung, 1999). As shown in figure 9, the reaction generates sorbitol by using aldose reductase to switch unused glucose form and oxidizes sorbitol to be fructose by using sorbitol dehydrogenase. During the pathway, NADH was produced from NAD^+ and multiple forms of dicarbonyl compounds were evoked (Brownlee, 2001). Hence, uncontrolled blood glucose in diabetic patient also happened because hexokinase can turn back that molecule by phosphorylating fructose to glycolysis pathway to form fructose-6-phosphate (Mayes, 1993). For another reason, the production of fructose is come from glucose auto-oxidation as shown in figure7. Moreover, the trend of high fructose consumption in Thailand also be expandable, fructose is replacement sucrose in sugary beverage that effect to cause

degenerative disease such as cardiovascular complication in diabetic patient (Bray, Nielsen, & Popkin, 2004). Fructose also is the interesting agent to observe the AGE reaction in *vitro* model because the rate of AGE reaction of fructose is much faster than glucose because of in amadori product rearrangement, the formaldehyde reaction (at C-1 and C-3) that increase the potential of fructated BSA-derived AGE in the higher rate than that glucose (ref). Some evidence explained about the Schiff base adduction, fructose attained steady-state earlier that why fructose got ability to increase the fluorescence AGE and rate differ than glucation (Suarez, Rajaram, Oronsky, & Gawinowicz, 1989).

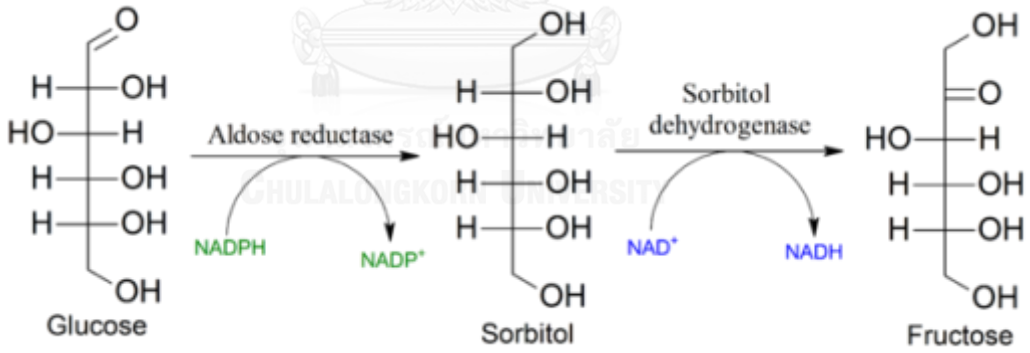


Figure 9. Sorbitol-aldose reductase pathway.

❖ **Methylglyoxal**

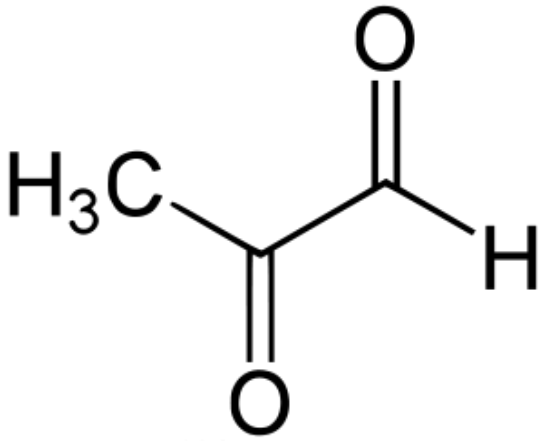


Figure 10. The chemical structure of methylglyoxal

(Paul Thornalley et al., 1999).

MG is one of glycating agent because of its structure that contains two group of dicarbonyl as shown in figure 8. It commonly found in both natural source and human body. External source, Coffee, alcoholic beverage as well as Manuka honey which is the major source of MG container and it found antibacterial activity but still unknown mechanism (Russell, 1996). In human body, there are 3 possible sources; first is formed in situ in the plasma. Second is a release from cells and third is outflow from injured cells (Kalapos, 2013). From recent studies, it has been shown the significant level in MG-derived AGEs in diabetic patients when compared to the healthy subjects (Figure 11). The accumulation of MG in plasma protein, renal glomeruli, retina and also peripheral nerve typically operated the progression in diabetic incurrent diseases (P Thornalley et al., 2003)

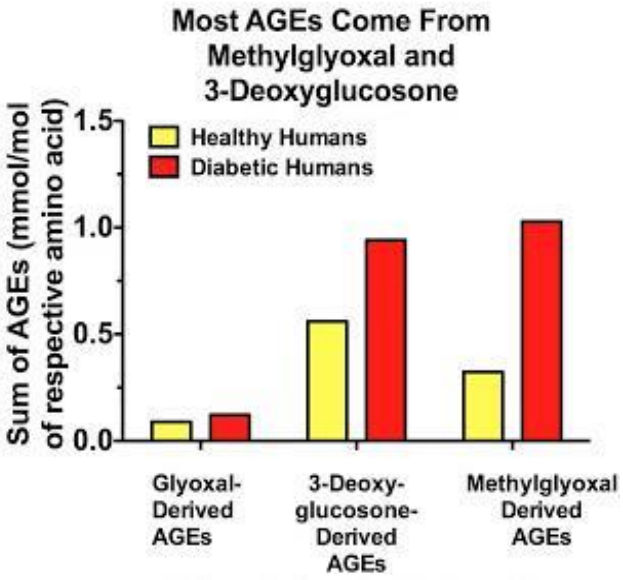


Figure 11. Concentrations of AGE-derived by GO, 3-DG and MG in healthy and diabetic subjects. (P Thornalley et al., 2003)

The high concentration of MG in the plasma accelerated to produce carbonyl stress in diabetic patient. That situation leads to cause protein, lipid and also DNA modification (P Thornalley et al., 2003)

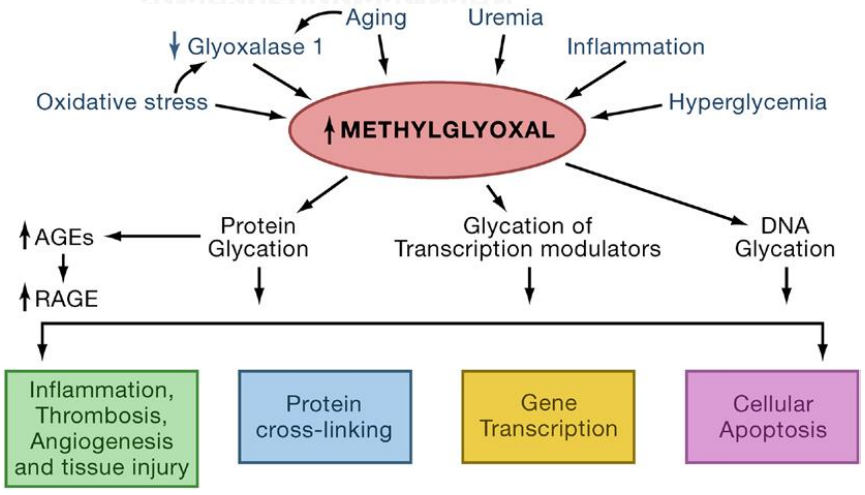


Figure 12. The relationship between MG on protein glycation and gene transcription (Ramasamy, Yan, & Schmidt, 2006)

Moreover, MG plays a role as an intermediate compound after glucose auto-oxidation, lipid peroxidation, and also protein oxidation as shown in figure 13 and it also related to proteome as well as genome. Exactly, protein glycation directly motivate protein cross-link structure that is the main effect to cause Alzheimer's disease. However, MG also provokes tumor cell apoptosis as shown in figure 12. Therefore, MG is known as the highest reactive compound to induce protein glycation in in *vitro* study (Ramasamy et al., 2006).

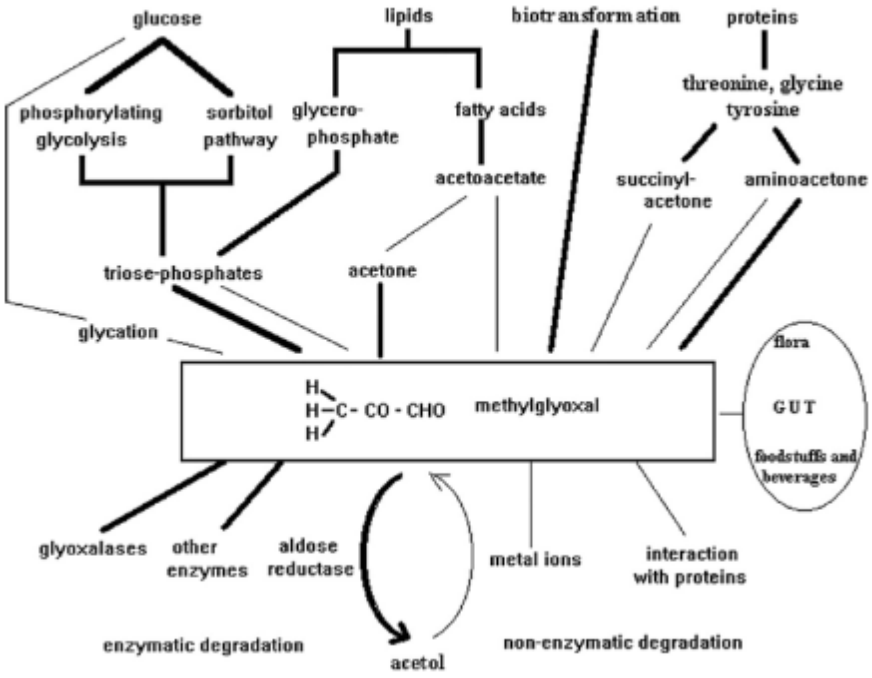


Figure 13. MG is the intermediate compound in several pathways.

2.5 Anti-glycating agent

2.5.1 Drug

❖ **Aminoguanidine**

Aminoguanidine (AG) also known as Pimagedine; is the first drug using as AGEs inhibitor (14). AG can inhibit AGEs formation by acting as protein binding with reducing sugar in initiation stage and trapping the reactive carbonyl groups in propagation stage of the reaction. That also prevent diabetic vascular complications in animal studies by decreasing AGEs formation via inhibit NOS as well as improving arterial wall properties with increasing vascular elasticity. In contrast to previous studies, it has been shown the clinical side effect such as flu-like symptoms, a rare vascularitis, gastrointestinal disturbances and anemia (Corbett et al., 1992). Glomerulonephritis was found when taking high dose (300mg/day) of AG while 150 mg/day was not associated with any toxicity. Thus, AG was withdrawn from the crucial phase III of clinical trials because of safety concerns and apparent lack of efficacy (Wardle, 1979).

❖ **Aspirin**

Aspirin, an analgesic to relieve the pain, has been shown the inhibitory effect on pentosidine formation in glucose-induced protein glycation in protein collagen model as well as in diabetic dog. The mechanism has been

investigated in chelating metal ion and scavenging the oxygen radical and metal ion (Watala et al., 2005).

2.5.2 Vitamin

❖ **Vitamin B6**

Pyridoxamine or commonly known as vitamin B6 was explicated as an AGE inhibitor. The mechanisms were described in the previous evidences about protein adduction in initiation stage and also trapped dicarbonyl compound as well as chelated metal ion. Moreover, vitamin B6 also scavenged ROS during AGE reaction. Although it was investigated in vitro model, it still lacked the data in in vivo study and human trial (Booth, Khalifah, & Hudson, 1996).

❖ **Vitamin C**

Ascorbic acid as well-known as vitamin C also plays a major role in the antioxidant effect. Moreover, it has been shown the inhibitory effect on protein glycation and lipid peroxidation in diabetic rat. Effects of vitamin E and vitamin C on non-enzymatic glycation and peroxidation in experimental diabetic rats. In addition, vitamin C is also called as an antioxidant vitamin to prevent dicarbonyl stress which is one of stage in AGE reaction. Antioxidant vitamins prevent oxidative and carbonyl stress in an animal model of obstructive sleep apnea (Wu et al., 2011).

❖ **Vitamin E**

Vitamin E or α - tocopherol is also well documented known as lipid soluble vitamin. It has been shown strong pharmacological properties such as anti-glycation effect on glycated hemoglobin in diabetic type II patients with modest dose supplementation (100IU/day) (Maxwell & Lip, 1997). The effect of modest vitamin E supplementation on blood glycated hemoglobin and triglyceride levels and red cell indices in type I diabetic patients.

2.5.3 Antioxidant

During oxidation occurred, free radical is the key factor damaged the function of the cell that is the serious effect for human life because oxidative stress play an important role to develop the progression in various diseases. Antioxidant is the molecule that blocks another free radical throughout that reaction. Thus, it also gives the beneficial effect on many pathways related to scavenging activity, chelating effect (Tiwari, 2001).

❖ **Polyphenol**

Polyphenol is the huge class of natural organic chemical compounds which contain aromatic benzene ring (phenyl ring) and hydroxyl group (-OH). Depended on that structure, the classification is divided into 5group as shown in figure14. One of division is focused on flavonoid. It is mostly

abundant in fruit, vegetable, cereal, nut, seed, and also herbs. The previous evidences have been shown abilities of polyphenol and its derivatives such as caffeic acid, ferulic acid, chlorogenic acids and cinnamic acid (Chen & Ho, 1997; Clifford, 1999).

The possible biochemical abilities are included metal ion chelating reaction, and also ROS scavenging activity.

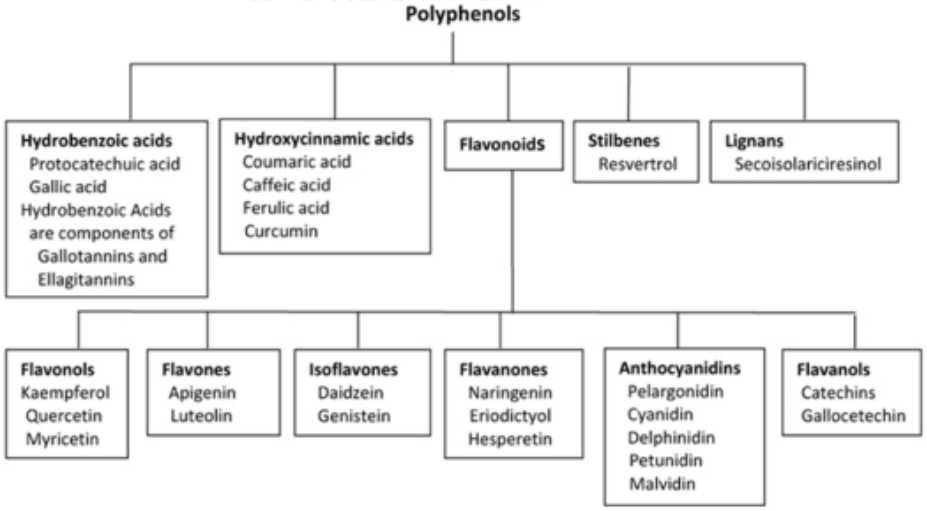


Figure 14. The classification of polyphenol (Okuda, 1999).

❖ **Flavonoid**

According to plant-rich in polyphenol especially in flavonoid has been reported about their pharmacological properties on antioxidant capacity, antihypertensive effect, anti-diabetic activity, anti-proliferation, hypolipidemic effect and also anti-glycation activity (Wu et al., 2011). Due to the primary structure of flavonoid (the benzene backbone ring and the group of hydroxyl), is the important keyword to speculated that as antioxidant capacity involve scavenging free radical activity, chelating the metal ion, and also binding to macromolecules or interaction with other kinds of antioxidants. There are many subclasses of flavonoid division such as Flavonols, flavones, flavanones, and flavanols.

❖ **Flavonols**

Flavonols structure contains 3 rings of hydroxyflavone backbone. It included rutin, quercetin, keamferol, myristein, rhamnetin, morin as well as galangin. In term of rutin, astragalin, and isoquercetin is classified into flavonol subgroup which is called flavonol glycoside. Multiple evidences have been shownpharmacological properties such as antiinflammation, antibacterial, antioxidant, and antithrombogenic properties (Matsuda, Wang, Managi, & Yoshikawa, 2003).

❖ **Flavones**

Flavone structure contained 2-phenylchromen-4-one-(2-phenyl-1-benzopyran-4- one). The main flavone represented in apiginin, luteolin as well as tangeritin which found in natural dietary such as green leafy vegetable and also citrus fruits. The scientific based have been found the strongly effect of flavone on antioxidant, anti-diabetic, anti-cancer, anti-inflammation, and also neurogenesis activity (La Casa, Villegas, Alarcon de La Lastra, Motilva, & Martın Calero, 2000).

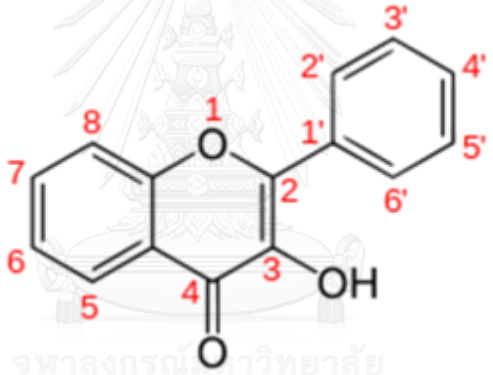


Figure 15. Flavonol backbone.

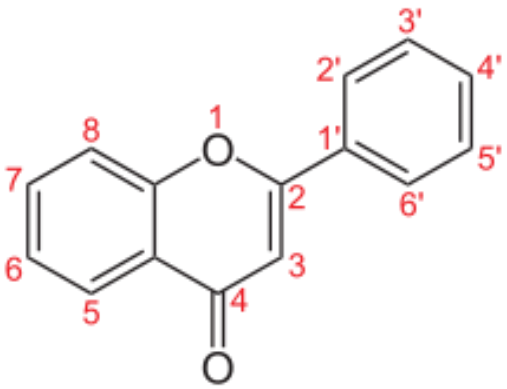


Figure 16. Flavone backbone

2.5.4 Polyphenol and anti-glycation effect

From the recent studies, there was various evidences support that plant rich in polyphenol has the beneficial effect on anti-glycation activity. Cinnamic and its derivatives in *Ilex paraguariensis*, *Chrysanthemum morifolium, and Chrysanthemum indicum* inhibit the production of fluorescence AGE and CML in *in vitro* model (Bartoli, Simontacchi, Guiamet, Montaldi, & Puntarulo, 1995; I.-S. Kim, Ko, Koppula, Kim, & Choi, 2011; Lunceford & Gugliucci, 2005) Ferulic acid was mostly found in rice, wheat, oat can protect against protein oxidation by reduced HSA α-helix structure that caused structural changes in protein. Moreover, it can inhibit AGE formation by approximately 90% in vitro systems. Gallic, chlorogenic and p-coumaric acid also showed the inhibitory effect on AGE formation and protein oxidation (Kähkönen et al., 1999). As the same way to flavonoid, it is the important naturally phytochemical compound containing in the plant kingdom. Quercetin is commonly known as flavonol aglycone which mostly presented in citrus fruit. Besides, it presented in main composition in guava leaf that effect on anti-glycation properties more than AG (Urios, Grigorova-Borsos, & Sternberg, 2007).

Interestingly, rutin is a subgroup of flavonoid that can inhibit AGE formation in collagen type I induced by glucose *in vitro* and also effect on lipoprotein glycation by increasing the resistance of LDL to HG/Cu (II) mediated oxidation (Cervantes-Laurean et al., 2006). Apiginin and luteolin also inhibit towards aldose reductase and AGEs formation (Cervantes-Laurean et al., 2006). In addition,

keamferol resulted to suppressed the AGE formation by modulated the attachment between AGE and AGE receptor which related to NF-KB activation which down regulated the pro-inflammatory genes through the suppression of AGE-induced NADPH oxidase activation (J. M. Kim, Lee, Kim, Yu, & Chung, 2010).

2.6 Moringa oleifera

Picture 1. Characteristic of drumstick tree.

Moringa oleifera or drumstick tree or horseradish tree as called as Marum in Thailand is the plant most widely cultivated species of a monogeneric family, the *Moringaceae* that is the edible plant native to Asia and South East Asia including Thailand. Numerous reports have shown the multi-part of the tree contain some nutritional and phytochemical properties especially in leaves contain more Vitamin A than carrots 4 times, more calcium than milk 4 times, more iron than spinach, more Vitamin C than oranges 7 times, and more potassium than bananas 2 times, and that the protein quality of Moringa leaves rivals that of milk and eggs. Including to carotenoid that represent in the leaves as well (Sreelatha & Padma,

2009). From that reason Moringa trees have been used to battle malnutrition especially among infants and nursing mothers in some countries and also promising as a food source when the dry season begin (Siddhuraju & Becker, 2003). Briefly, from nutritional properties of Moringa tree may be called as multi-purpose medicinal plant which contains nutritional and socio-economic values.

2.6 Pharmacological properties of *Moringa oleifera*

❖ Antioxidant properties.

Many previous studies have shown about the antioxidant activity. It depends on the extraction process such as aqueous extract, methanol or ethanol extract as well as the part of the tree. Moringa seed, leaves and flower contained high level of gamma and **α**-tocopherol which is the most active an abundant constituent that can reduce lipid peroxidation (Ahmed, 2005). Hydroalcoholic extract of *Moringa Oleifera* pods can increase the levels of cytochrome P_{450}, catalase and glutathione-peroxidase. For Moringa leaves have shown about phytochemical analysis such as polyphenolic compounds presented in gallic acid, chlorogenic acid, ellagic acid, ferulic acid, and flavonoids contents like keamferol, quercetin and rutin by HPLC techniques (Bharali, Tabassum, & Azad, 2003; Driskell, Neese, Bryant, & Bashor, 1982; Verma, Vijayakumar, Mathela, & Rao, 2009a). Both *in vitro* and *in vivo ,* the aqueous extract of leaf was able to show DPPH,

superoxide, hydroxyl radical scavenging, ferrous ion chelation and lipid peroxidation in a concentration-dependent manner because of theirs antioxidant (Adisakwattana & Chanathong, 2011; Verma et al., 2009a).

Thus, this is the reason how the *Moringa oleifera* can be used as the therapeutic agent for hypoglycemic, anti- diabetic and antihypertensive.

❖ **Antihypertensive properties.**

On the basis of their pharmacological properties of *Moringaoleifera,* the ethanolic of Moringa pod 30mg/kg has the antihypertensive effect from thiocarbamate and isothiocyanate glycosides (Faizi, Siddiqui, Saleem, Aftab, & Shaheen, 1998). There are another phytochemical compounds containing in *Moringa oleifera* Crude aqueous leaf extract of Moring*a* family has blood pressure lowering effect among in systolic, diastolic and mean arterial blood pressure in the dose dependent manner (10, 20, 30, 40 mg/kg) in normotensive anaestitized guinea pigs (Sulaiman et al., 2008). For another reason, *Moringa oleifera*; plant rich in flavonoid such as quercetin, keamferol also have the beneficial effect on reducing blood pressure.

❖ **Hypolipidemic effect.**

Regarding to the studies about hypercholesterolemia, *Moringa oleifera* leaf extract can inhibit alpha-glucosidase, cholesterol esterase, formation of cholesterol micellization, and bile acid binding to lowering lipid profile involving this mechanism *In vitro* study (Adisakwattana & Chanathong, 2011). In animal study, administration of the crude leaf extract of *Moringa oleifera* along with high-fat diet decreased the high-fat diet-induced increases in serum, liver, and kidney cholesterol levels by 14.35, 6.40% and 11.09% respectively (Ghasi, Nwobodo, & Ofili, 2000). For clinical trial, *Moringa oleifera* leaf extract can reduce total cholesterol, non-HDL-C by having orally 4.6g per day for 50days (Nambiar, Guin, Parnami, & Daniel, 2010) and lower the level of total cholesterol, LDL-C and VLDL-C while having 8g per day for 40 days by orally in form of tablets (Arun Giridhari, Malathi, & Geetha, 2011). From this reason, it may be supported to use the *Moringa oleifera* leaf extract as the therapeutic agent for dyslipidemia.

❖ **Anti-inflammation and anti-proliferation properties.**

From the roots of *Moringa oleifera* isolation can inhibited the production of TNF-alpha and IL-2 which is the pro-inflammatory marker. Apart from the leaf extract and flower, *M. oleifera* leaves have been modulated humoral and cellular immunity in rats and mice (Sharifudin et

al., 2013; Sulaiman et al., 2008). Some studies have exhibited strong anti-inflammatory properties in rodent models of chemically induced inflammation of the paw (Faizi et al., 1998). From the previous study, it provides a biochemical mechanism underlying the usage of *Moringa oleifera* leaf extract as a therapeutic agent in lung cancer therapy (Gupta et al., 2010).

❖ **Anti-diabetic properties.**

Nowadays, medicinal plants are popular to use as the drug to lower the level of glucose in the blood because of their pharmacological effect. *Moringa oleifera* leaf extract is the most widely known as diabetic therapeutic plant that also have shown many previous studies among *in vitro, in vivo* and clinical trials. Inhibiting of α–glucosidase is one of mechanism involving anti-diabetic activity (Adisakwattana & Chanathong, 2011). When treated Streptozotocin (STZ)-inducing rats by *Moringa oleifera aqueous* leaf extract have shown a daily dose of 300mg/kg-body weight reduced fasting plasma glucose and post-prandial glucose by 69and 51%, respectively relative to untreated controls (Ghasi et al., 2000). Additionally, 3 months supplementation with *Moringa oleifera* leaf tablets in human study can decrease blood glycated hemoglobin (HbA1c) by 0.4% point relative to the baseline (Jaiswal et al., 2009)The treatment of *Moringa oleifera* leaf

extract can lower the glycemic response in post-prandial plasma glucose that indicating to hypoglycemic effect (Arun Giridhari et al., 2011). If the *Moringa oleifera* leaf extract can effect on lowering blood glucose level both *in vitro* and *in vivo* study, it may be used as the therapeutic for the degenerative disease of diabetes mellitus.

2.7 Safety and toxicology of *Moringa oleifera*

In term of toxicity of the *Moringa oleifera* leaf extract, the leaf extract can protect liver damage in rats given an over dosage of acetaminophen (Sharifudin et al., 2013). Safety evaluation studies showed no toxicity of the extracts up to a dose of 100 mg/kg body weight (Goh & Cooper, 2008). In the same way, the supra-supplementation levels of *Moringa oleifera* is 3,000 mg/kg body weight. However, an intake level below 1,000 mg/kg body weight is safe by feeding in *in vivo* study (Awodele, Oreagba, Odoma, Teixeira da Silva, & Osunkalu, 2012). From the previous studies that shown many beneficial effects of *Moringa oleifera* aqueous leaf extract demonstrating in human study, in vitro study and also in vivo study in several disease especially in the major of diabetes mellitus, but no study has been investigated about anti-glycation effect. Resulting from this study, this research will be the first study to focus on the anti-glycation properties of *Moringa oleifera* aqueous leaf extract.

CHAPTER III

MATERIALS AND METHOD

3.1 Material

❖ **Plant material**

Moringa oleifera leaf was collected in December of 2013 from local areas (Nongkhame district) of Bangkok, Thailand. The plant was authenticated at Department of Botany, Faculty of Science, and Chulalongkorn University, Thailand. The herbarium number of the plant is A014172 (BCU).

❖ Chemical	Company
1-deoxy-1-morpholino-D-fructose (DMF)	Sigma-Aldrich CO. (St. Louis, MO, USA)
2, 4-dinitrophenylhydrazine (DNPH)	Ajax finechem (Taren Point, Australia)
5, 50-dithiobis-(2-nitrobenzoic acid) (DTNB)	Calbiochem (Darmstadt, Germany)
Acetonitile	Merck (Darmstadt, Germany)
Aluminium chloride	Merck (Darmstadt, Germany)
Aminoguanidine	Sigma-Aldrich CO. (St. Louis, MO, USA)

Chemical	Company
Bovine Serum Albumin	Sigma-Aldrich CO. (St. Louis, MO, USA)
Catechin	Sigma-Aldrich CO. (St. Louis, MO, USA)
Ethanol	Merck (Darmstadt,Germany
Ethyl acetate	Fisher (Loughborough, LE, UK)
Folin reagent	Sigma-Aldrich CO. (St. Louis, MO, USA)
Formic acid	Merck (Darmstadt, Germany)
Fructose	Fisher (Loughborough, LE, UK
Gallic acid	Fluka (St. Louis, MO, USA)
Glucose	Ajax finechem (Auckland, New Zealand)
Guanidine hydrochloride	Calbiochem (Darmstadt, Germany)
Hydrochloric acid (HCL)	Loba chemie (Mumbai, India)

Chemical	Company
L-cysteine	Sigma-Aldrich CO. (St. Louis, MO, USA)
Methylglyoxal solution (MGO)	Sigma-Aldrich CO. (St. Louis, MO, USA)
Monosodium phosphate (NaH_2PO_4)	Qrec chemical co, Ltd. (New Zealand)
Disodium phosphate (Na_2HPO_4)	Ajax finechem (Auckland, New Zealand)
Sodium chloride (NaCl)	Ajax finechem (Auckland, New Zealand)
Sodium carbonates (Na_2Co_3)	Ajax finechem (Auckland, New Zealand)
Sodium hydrogen carbonates ($NaHCO_3$)	Qrec chemical co, Ltd. (New Zealand)
Sodium nitrite ($NaNO_2$)	Qrec chemical co, Ltd. (New Zealand)
Sodium hydroxide (NaOH)	Ajax finechem (Auckland, New Zealand)
Sodium azide (NaN_3)	Merck (Darmstadt, Germany)
Thioflavin T	Sigma-Aldrich CO. (St. Louis, MO, USA)

Laboratory equipment	Company
Spectrofluorometer	Perkin Elmer (Waltham, MS, USA)
Spectrophotometer	Perkin Elmer (Waltham, MA, USA)
High – performance liquid chromatography (HPLC)	Shimadzu Corporation (Kyoto, Japan)
pH meter	Thermo Scientific, Inc. (Waltham, MA, USA)
Laboratory refrigerator	Sanden intercool (Thailand)
Orbital shaker	Labnet international, Inc. (Edison, NJ, USA)
Vortex	Gemmy industrial corp. (Taipei, Taiwan)
Spray dry machine	Eyela world (Tokyo, Japan)

❖ **Miscellaneous**

CML ELISA assay kit	Cell Biolabs, INC (San Diego, CA, USA)
Distilled water	
Auto pipette	
Desiccator, Plastic ware	

3.2 Chemical preparations.

3.2.1 Preparation of *Moringa oleifera* leaf extract

Chemical

Moringa oleifera leaf	250	g
Distilled water	3	L

Method

Step1. The dried *Moringa oleifera* leaf (250g) was weighted and boiled with 3 lit of distilled water 2 times for 3h at 97°C.

Step2. After that, pooled extracts was sieved though the cheesecloth and kept in the refrigerator (4°C) overnight.

Step3. The solution filtered through Whatman No.1 filter paper under vacuum.

Step4. Kept the solution at the room temperature before subjected to a spray dryer SD-100 (Eyela world, Tokyo Rikakikai Co., LTD, Japan) to obtain the extract powder. The spray drying condition, inlet and outlet air temperature were set at 160 °C and 90 °C, respectively.

3.2.2 Preparation of Phosphate buffer saline (0.1 M PBS)

Chemical (10x (0.1 M) PBS, 500 mL)

NaCl	43	g
Na_2HPO_4	11.62	g
NaH_2Po_4	2.71	g

Distilled water

Method

Step1. Together weighted the Na_2HPO_4 and NaH_2Po_4 in the same beaker as well as dissolved the solution with distilled water.

Step2. NaCl was dissolved in another beaker with distilled water.

Step3. Pooled the solution in the beaker and added distilled water approximately adjusting at 400 mL.

Step4. Magnetic stirrer was used for dissolved it well.

Step5. The buffer was adjusted pH at 7.4.

Step6. Volumetric flask was used to adjusted the volume at 500 mL

Step7. The solution was diluted 10-folds before used. In order to prepare 1x PBS (0.1 M PBS) (1000 mL), mixed the solution in this ratio distilled water: 10x PBS = 900: 100 mL. After homogeneous, kept the solution at the room temperature in Duran bottle.

3.2.3 Preparation of bovine serum albumin (BSA)

Chemical

BSA	2	g
0.1 M PBS pH 7.4	100	mL

Method

Step1. BSA (2 g) was weighted and dissolved in 100 mL of PBS pH 7.4 adjusting volume by using volumetric flask.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.4 Preparation of 0.5 M glucose solution

Chemical

Glucose	49.5	g
0.1 M PBS pH 7.4	250	mL

Method

Step1. Glucose (49.5 g) was weighted and dissolved in 25 mL of PBS pH 7.4 adjusting volume by using volumetric flask.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.5 Preparation of 0.5 M fructose solution

Chemical

Fructose	49.5	g
0.1 M PBS pH 7.4	250	mL

Method

Step1. Fructose (49.5 g) was weighted and dissolved in 25 mL of PBS pH 7.4 adjusting volume by using volumetric flask.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.6 Preparation of 1 mM Methylglyoxal solution

Chemical

40% Methylglyoxal solution	66	µL
0.1 M PBS pH 7.4	100	mL

Method

Step1. Methylglyoxal solution was mixed with 100 mL PBS adjusting volume by using volumetric flask.

Step2. The mixed solution was inversed 2-3 times for dissolved it well.

3.2.7 Preparation of Aminoguanidine (AG)

Chemical (stock concentration = 25 mg/mL)

AG	250	mg
0.1 M PBS pH 7.4	10	mL

Method

Step1. Aminoguanidine (250 mg) was weighted and dissolved in 10 mL of PBS pH 7.4 adjusting volume by using volumetric flask.

Step2. Magnetic stirrer was used for dissolved the solution well.

Step3. The solution was diluted to be 1 mg/mL by using 40 µL in the tube of glycated protein.

3.2.8 Preparation of *Moringa oleifera* solution

Chemical (stock concentration = 100 mg/mL)

Moringa oleifera aqueous leaf extracts (MOE)	1.5	g
0.1 M PBS pH 7.4	15	mL

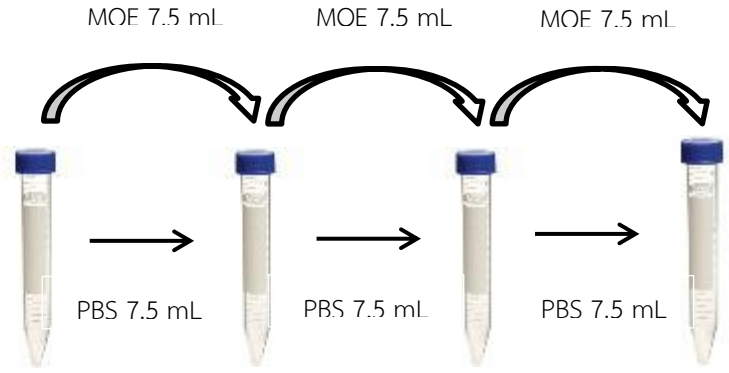
Method

Step1. MOE (1.5 g) was weighted in the conical tube 15 mL.

Step2. PBS pH 7.4 (15 mL) was added into the tube and vortex was used to dissolve the solution clearly.

Step3 2- fold dilution was done in conical tube 15 mL by starting from stock solution (100 mg/mL = 2, 1.5, 1, 0.5 mg/mL)

Stock concentration



100 mg/mL 50 mg/mL 25 mg/mL 12.5 mg/mL

3.2.9 Preparation of Folin-Ciocalteu reagent

Chemical

10x Folin-Ciocalteu reagent	2.5	mL
Distilled water		

Method

Step1. 10 x Folin-Ciocalteu reagent (2.5 mL) was diluted by distilled water adjusting the volume in volumetric flask 25 mL.

Step2. The solution was inversed 2-3 times for well dissolving.

*The reagent should keep in the room temperature with darkness before used.

3.2.10 Preparation of 7.5% Na_2CO_3

Chemical

Na_2CO_3	7.5	g
Distilled water		

Method

Step1. Na_2CO_3 (7.5 g) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 100 mL.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.11 Preparation of gallic acid as a standard

Chemical (stock concentration)

Gallic acid	5	mg
Distilled water		

Method

Step1. Gallic acid (5 mg) was weighted on the weighting paper and dissolved with distilled water in micro centrifuge tube 1mL.

Step2. Vortex was used to mix the solution clearly.

3.2.12 Preparation of 5% $NaNO_2$

Chemical

$NaNO_2$	7.5	g
Distilled water		

Method

Step1. $NaNO_2$ (7.5 g) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 100 mL.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.13 Preparation of 10% $AlCl_3$

Chemical

$AlCl_3$	10	g
Distilled water		

Method

Step1. $AlCl_3$ (10 g) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 100 mL.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.14 Preparation of 1 M NaOH

Chemical

NaOH 4 g

Distilled water

Method

Step1. NaOH (4 g) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 100 mL.

Step2. Magnetic stirrer was used for dissolved the solution well.

3.2.15 Preparation of catechin standard as a standard

Chemical (stock concentration)

Catechin 300 mg

Distilled water

Method

Step1. Catechin (0.3 mg) was weighted on the weighting paper and dissolved with ethanol in micro centrifuge tube 1mL.

Step2. Vortex was used to mix the solution clearly.

3.2.16 Carbonate buffer pH 10.3

Chemical

Na_2CO_3	17.75	mL
$NaHCO_3$	7.25	mL

Method

Step1. Na_2CO_3 and $NaHCO_3$ were mixed together in the beaker.

Step2. Distilled water 50 mL was added into that solution.

Step3. The buffer was adjusted pH at 10.3

Step4. Volumetric flask was used to adjust the final volume at 100 mL.

3.2.17 Preparation of 0.5 mM NBT reagent

Chemical

NBT	20.5	mg
Carbonate buffer pH 10.3	50	mL

Method

Step1. NBT (20.5 mg) was weighted on the weighting paper and dissolved with carbonate buffer pH 10.3 in volumetric flask reach to 50 mL.

3.2.18 Preparation of DMF

Chemical

DMF	2.49	mg
0.1 M PBS pH 7.4		

Method

Step1. DMF (2.49 mg) was weighted on the weighting paper and dissolved with 0.1 M PBS pH 7.4 in volumetric flask reach to 10 mL.

3.2.19 Preparation of 6 M DTNB reagent

Chemical

DTNB	118.84	mg
0.1 M PBS pH 7.4	50	mL

Method

Step1. DTNB (118.84 mg) was weight on the weighting paper and dissolved with PBS pH 7.4 in volumetric flask reach to 50 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

3.2.20 Preparation of L-cysteine

Chemical (1,000,000x L-cysteine)

L-cysteine	12	mg
0.1 M PBS pH 7.4	10	mL

Method

Step1. L-cysteine (12 mg) was weight on the weighting paper and dissolved with PBS pH 7.4 in volumetric flask reach to 10 mL.

Step2. The solution was diluted from 1,000,000X to 10,000x.

Step3. Then, 2-fold dilution was started from

10,000x → 5,000x → 2,500x →1,250x → 625x→ 0

The final concentration was in the range from 0 -10 μM.

3.2.21 Preparation of 2.5 M HCl

Chemical

11.45 M Conc. HCl	218.32 mL
Distilled water	

Method

Step1. Distilled water was poured in the cylinder 1,000 mL

Step2. HCl (218.32 mL) was added into the water and did in the laboratory cabinet.

Step3. The volumetric flask was used for adjust the volume at 1,000 mL

3.2.22 Preparation of 10 mM DNPH reagent

Chemical

DNPH	495.3	mg
2.5 M HCl	250	mL

Method

Step1. DNPH (495.3 mg) was weight on the weighting paper and dissolved with 2.5 M HCl in volumetric flask reach to 250 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

*The powder of DNPH was quite difficultly dissolved. Thus, DNPH were pulverized before used.

3.2.23 Preparation of 20% (w/v) TCA

Chemical

TCA	50	g
Distilled water		

Method

Step1. TCA (50 g) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 250 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

3.2.24 Preparation of Ethanol : Ethyl acetate (1:1 v/v)

Chemical

Ethanol	1	L
Ethyl acetate	1	L

Method

Step1. Ethanol and ethyl acetate was estimated by using cylinder 1,000mL.

Step2. Both of that solution was mixed together in the Duran bottle 2 L.

3.2.25 Preparation of 6M Guanidine hydrochloride

Chemical

Guanidine hydrochloride	143.25mg
Distilled water	

Method

Step1. Guanidine hydrochloride (143.25 mg) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 250 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

*The powder was quietly saturated agent. Thus, distilled water was gently added in the flask.

3.2.26 Preparation of Tht reagent

***Chemical* (10x Tht)**

Tht	10	mg
0.1 M PBS pH 7.4		

Method

Step1. Tht (10 mg) was weighted on the weighting paper and dissolved with PBS pH 74 in volumetric flask reach to 50 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

3.2.27 Preparation of reduced-BSA in CML ELISA assay kit

Chemical

Reduced BSA	100	µL
0.1 M PBS pH 7.4		

Method

Step1. Reduced BSA 100 µL was dissolved with PBS pH 7.4 reach to 10 mL in volumetric flask.

Step2. The mixed solution was inversed 2-3 times for well dissolving.

3.2.28 Preparation of wash buffer in CML ELISA assay kit

Chemical

10x Wash buffer	25	mL
Distilled water		

Method

Step1. 10x wash buffer (25 mL) was diluted with distilled water 225 mL to reach the final volume at 250 mL by using volumetric flask.

Step2. The mixed solution was inversed 2-3 times for well dissolving.

3.2.29 Preparation of Anti-CML antibody

Chemical (1:1000 dilution in assay dilutant)

Anti-CML antibody	5	µL
Assay dilutant	4.995	mL

Method

Step1. Anti-CML antibody (5 µL) was mixed with 4.995 mL of assay dilutant in conical tube 15 mL by using vortex.

3.2.30 Preparation of anti-HRP conjugated antibody

Chemical (1:1000 dilution in assay dilutant)

Anti-HRP conjugated antibody	5	µL
Assay dilutant	4.995	mL

Method

Step1. Anti-HRP conjugated antibody (5 µL) was mixed with 4.995 mL of assay dilutant in conical tube 15 mL by using vortex.

3.2.31 Preparation of Moringa solution for HPLC

Chemical

Moringa oleifera aqueous leaf extracts (MOE)	4	mg
Methanol (HPLC grade)	1	mL

Method

Step1. MOE (4 mg) was weighted in micro centrifuge tube and dissolved with1 mL of methanol.

Step2. Mixed the solution by using vortex.

Step3. PES syringe filter was used to filter the extract solution into the amber.

3.2.32 Preparation of 0.1% formic acid in distilled water (pH 2.4)

Chemical

Formic acid	1	mL
Distilled water		

Method

Step1. Formic acid (1 mL) was added into distilled water which approximately estimated in cylinder 950 mL.

Step2. The solvent was adjust the pH at 2.4

Step3. Then, the solvent was adjusted the volume in volumetric flask at 1,000 mL.

3.2.33 Preparation of 0.1% formic acid in acetonitile

Chemical

Formic acid	1	mL
Acetonitile (HPLC grade)		

Method

Step1. Formic acid (1 mL) was added into acetonitile which estimated in cylinder reach to 1000 mL

Step2. Mixed the solution in the Duran bottle

3.3 Method

3.3.1 Determination of phytochemical analysis

❖ **Total phenolic compound**

Chemical

Moringa oleifera aqueous leaf extracts (MOE)

Folin-Ciocalteu reagent

7.5% Na_2CO_3

Gallic acid

Distilled water

Method

Step1. The extract powder was dissolved in distilled water at a final concentration of 1.25, 2.5, 5 and 10 mg/mL.

Step2. That extract solution (10 µL) was gently mixed with freshly prepared Folin-Ciocalteu reagent (75 µL) and kept in the dark at room temperature.

Step3. After 5 minutes of incubation, 75 µL of 7.5% Na_2CO_3 was added to stop the reaction and allowed to stand for 30 min at room temperature in dark.

Step4. The mixtures were measured at 725 nm by UV spectrophotometer.

Step5. Gallic acid (0.025 – 0.4 mg/ml) was used as the standard curve and expressed as mg gallic acid equivalents/g dry extract.

❖ **Total flavonoid content**

Chemical

Moringa oleifera leaf extracts (MOE)

5% $NaNO_2$

10% $AlCl_3$

1 M NaOH

Catechin

Distilled water

Method

Step1. The extract powder was dissolved in distilled water at a final concentration of 1.25, 2.5, 5 and 10 mg/mL.

Step2. That extract solution (100 µL) was mixed with 30 µL of 5% $NaNO_2$ and allowed to stand for 5 min at room temperature.

Step3. Then, 30 µL of 10% $AlCl_3$ was added to start the reaction.

Step4. After 6 minutes of incubation, the reaction mixtures were treated with 200 µL of 1 M NaOH to stop the reaction and total volumes were made up to 1 mL with distilled water.

Step5. The absorbance was measured at 510 nm immediately using spectrophotometer.

Step6. Catechin (0-300 mg/mL) was used as the standard for the calibration curve and expressed the data as mg catechin equivalents/g dry extract.

❖ **HPLC identification**

Chemical

Moringa aqueous leaf extracts (MOE)

Solvent A: 0.1% formic acid in DW (pH 2.4)

Solvent B: 0.1% formic acid in acetonitile

Standards
- Caffeic acid
- Cinnamic acid
- Ferulic acid
- Quercetin
- Keamferol
- Rutin

Method

Step1. The HPLC system Shimadzu LC-10A (Kyoto, Japan) was equipped with dual pump LC-10A binary system, UV detector SPD – 10A. Inertsil ODS-3, C18 column (4.6 *x* 250 mm, 5µm) was used.

Step2. The binary mobile phased for chromatographic separation consisted of:

pump A: 0.1% formic acid (v/v) in water pH 2.4

pump B: 0.1% formic acid (v/v) in acetonitile

For following gradient:
0-2 min: 5%
32% B in 15 min
50% B in 30 min
75% B in 45 min
50% B in 47 min
5% B in 55 min

Step3. The injection volume was 10 µL and wavelength was set at 370 nm. Flow rate was set at 1 ml/min. All Standards (1mg/mL) were dissolved in absolute methanol and filtered with polyethersulfone membrane (PES).

3.3.2 Determination of inhibitory effect of MOE on glucose, fructose and methylglyoxal-induced protein glycation *in vitro*.

Chemical

BSA

0.5 M Glucose

0.5 M Fructose

1 mM Methylglyoxal

10 mg/mL Aminoguanidine

MOE (0.5, 1, 1.5, 2 mg/mL)

0.1 M PBS pH 7.4

Method

Step1. The incubation setting was divided into 2 groups: blank and test to determine the inhibitory effect on AGE formation. The constituents in each group were presented in table1.

Table 1. The chemical were presented in blank and test group.

Sample	Blank	Test
Blank	PBS	PBS+BSA
Negative	PBS + Glu/Fr/MGO	BSA+ Glu/Fr /MGO
Positive	PBS+ Glu/Fr/MGO+AG	BSA+ Glu/Fr /MGO
Treatment	PBS+ Glu/Fr/MGO+MOE	BSA+ Glu/Fr/MGO

Step2. The difference volumes of the chemicals were added into the 1 mL of micro centrifuge tube which presented in table2-3. The final concentration of BSA was10 mg/mL, Aminoguanidine was 1mg/mL, fructose and glucose were 0.5 M, in contrast to the methylglyoxal, and it was 1 mM. Furthermore, MOE was 0.5, 1.5, 2 mg/mL. All measurements were done in triplicate.

Table 2. The chemical were presented in blank group.

Blank group	PBS (µL)	Glucose/ Fructose/ MGO (µL)	BSA (µL)	AG (µL)	MOE (µL)
Blank	1000	-	-	-	-
Negative	540	460	-	-	-
Positive	500	460	-	40	-
Treatment	500	460	-	-	40

Table 3. The chemical were presented in test group.

Test group	PBS (µL)	Glucose / Fructose/ MGO (µL)	BSA (µL)	AG (µL)	MOE (µL)
Blank	500	-	500	-	-
Negative	40	460	500	-	-
Positive	-	460	500	40	-
Treatment	-	460	500	-	40

Step3. After finished the chemical preparation, all sample were incubated at 37°C in the difference periods following the table 4.

Table 4. The incubation period of AGE prepared sample.

	Incubation period
0.5 M Glucose	7, 14, 21, 28 day
0.5 M Glucose	7, 14, 21, 28 day
1 mM MGO	3, 7, 14 day

Step4. To determine the inhibitory effect, the samples in each period were detected the formation of AGE, fructosamine, protein oxidation and protein aggregation.

3.3.3 Determination of Amadori product and AGE formation.

❖ **Fructosamine content**

Fructosamine was an Amadori product presenting in the initiation stage of non-enzymatic glycation which occurring between reducing sugars and amino groups of proteins (Armbruster, 1987).

Chemical

Glycated sample

0.5 mM NBT

Carbonate buffer

DMF

Method

Step1. Glycated samples (10 µL) were placed into 96 well-plates.

Step2. The reagent were added in the sample

Blank : Carbonate buffer	90	µL
Test : 0.5 mM NBT	90	µL

Step3. Spectrophotometer was set the temperature at 37°C. The setting point was divided to 2 times interval following these steps:

Temperature set point	: 37°C
Delay	: 10 minutes
Shake	: 0.03 second
Read	: 590 nm
Delay	: 5 minutes

Shake : 0.03 second

Read : 590 nm

Step4. DMF (0-10 mM) was used as the standard for the calibration curve.

❖ **Fluorescence AGE**

The fluorescent AGE was the irreversible product presenting in the late stage of the reaction. Pentosidine was the product mostly detected by spectrofluorometer at excitation and emission wavelengths of 355 nm and 460 nm, respectively (Adisakwattana et al., 2010).

Chemical

Glycated sample

Method

Step1. Glycated samples (50 µL) were placed into 96 well-plate.

Step2. The samples were measured by spectrofluorometer by setting excitation : 355 nm.

emission : 460 nm.

Step3. All measurements were done in triplicate. The percentage Inhibitions of each sample were calculated from:

$$\%\text{inhibition} = \frac{\text{Fluorescence}_{\text{Neg}} - \text{Fluorescence}_{\text{Pos.}}}{\text{Fluorescence}_{\text{Neg}}}$$

❖ **Non-fluorescence**

Non-fluorescent AGEs, N^{ε}-carboxymethyl lysine; CML, is the most abundant product of glycation reaction. Commercially available ELISA kit will be used for measurement of CML formation (OxiSelectTM N^{ε}-(carboxymethyl) lysine (CML) ELISA Kit, Cell Biolabs, CA, USA).

Chemical

10-fold of sample dilution in reduced-BSA

1x PBS

Assay dilutant

Wash buffer

1:1000 anti-CML Antibody

1:100 anti-HRP Conjugated Antibody

Substrate solution

Stop solution

***Method* (Sample dilution)**

Step1. Glycated BSA 20 µl were diluted with 1980 µl of 1xPBS.

Step2. 10-fold of sample from step 1 was diluted in reduced-BSA (10 µg/mL) by following these ratio

1 : 9

15 µL : 135 µL

***Method* (CML assay)**

Step1. The diluted samples (100 µL) were placed in 96 well plates.

Step2. All samples were incubated at 37°C for 2 hours.

Step3. 1x PBS (250 µl) was used to remove unbound substances for twice in each well.

Step4. Assay dilutant (200 µl) was added in the plate then incubated at 25°C for 2 hours on an orbital shaker.

Step5. Wash buffer (250 µl) was washed each well for 3 times.

Step6. Diluted anti-CML antibody (100 µl) was added and incubated for an hour at 25°C on an orbital shaker.

Step7. Wash buffer (250 µl) was washed each well for 3 times.

Step8. Diluted anti-HRP conjugated antibody (100 µl) was added in the plate to coat the strip well and put it into an orbital shaker for 1 hour at 25°C.

Step9. The wells were washed again by using 250 µl of wash buffer for 5 times to remove unbound HRP conjugated antibody.

Step10. Substrate solution was warmed at the room temperature and placed100 µl into the wells for 20 minutes on an orbital shaker to trap with bound HRP conjugated antibody.

Step11. Stop solution (100 µl) was placed into the plate to stop the reaction.

Step12. The absorbance was immediately determined at 450 nm with spectrophotometer. CML-BSA provided in the assay kit was used as the standard (concentration range 0-6.25 ng/mL).

❖ **Protein oxidation**

- **Protein carbonyl content**

Protein carbonyl group is the marker of oxidative damage of BSA according to a slightly modified method of Levine and colleagues **(Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003)**

Chemical

Glycated sample

10 mM DNPH

20% TCA

2.5 M HCl

Ethanol: Ethyl acetate (1:1 (v/v))

6 M Guanidine hydrochloride

Bradford reagent

0.1M PBS (pH 7.4)

Method

Step1. Glycated sample (100 µl) were incubated with

Blank	: 10 mM DNPH in 2.5M HCl	400 µL
Test	: 2.5M HCl	400 µL

in micro centrifuge tube in the darkness place at room temperature for 60 minutes.

*The samples were vortex every 15 minutes.

Step2. 20% TCA (500 µL) were kept in the refrigerator and placed in the tube to precipitate protein in the sample and stand on ice for 5 minutes.

Step3. After 5 min, protein precipitation will be centrifuged at 10,000 rpm (4 ℃) for 10 minutes.

Step4. The supernatant were removed and 500 µl of ethanol: ethyl acetate was added into the tube.

Step5. The stirring rod was used to spin 5 times to wash the protein pellet.

Step6. 6 M guanidine hydrochloride (250 µL) was used to re-dissolve protein.

Step7. The re-dissolve protein (110 µL) was determined by spectrophotometer at 370 nm to detect the carbonyl content.

Step8. Bradford reagent was used to determine the protein representing in carbonyl group by following these step.

Method (To determine the protein in the sample)

Step1. The samples were diluted 10-fold by 0.1 M PBS following these ratio 10:90 µL

Step2. The samples (5 µL) ware incubated with Bradford reagent (200 µL) for 5 minutes at room temperature.

Step3. The absorbance was immediately determined at 95 nm with spectrophotometer. The concentration of protein carbonyl content will be calculated using an absorption coefficient of

22,000 $M^{-1}cm^{-1}$ and expressed as nmol carbonyls/mg protein.

- **Protein thiol group**

Protein thiol group is the organosulfur compound containing disulfide bond that can be used for indicating the oxidation of protein during glycation reaction. The determination of free thiol group will be performed according to Ellman's assay (Ellman, 1959).

Chemical

Glycated sample

6 mM DTNB

0.1 M PBS (pH 7.4)

L-cysteine

Method

Step1. Glycated samples (10 µL) were placed into 96 well-plates.

Step2. The reagent were added in the sample

Blank	: 0.1M PBS, pH 7.4	90	µL
Test	: 6 mM DTNB	90	µL.

Step3. The samples were incubated at the room temperature for 15 minutes.

Step4. Spectrophotometer was set the absorbance at 410 nm.

Step5. L-cysteine (0-10 µM) was used as the standard and the concentration of free thiol group will be expressed as µmol/ mg protein

❖ **Protein aggregation**

Thioflavin T reagent (Tht) used to detect amyloid cross structure during AGE reaction (Hudson, Ecroyd, Kee, & Carver, 2009).

Chemical

Glycated sample

Tht reagent

0.1M PBS pH 7.4

Method

Step1. Glycated samples (50 µL) were placed into 96 well-plates.

Step2. The reagent were added in the sample

Blank : 0.1M PBS, pH 7.4 50 µL

Test : 64 µM Tht reagent in 0.1M PBS (pH 7.4)50 µL.

Step3. The samples were incubated at the room temperature for an hour.

Step4. Fluorescence intensity was measured at

Excitation wavelength h : 435 nm

Emission wavelength : 485 nm.

3.4 Statistical analysis

Data were reported based on triplicate results (n=3) as mean±SEM. All data were determined by using one-way ANOVA by DUNCAN post-hoc test for multiple comparisons. *P*-value < 0.05 will be considered statistically significant in all tests.

CHAPTER IV

RESULTS

4.1 Phenolic and Flavonoid contents in *Moringa Oleifera* leave extract

4.1.1 Phenolic contents

Phenolic compounds constituent in MOE extract was measured by using Folin's ciocalteu method. As shown in table 5, the results showed that MOE contained polyphenolic compound about 38.56±1.50 mg gallic acid/ g dry extract.

4.1.2 Flavonoid contents

As shown in table 5, total flavonoid content of MOE extract was measured by using $AlCl_3$ colorimetric method. The results showed that MOE contained total flavonoid content about 23.12±0.80 mg catechin / g dry extract.

Table 5. Phenolic and flavonoid quantification

	Phenolic compound (mg gallic acid/ g dry extract)	Flavonoid content (mg catechin/ g dry extract)
MOE	38.56±1.50	23.12±0.80

Data were expressed as mean ± SEM (n = 3).

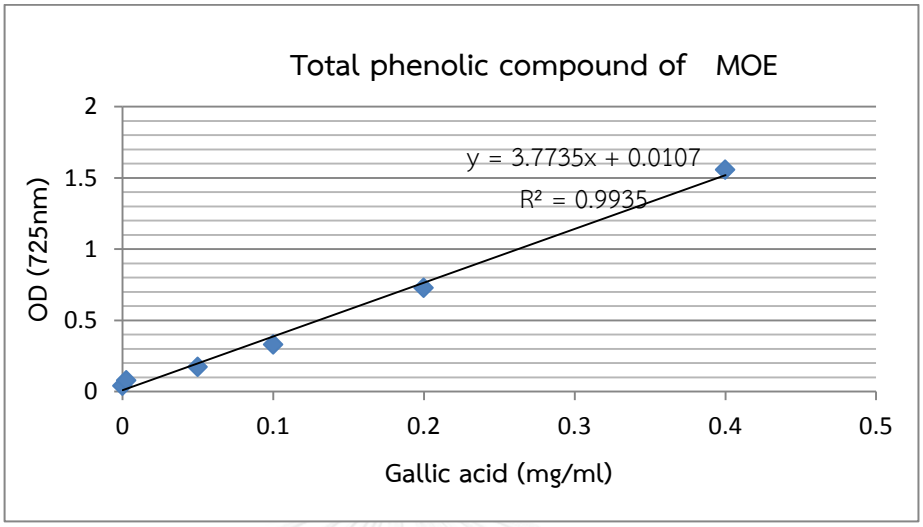


Figure 17. The standard curve of gallic acid.

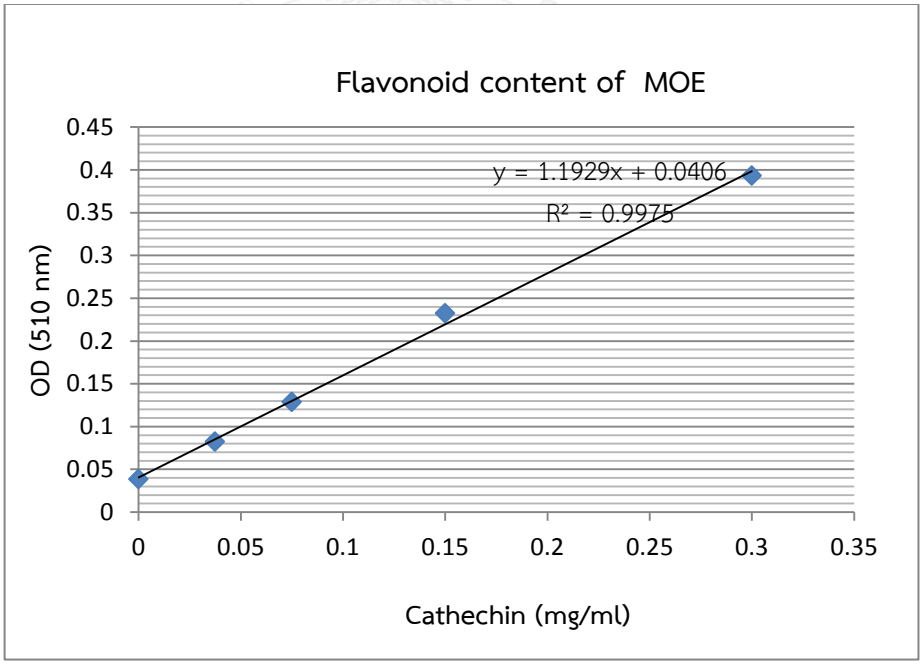


Figure 18. The standard curve of Cathechin.

4.1.3 HPLC identification

Polyphenolic compound and flavonoid in MOE were identified by HPLC. Rutin, quercetin, and keamferol were used as standards for flavonoid quantification, whereas ferulic acid was used as a standard compound for phenolic quantification. HPLC chromatogram of MOE was shown in figure 20. To confirm the peak of that chromatogram, phenolic and flavonoid standard were added into the system to detect the similar peak and area as a standard which showed in figure 21. All standard (4 µg/mL) peaks were represented in the figure22 to specify and verify.

The concentration of phenolic and flavonoid was compared to the standard curve as shown in the figure 21-24. The highest amount of active compound in MOE was ferulic acid (225.53±1.27 µg/mL). The lowest amount of active compound in MOE was keamferol (0.15±0.04 µg/mL). On the other hand, rutin and quercetin was 0.09±0.05 µg/mL and 0.38±0.04 µg/mL, respectively.

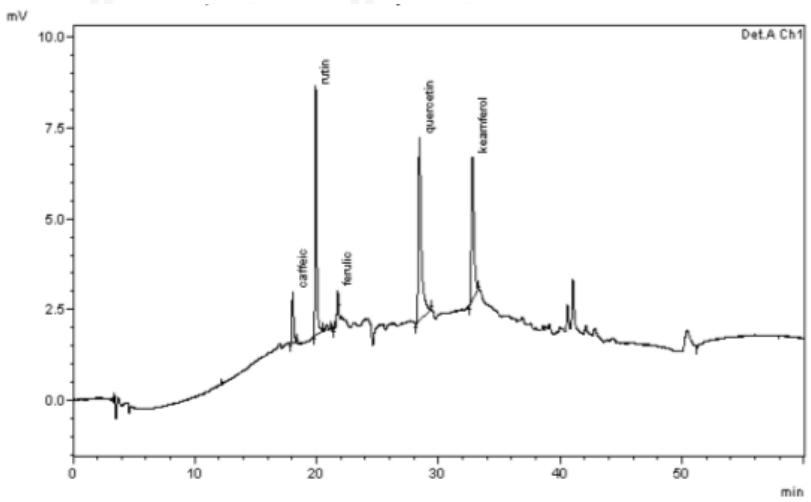


Figure 19. The chromatogram of phenolic and flavonoid standard.

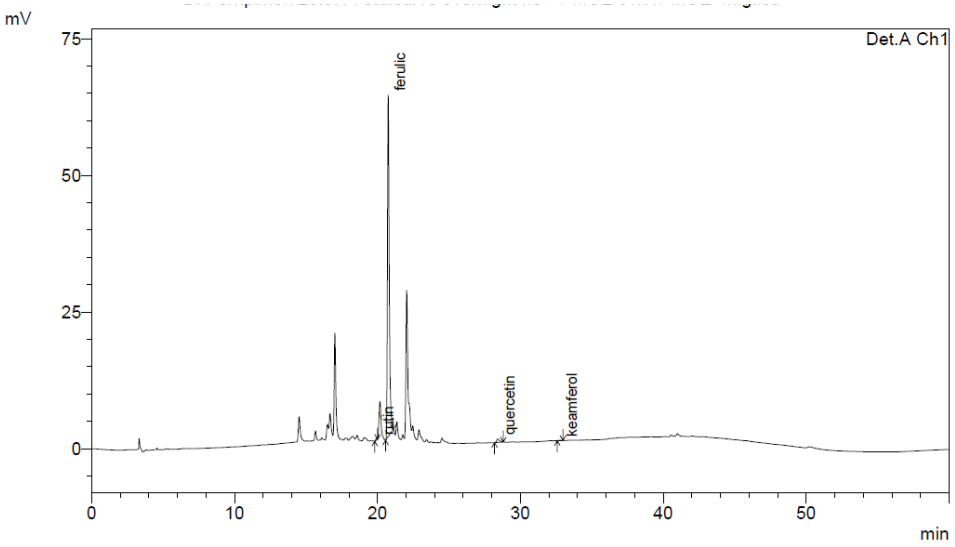


Figure 20. The HPLC chromatogram of MOE**.**

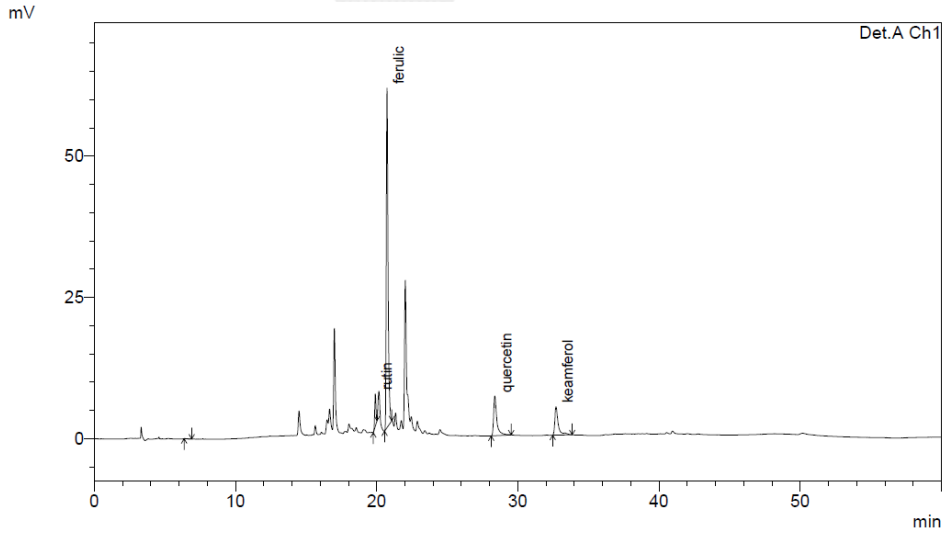


Figure 21. The chromatogram of spike MOE

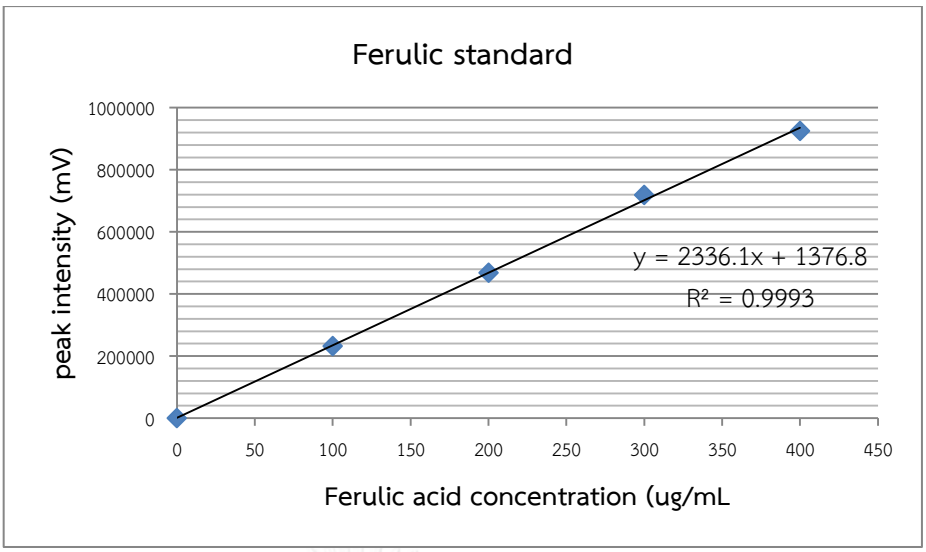


Figure 22. The standard curve of ferulic acid

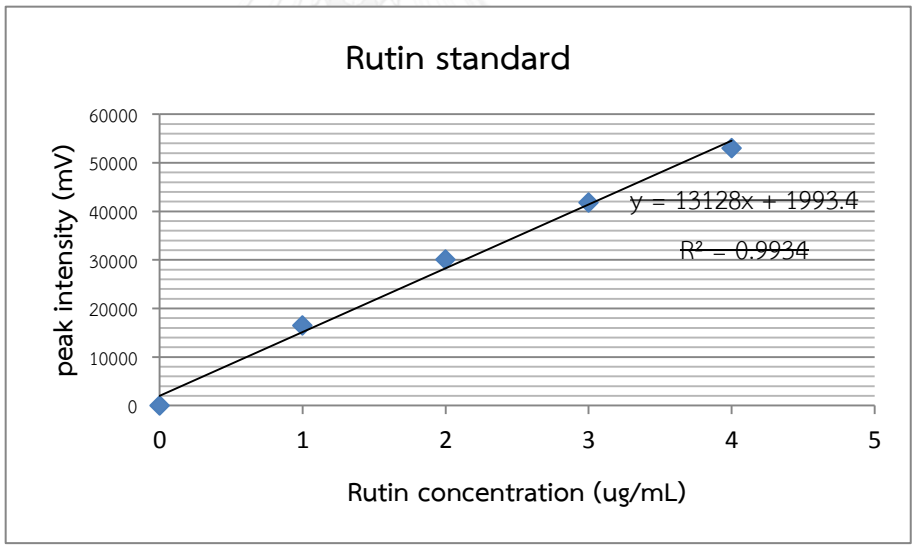


Figure 23. The standard curve of rutin

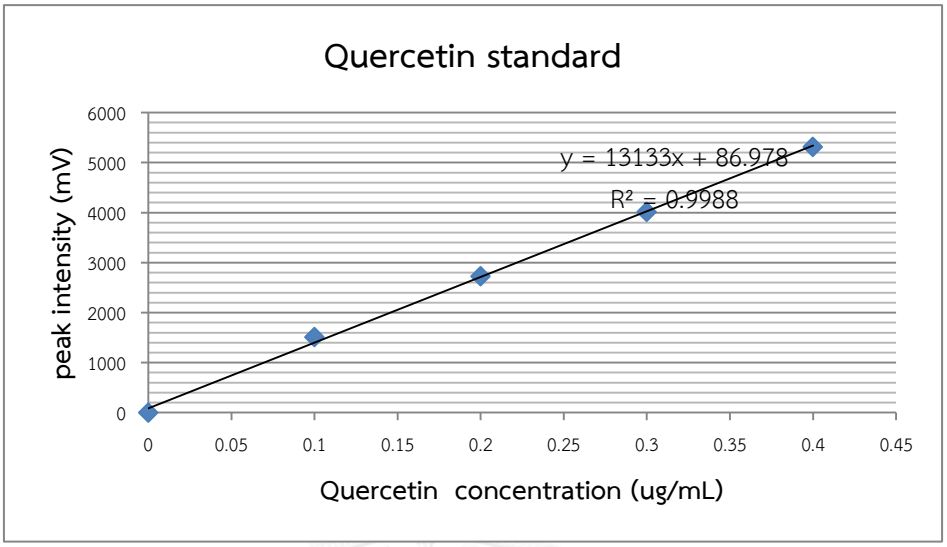


Figure 24. The standard curve of quercetin

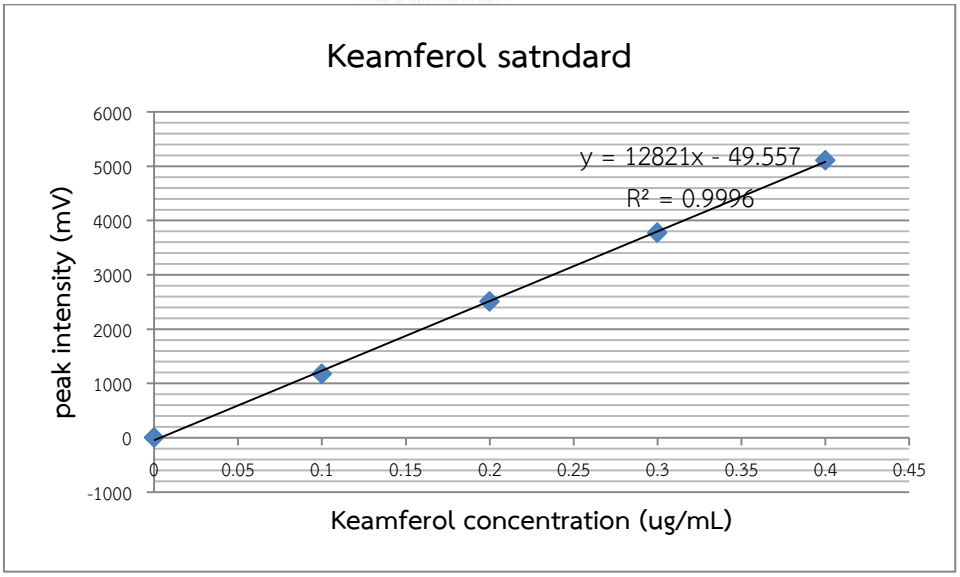


Figure 25. The standard curve of keamferol

4.2 The inhibitory effect of MOE on AGE formation

❖ The investigation of fluorescence AGE

As shown in Fig., 27-28 the fluorescence AGE formation of BSA incubated with glucose and fructose was represented as a negative control for 7, 14, 21, and 28 days. Glucose and fructose markedly increased the fluorescence AGE formation by 3.25 and 5.76 -fold, respectively when compared to BSA at day 28 of incubation. The negative control increased in each time-dependent during the experimental period. After MOE was added in the BSA/glucose and BSA/fructose system, it has been showed that the addition of MOE in glucose and fructose systems had significantly reduced the fluorescence AGE level in a dose-dependent manner. At day 28, when compared to negative control, the treatment of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) exhibited the percentage of inhibition on fluorescence AGE by 14.52%, 26.22%, 33.24%, 40.65%, respectively in glucose system and 45.82%, 54.06%, 58.47%, 65.43%, respectively in fructose system.

In contrast, the addition of AG (1.0 mg/mL) into the systems inhibited fluorescence AGEs by 73.12% in glucose system and 88.15% in fructose system at the end of incubation. Thus, the results manifested that MOE has less potent in the inhibition of fluorescence AGE formation when compared to AG at the same concentration (1.0 mg/mL).

In order to examine the fluorescence AGE formation in BSA/MG system, BSA incubated with MG was used as a negative control for 3, 7 and 14 days. The experimental period was different from other systems because MG had a high rate of the glycation reaction due to its structure. Therefore,

the incubation period of MG was ended at day 14.MG significantly increased the fluorescence AGE by 6.52-folds when compared to BSA at the end of incubation period.

Moreover, AGE formation was inhibited in a dose-dependent manner throughout the experimental periods when MOE (0.5-2.0 mg/mL) was added into the systems. At day 14 of incubation period, MOE (0.5, 1.0, 1.5, 2.0 mg/mL) inhibited AGE formation ranged from 22.14%, 25.20%, 25.36%, and 29.40%, respectively whereas AG (1.0 mg/mL) inhibited AGE formation about 66.83%.

Thus, the results manifested that MOE has less potent in the inhibition of AGE formation when compared to AG at the same concentration (1.0 mg/mL).

Table 6. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fluorescence AGEs in the BSA/glucose system.

	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	76.77 ± 11.14	56.98 ± 16.37	58.43 ± 7.51	65.00 ± 7.42	76.31 ± 6.33
Day 14	74.08 ± 18.56	36.06 ± 16.76	43.71 ± 18.34	48.05 ± 10.84	53.41 ± 18.58
Day 21	73.47 ± 1.00	27.48 ± 26.36	37.00 ± 22.50	40.79 ± 17.69	45.23 ± 12.12
Day 28	73.12 ± 11.17	14.52 ± 12.99	26.22 ± 48.29	33.24 ± 23.63	40.65 ± 34.85

Data were expressed as mean ± SEM (n = 3).

Table 7. The fluorescence intensity of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fluorescence AGEs formation in the BSA/glucose system.

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	508.00 ± 13.65	1010.33 ± 3.93[a]	234.67 ± 11.14[b]	434.67 ± 16.37[b]	420.00 ± 7.51[b]	353.67 ± 7.42[b]	239.33 ± 6.33[b]
Day 14	593.00 ± 4.98	1298.67 ± 9.77[a]	336.67 ± 18.56[b]	830.33 ± 16.76[b]	731.00 ± 18.34[b]	674.67 ± 10.84[b]	605.00± 18.58[b]
Day 21	532.67 ± 2.33	1662.00 ± 9.54[a]	441.00 ± 1.00[b]	1205.33 ± 26.36[b]	1047.00 ± 22.50[b]	984.00 ± 17.69[b]	910.33 ± 12.12[b]
Day 28	571.67 ± 34.34	1857.33 ± 14.68[a]	499.33 ± 11.17[b]	1587.67 ± 12.99[b]	1370.33 ± 48.29[b]	1240.00 ± 23.63[b]	1102.33 ± 34.85[b]

Data were expressed as mean ± SEM (n = 3). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/glucose.

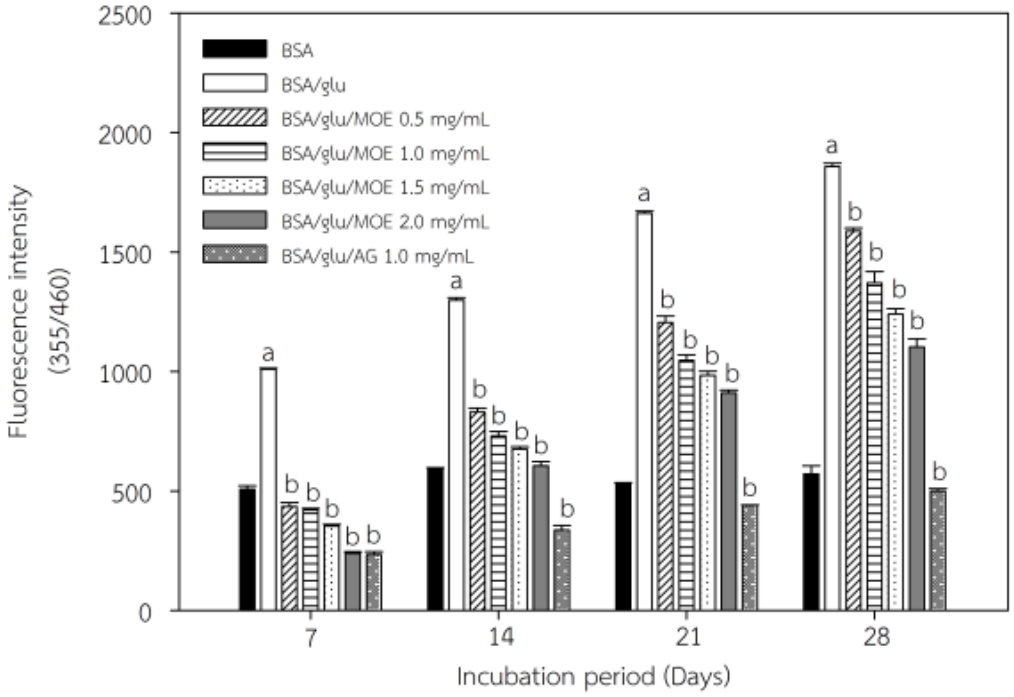


Figure 26. The effect of MOE (0.5 -2.0 mg/mL) on the fluorescence AGE formation in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). $^{a}P< 0.05$ compared to BSA, $^{b}P< 0.05$ compared to BSA/glucose.

Table 8. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fluorescence AGEs in the BSA/fructose system.

	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	87.13 ± 9.24	69.54 ± 3.33	76.00 ± 5.36	82.20 ± 4.84	90.59 ± 8.09
Day 14	87.21 ± 6.43	57.80 ± 10.54	63.41 ± 7.00	71.42 ± 8.50	76.89 ± 5.20
Day 21	87.91 ± 4.36	51.17 ± 2.40	58.09 ± 7.81	66.32 ± 9.17	67.90 ± 6.12
Day 28	88.15 ± 4.58	45.82 ± 8.62	54.06 ± 4.37	58.47 ± 4.18	65.43 ± 6.00

Data were expressed as mean ± SEM (n = 3).

Table 9. The fluorescence intensity of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fluorescence AGEs formation in the BSA/fructose system

	BSA	BSA+fr	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	529.67 ± 3.93	2129.67 ± 4.37 [a]	274.00 ± 9.24 [b]	648.67 ± 3.33 [b]	510.33 ± 5.36 [b]	377.33 ± 4.84 [b]	200.33 ± 8. [b]
Day 14	602.00 ± 3.00	2656.67 ± 10.02 [a]	339.67 ± 6.43 [b]	1121.00 ± 10.54 [b]	972.00 ± 7.00 [b]	759.33 ± 8.50 [b]	614.00 ± 5.20 [b]
Day 21	590.00 ± 0.58	2954.00 ± 4.02 [a]	357.00 ± 4.36 [b]	1442.33 ± 2.40 [b]	1238.00 ± 7.81 [b]	995.00 ± 9.17 [b]	948.33 ± 6.12 [b]
Day 28	584.00 ± 3.06	3366.67 ± 6.39 [a]	399.00 ± 4.58 [b]	1824.00 ± 8.62 [b]	1546.67 ± 4.37 [b]	1398.33 ± 4.18 [b]	1164.00 ± 6.00 [b]

Data were expressed as mean ± SEM (n = 3). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/fructose.

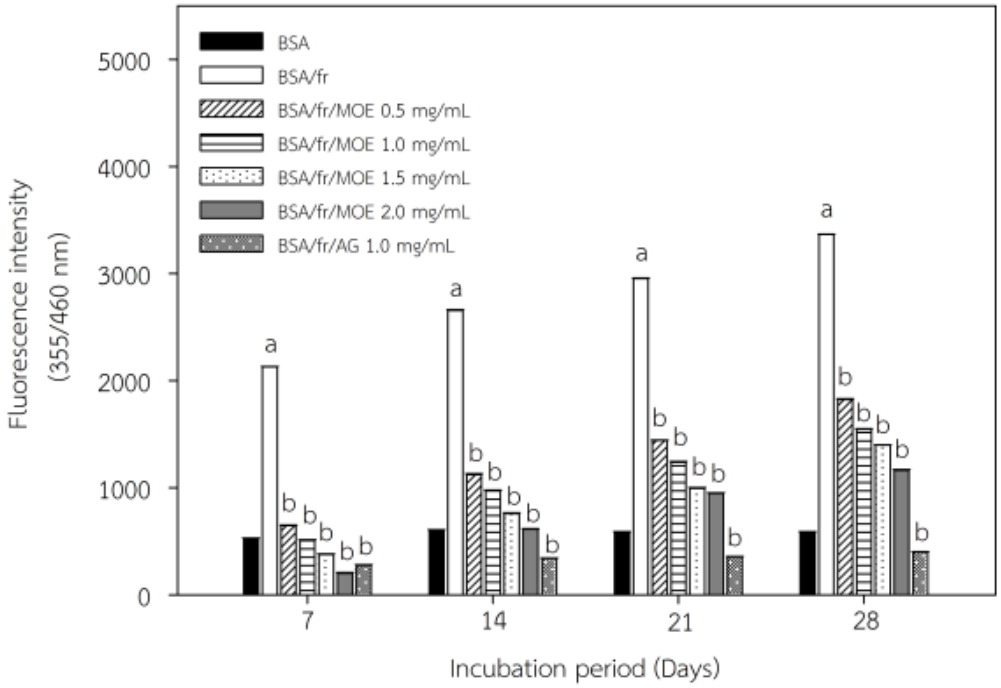


Figure 27. The effect of MOE (0.5 -2.0 mg/mL) on the fluorescence AGE formation in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose.

Table 10. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fluorescence AGEs in the BSA/MG system

	BSA+MG+AG (1.0 mg/mL)	BSA+MG+MOE (0.5mg/mL)	BSA+MG+MOE (1.0 mg/mL)	BSA+MG+MOE (1.5 mg/mL)	BSA+MG+MOE (2.0 mg/mL)
Day 3	65.85 ± 4.51	45.76 ± 10.82	46.55 ± 15.19	50.99 ± 1.00	55.96 ± 16.09
Day 7	68.49 ± 11.05	39.63 ± 16.04	40.46 ± 9.24	41.31 ± 11.32	47.24 ± 4.91
Day 14	66.83 ± 4.04	22.15 ± 4.41	25.21 ± 12.12	25.37 ± 17.21	29.40 ± 16.09

Data were expressed as mean ± SEM (n = 3).

Table 11. The fluorescence intensity of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fluorescence AGEs formation in the BSA/MG system.

	BSA	BSA+MG	BSA+MG+AG (1.0 mg/mL)	BSA+MG+MOE (0.5mg/mL)	BSA+MG+MOE (1.0 mg/mL)	BSA+MG+MOE (1.5 mg/mL)	BSA+MG+MOE (2.0 mg/mL)
Day 3	599.67 ± 5.05	2425.34 ± 2.85 [a]	830.33 ± 4.51 [b]	1342.67 ± 10.82 [b]	1262.67 ± 15.19 [b]	1178.67 ± 1.00 [b]	1071.00 ± 16.09 [b]
Day 7	606.00 ± 1.74	3270.67 ± 10.48 [a]	989.33 ± 11.05 [b]	1976.33 ± 16.04 [b]	1944.67 ± 9.24 [b]	1899.67 ± 11.32 [b]	1743.67 ± 4.91 [b]
Day 14	606.34 ± 7.13	3954.34 ± 1.46 [a]	1290.67 ± 4.04 [b]	3077.00 ± 4.41 [b]	2967.00 ± 12.12 [b]	2937.33 ± 17.21 [b]	2815.67 ± 16.09 [b]

Data were expressed as mean ± SEM (n = 3). [a] $P < 0.05$ compared to BSA, [b] $P < 0.05$ compared to BSA/MG.

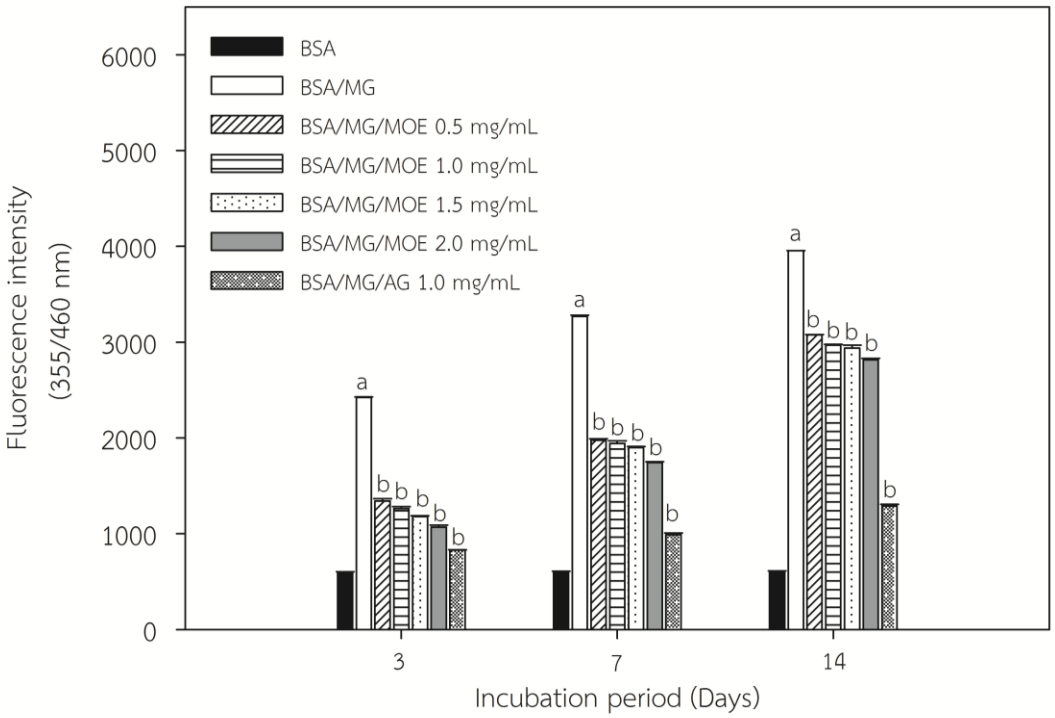


Figure 28. The effect of MOE (0.5 -2.0 mg/mL) on the fluorescence AGE formation n BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*<0.05 compared to BSA, [b]*P*<0.05 compared to BSA.

❖ **The investigation of non-fluorescence AGE**

A production of non-fluorescence AGE, N^{ε}-(carboxymethyl) Lysine (CML) was measured at day 28 of incubation. Glucose and fructose was remarkable to increase CML concentration by 3.10, 8.60-folds, respectively when compared to BSA at the end of incubation period as shown in Fig. 27-28. After MOE (1.0 mg/mL) presented in the BSA/glucose and BSA/fructose system, the percentage inhibition of CML formation by MOE was 31.01% in glucose system, and 66.81% in fructose system. In contrast to the system with AG, it was also able to decrease the level of CML as well. After the incubation period, AG (1.0 mg/mL) significantly decreased the accumulation of CML by 44.16% in glucose, 72.45% in fructose system.

Apart from BSA/MG system, MG dramatically increased CML formation in BSA system by 11.76-fold when compared to BSA at day 14 of incubation while the system with MOE (1.0 mg/mL) markedly decreased CML formation as shown in Fig.29. The extract inhibited CML formation by 67.97% whereas AG (1.0 mg/mL) inhibited CML formation by 72.48%.

Accordingly, when compared at the same concentration (1 mg/mL) of MOE and AG among three systems, the results have been shown that AG had a higher potential effect on CML formation than MOE after experimental period.

Table 12. The percentage inhibition of MOE (1.0mg/ml) and Aminoguanidine (1.0 mg/ml) on non-fluorescence AGEs in the BSA/glucose, BSA/fructose, and BSA/methylglyoxal system

	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (1.0 mg/mL)
Day 28	44.16 ± 0.08	31.01 ± 0.12
	BSA+fr+AG (1.0 mg/mL)	**BSA+fr+MOE (1.0 mg/mL)**
Day 28	72.45 ± 0.08	66.81 ± 0.10
	BSA+MG+AG (1.0 mg/mL)	**BSA+MG+MOE (1.0 mg/mL)**
Day 14	72.48 ± 0.10	67.97 ± 0.08

Data were expressed as mean ± SEM (n = 3).

Table 13. The effect of MOE (1.0 mg/ml) and Aminoguanidine (1.0 mg/ml) on non-fluorescence AGE in the BSA/glucose, BSA/fructose, and BSA/methylglyoxal system

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (1.0 mg/mL)
Day 28	3.03 ± 0.14	9.39 ± 0.08 [a]	5.24 ± 0.08 [b]	6.48 ± 0.12 [b]

Data were expressed as mean ± SEM (n = 3). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/glucose.

	BSA	BSA+fr	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (1.0 mg/mL)
Day 28	3.03 ± 0.14	26.06 ± 0.10 [a]	7.17 ± 0.08 [b]	8.64 ± 0.10 [b]

Data were expressed as mean ± SEM (n = 3). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/fructose.

	BSA	BSA+MG	BSA+MG+AG (1.0 mg/mL)	BSA+MG+MOE (1.0 mg/mL)
Day 14	3.03 ± 0.14	35.66 ± 0.08 [a]	9.81 ± 0.10 [b]	11.42 ± 0.08 [b]

Data were expressed as mean ± SEM (n = 3). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/MG.

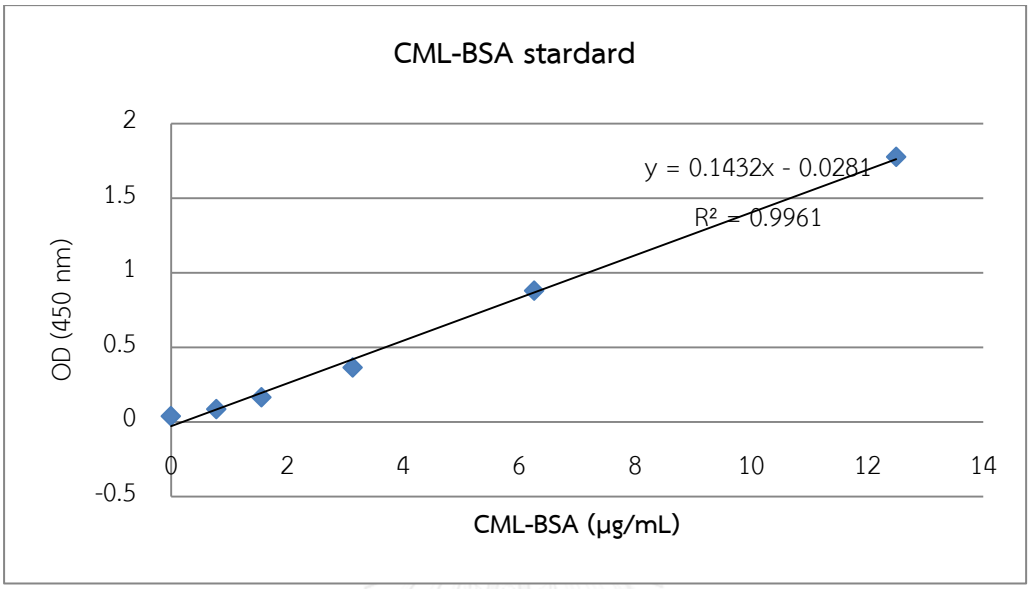


Figure 29 The standard curve of CML-BSA.

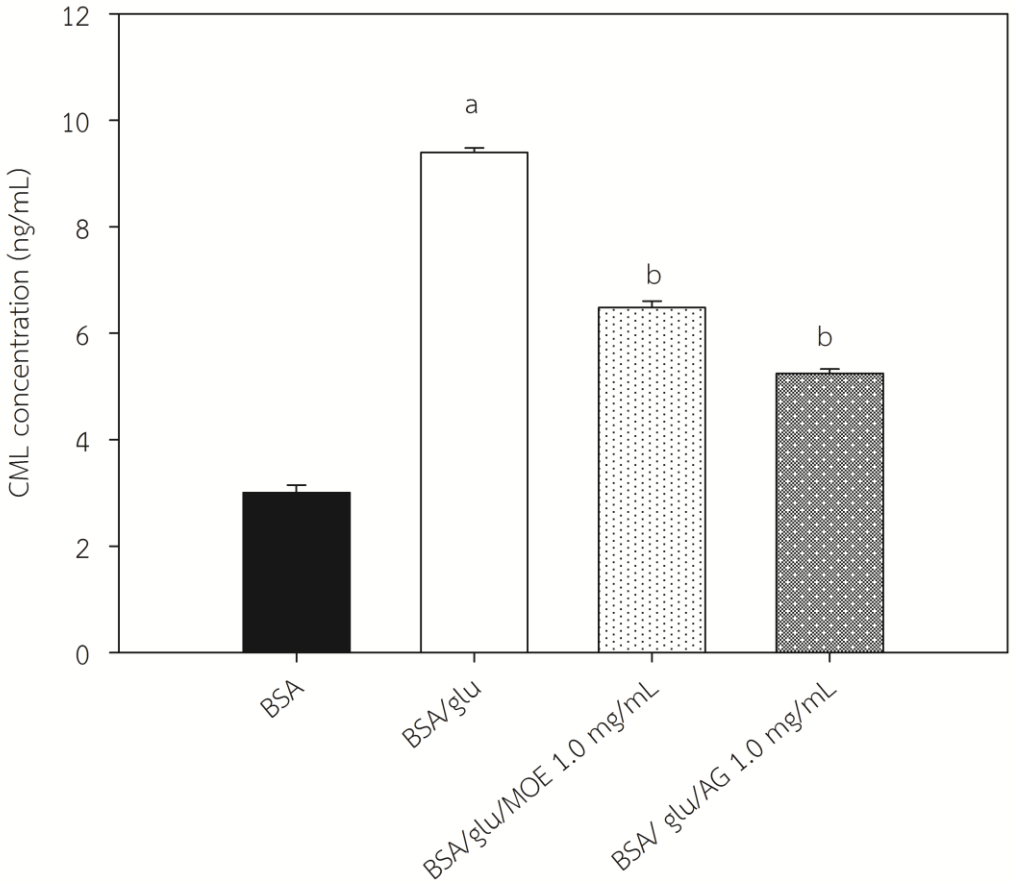


Figure 30. The effect of MOE (1.0 mg/mL) on N^{ε}- carboxymethyl lysine; CML (Non-fluorescence AGE) formation in BSA incubated with glucose model. Each value represented the mean±SEM (n=3).

[a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/glucose.

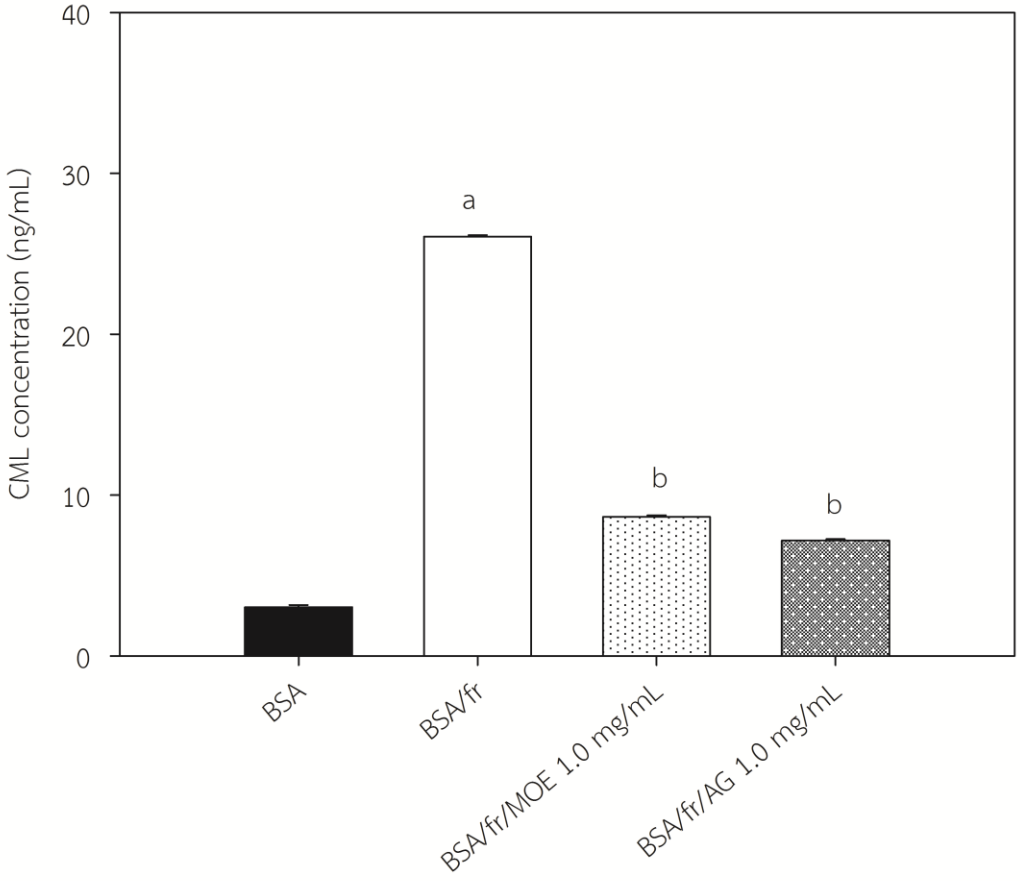


Figure 31. The effect of MOE (1.0 mg/mL) on N^{ε}- carboxymethyl lysine; CML (Non-fluorescence AGE) formation in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose.

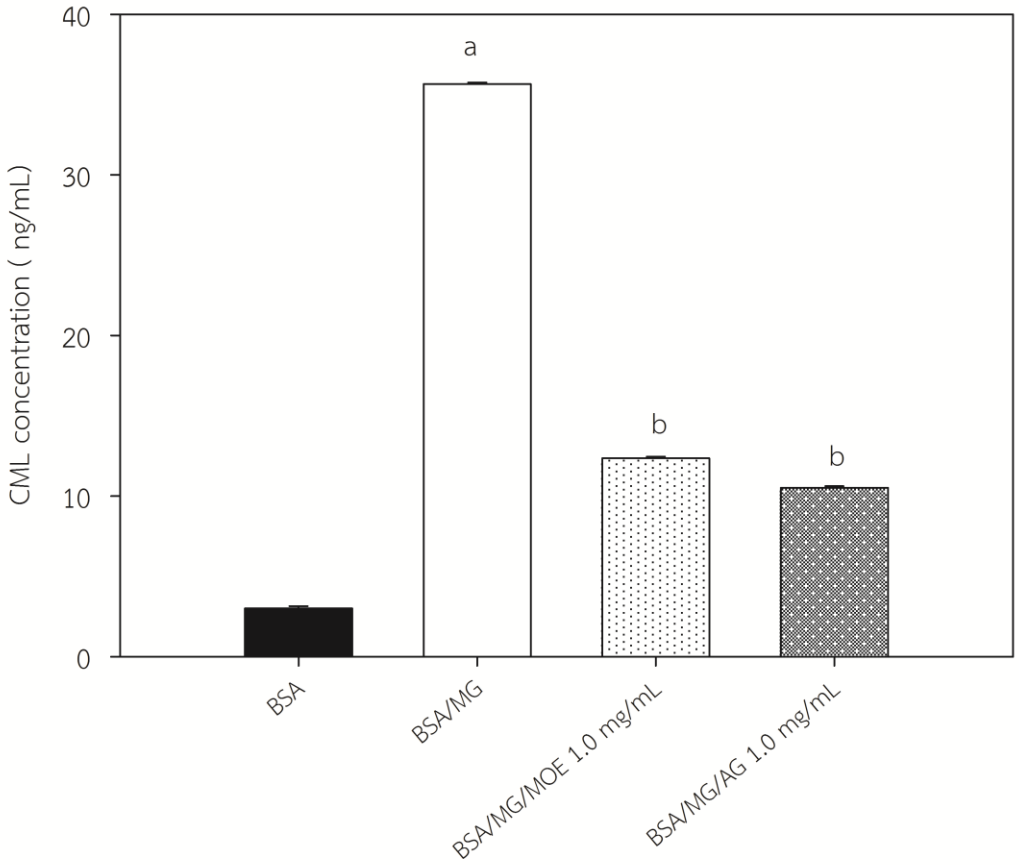


Figure 32. The effect of MOE (1.0 mg/mL) on N^{ε}- carboxymethyl lysine; CML (Non-fluorescence AGE) formation in BSA incubated with MG model. Each value represented the mean±SEM (n=3).

[a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/MG

❖ **The investigation of fructosamine level**

The fructosamine level was the indicator of Amadori product in initiation stage of AGE reaction. It was occurred when incubated glucose and fructose in the BSA system as shown in figure 31, 33. BSA/glucose and BSA/fructose represented as a negative control. In contrast to the system with AG, it represented as a positive control in the reaction. The effect of MOE was determined on day 7, 14, 21, 28. The increased of fructosamine level was depend on time-dependent manner. At the end of incubation, glucose and fructose induced fructosamine formation by 39.60, 5.2-folds, respectively when compared to BSA at the end of incubation period.

Apart from a lowered of fructosamine level, the various concentration of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) was investigated. The results showed that the addition of MOE in glucose and fructose system had a significantly lowered the accumulation of fructosamine in a dose-dependent manner. At day 28, when compared to negative control, the treatment of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) exhibited the ability to reduce fructosamine level by 71.21%, 76.76%, 79.29%, 80.30, respectively in glucose system and 18.96%, 29.31%, 31.03%, 49.56%, respectively in fructose system.

Additionally, AG (1 mg/mL) also showed a significantly decreased the level of fructosamine by 42.90%in glucose and 43.10% in fructose system. To compare the same concentration of 1 mg/mL at the end of incubation,

the results showed that MOE had a high potential effect on fructosamine accumulation than AG both in glucose and fructose system.

Table 14. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fructosamine level in the BSA/glucose system.

	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	4.76 ± 0.03	72.82 ± 0.02	81.55 ± 0.03	85.44 ± 0.01	95.15 ± 0.03
Day 14	12.10 ± 0.03	69.35 ± 0.07	71.77 ± 0.09	78.23 ± 0.10	85.48 ± 0.06
Day 21	28.00 ± 0.06	72.00 ± 0.00	74.67 ± 0.09	77.33 ± 0.03	80.00 ± 0.00
Day 28	43.59 ± 0.07	62.31 ± 0.00	67.95 ± 0.09	70.51 ± 0.03	81.54 ± 0.00

Data were expressed as mean ± SEM (n = 3).

Table 15. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fructosamine level in the BSA/glucose system.

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	0.14 ± 0.01	4.90 ± 0.03[a]	4.67 ± 0.03[b]	1.33 ± 0.02[b]	0.90 ± 0.03[b]	0.71 ± 0.01[b]	0.24 ± 0.03[b]
Day 14	0.10 ± 0.03	5.90 ± 0.01[a]	5.19 ± 0.03[b]	1.81 ± 0.07[b]	1.67 ± 0.09[b]	1.29 ± 0.10[b]	0.86 ± 0.06[b]
Day 21	0.10 ± 0.03	7.14 ± 0.00[a]	5.14 ± 0.06[b]	2.00 ± 0.00[b]	1.81 ± 0.09[b]	1.62 ± 0.03[b]	1.43 ± 0.00[b]
Day 28	0.10 ± 0.03	9.29 ± 0.00[a]	5.24 ± 0.07[b]	2.57 ± 0.00[b]	2.05 ± 0.09[b]	1.81 ± 0.03[b]	1.71 ± 0.00[b]

Data were expressed as mean ± SEM (n = 3).). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/glucose.

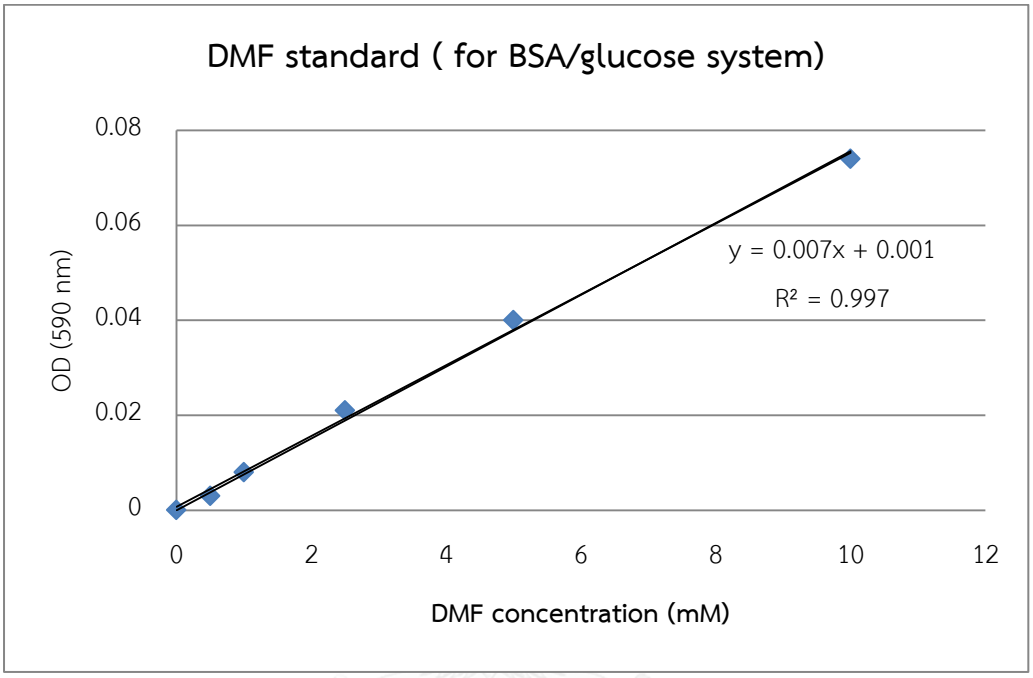


Figure 33. DMF standard curve for fructosamine in BSA/glucose system.

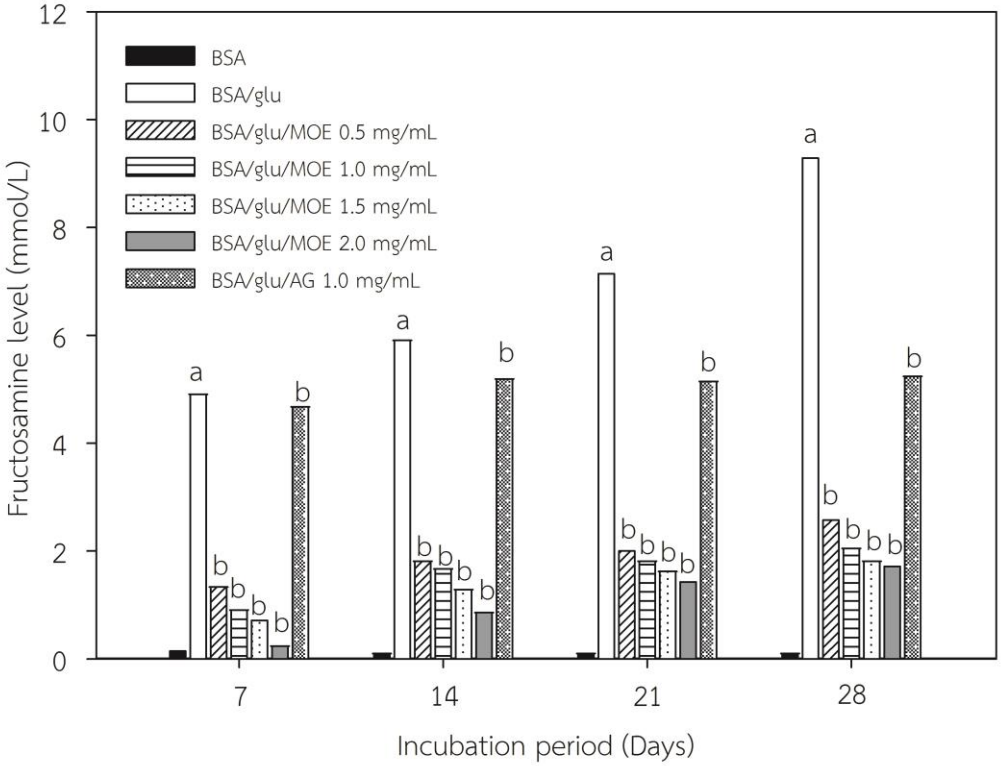


Figure 34. The effect of MOE (0.5 -2.0 mg/mL) on fructosamine level in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/glucose.

Table 16. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on fructosamine level in the BSA/fructose system.

	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	52.85 ± 0.01	28.57± 0.06	52.38 ± 0.03	71.42 ± 0.05	80.95 ± 0.03
Day 14	51.28 ± 0.03	2.56 ± 0.08	46.15 ± 0.01	51.28 ± 0.03	66.66 ± 0.06
Day 21	44.17 ± 0.03	2.30 ± 0.01	23.23 ± 0.01	41.84 ± 0.08	55.80 ± 0.06
Day 28	45.45 ± 0.06	0.39 ± 0.01	20.09 ± 0.01	32.72 ± 0.03	50.90 ± 0.06

Data were expressed as mean ± SEM (n = 3).

Table 17 The effect of MOE (0.5-2.0 mg/ml) and Aminoguanidine 1.0 mg/ml on fructosamine level in the BSA/glucose system

	BSA	BSA+fr	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	0.14	2.33 ± 0.05[a]	1.33 ± 0.01[b]	1.67 ± 0.06[b]	1.11 ± 0.03[b]	0.67 ± 0.05[b]	0.44 ± 0.03[b]
Day 14	0.10	4.33 ± 0.00[a]	2.11 ± 0.03[b]	4.22 ± 0.08[b]	2.33 ± 0.01[b]	2.11 ± 0.03[b]	1.44 ± 0.06[b]
Day 21	0.10	4.78 ± 0.03[a]	2.67 ± 0.03[b]	4.67 ± 0.01[b]	3.67 ± 0.01[b]	2.78 ± 0.08[b]	2.11 ± 0.06[b]
Day 28	0.10	6.11± 0.03[a]	3.33 ± 0.06[b]	4.93 ± 0.01[b]	4.33 ± 0.01[b]	4.11 ± 0.03[b]	3.00 ± 0.06[b]

Data were expressed as mean ± SEM (n = 3). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/fructose.

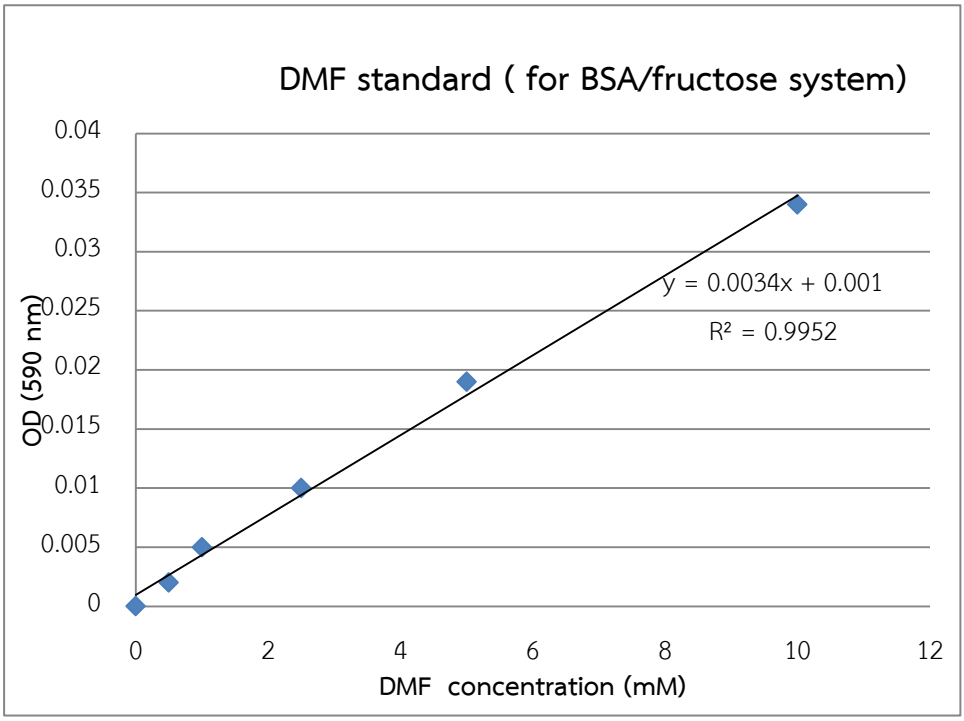


Figure 35. DMF standard curve for fructosamine in BSA/fructose system

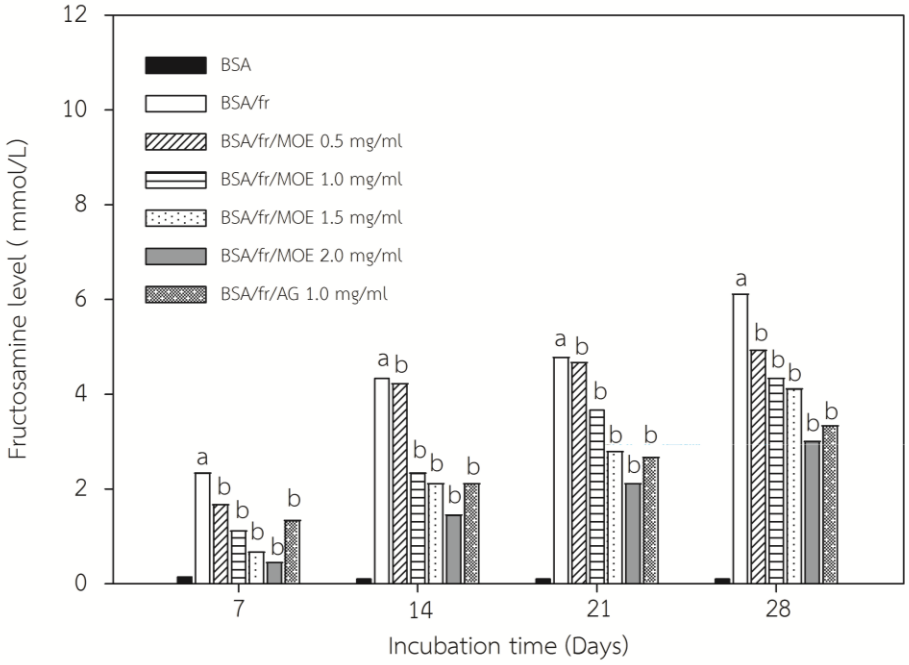


Figure 36. The effect of MOE (0.5 -2.0 mg/mL) on fructosamine level in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose.

4.3 The investigation of protein oxidation

❖ The formation of protein thiol group

In order to access the protein oxidation mediated by glycation process, the level of protein thiol group was used for determination at day 7, 14, 21, 28 in glucose and fructose system. BSA/glucose and BSA/fructose were represented as a negative control whereas the system with AG was represented as a positive control. At the end of incubation, the negative control significantly depleted protein thiol groups by 3.88, 13.20-folds, respectively when compared to BSA alone. As shown in figure 35, 37 the addition of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) increased the protein thiol level by 1.08, 1.08, 1.09, 1.10-folds in glucose system whereas the addition of MOE increased the protein thiol level by 1.05, 1.07, 1.08, 1.10-folds in fructose system. In addition, 1 mg/mL of AG showed a significant increased protein thiol group formation for 1.09-folds in glucose system and 1.14-folds in fructose system. At the same concentration (1.0 mg/mL) as MOE, AG had higher potential effect on elevating protein thiol group than MOE.

To determine the BSA/MG system, the incubation time was 7, 14, 21 days. MG had a significant depleted the level of protein thiol group by 14.67-folds which was the fastest glycating inducer when compared to glucose and fructose. After MOE in various concentration (0.5, 1.0, 1.5, 2.0 mg/mL) was added into the system, the results found that MOE protected the protein thiol group depletion in dose-dependent manner by 1.89, 3.67, 5.67, 7.33-folds, respectively when compared to negative control at the end of experimental period while AG (1mg/mL) significantly increased

protein thiol group by 12.22-folds. When compared MOE and AG at the same concentration (1 mg/mL), AG was able to present a higher ability in elevating the formation of protein thiol group than MOE as shown in figure 39.

Table 18. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on protein thiol group in the BSA/glucose system.

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	0.4 ± 0.06	0.34 ± 0.03[a]	0.38 ± 0.01[b]	0.30 ± 0.01[b]	0.33 ± 0.03[b]	0.35 ± 0.01[b]	0.39 ± 0.06[b]
Day 14	0.4 ± 0.03	0.24 ± 0.01[a]	0.34 ± 0.06[b]	0.28 ± 0.05[b]	0.31 ± 0.05[b]	0.33 ± 0.06[b]	0.35 ± 0.03[b]
Day 21	0.4 ± 0.06	0.18 ± 0.03[a]	0.29 ± 0.06[b]	0.26 ± 0.03[b]	0.29 ± 0.06[b]	0.31 ± 0.03[b]	0.31 ± 0.05[b]
Day 28	0.4 ± 0.01	0.10 ± 0.06[a]	0.28 ± 0.08[b]	0.24 ± 0.03[b]	0.27± 0.00[b]	0.29 ± 0.03[b]	0.30 ± 0.01[b]

Data were expressed as mean ± SEM (n = 3). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/glucose

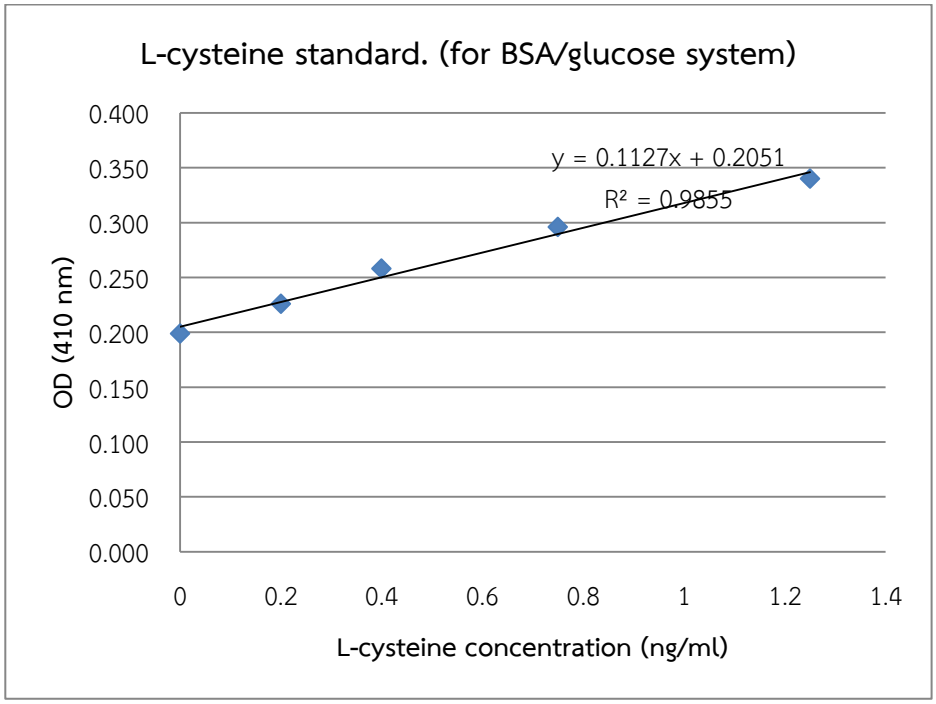


Figure 37. L-cysteine standard curve for BSA/glucose system.

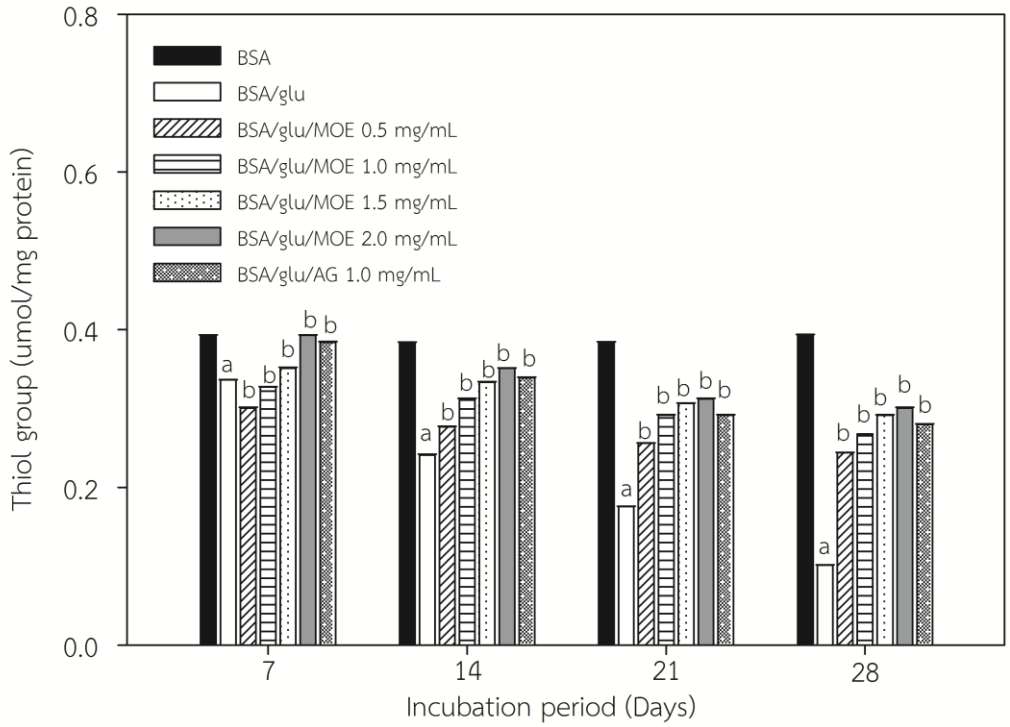


Figure 38. The effect of MOE (0.5 -2.0 mg/mL) on fructosamine level in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/glucose.

Table 19. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on protein thiol group in the BSA/fructose system.

	BSA	BSA+fr	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	0.4±0.00	0.30 ± 0.00[a]	0.33 ± 0.01[b]	0.34 ± 0.01[b]	0.36 ± 0.01[b]	0.37 ± 0.01[b]	0.39 ± 0.01[b]
Day 14	0.4±0.01	0.25 ± 0.01[a]	0.33 ± 0.01[b]	0.31 ± 0.01[b]	0.33 ± 0.01[b]	0.35 ± 0.01[b]	0.39 ± 0.01[b]
Day 21	0.4±0.01	0.24 ± 0.01[a]	0.34 ± 0.01[b]	0.29 ± 0.01[b]	0.31 ± 0.01[b]	0.31 ± 0.01[b]	0.36 ± 0.01[b]
Day 28	0.4±0.01	0.22 ± 0.01[a]	0.34 ± 0.01[b]	0.26 ± 0.01[b]	0.28 ± 0.01[b]	0.31 ± 0.01[b]	0.34 ± 0.01[b]

Data were expressed as mean ± SEM (n = 3). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/fructose.

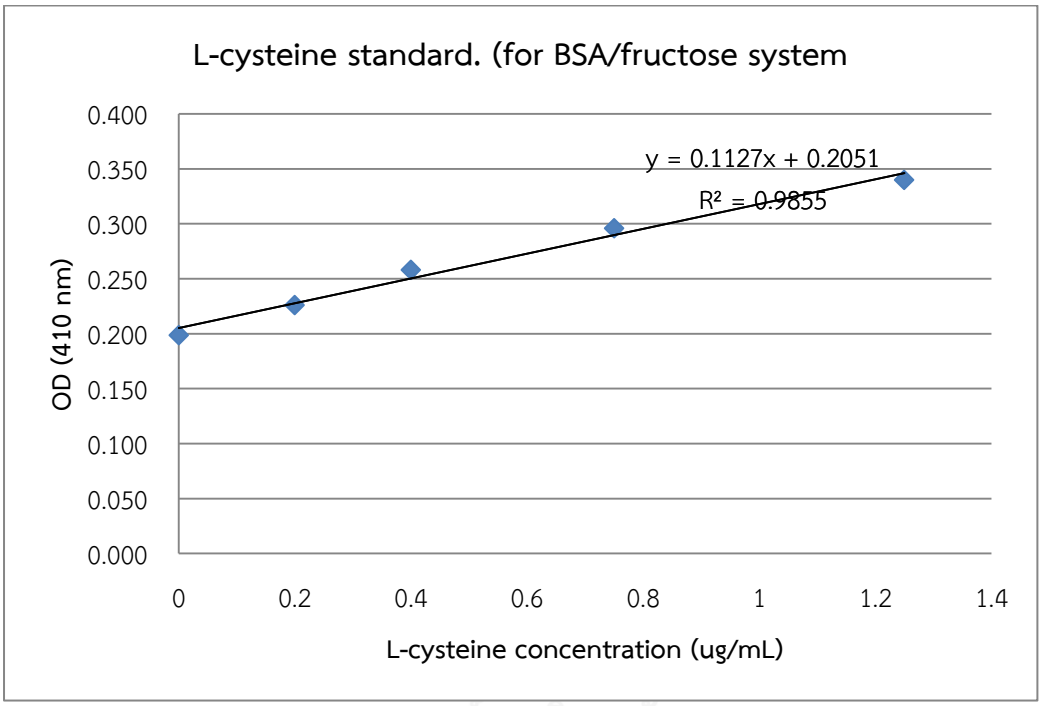


Figure 39. L-cysteine standard curve for BSA/fructose system.

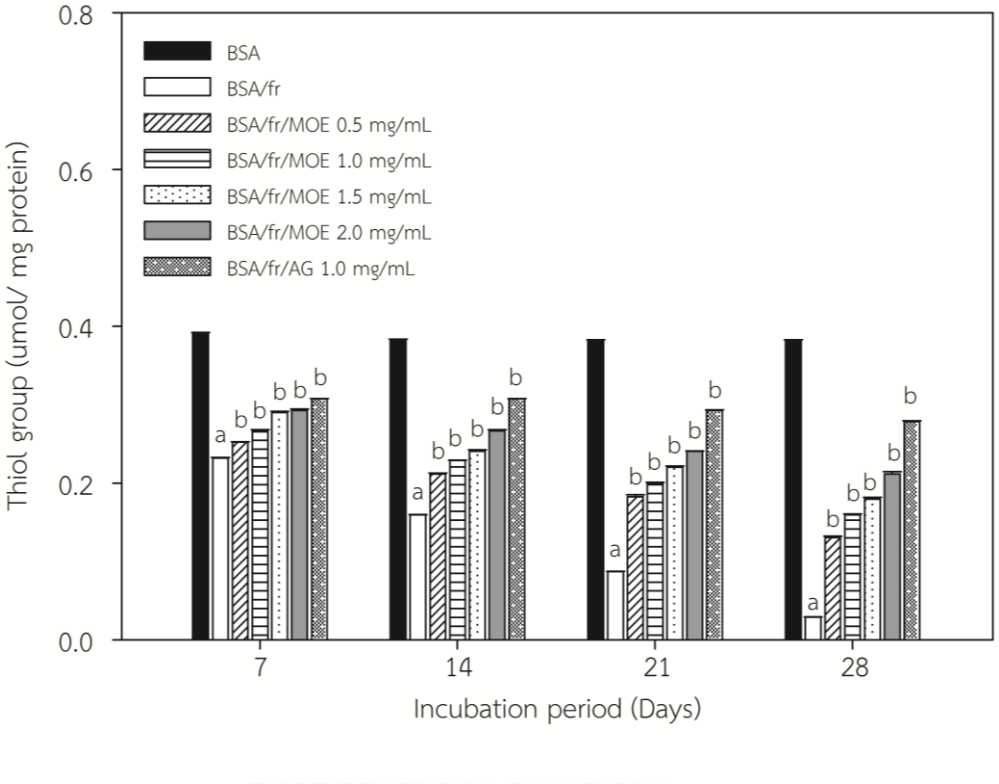


Figure 40. The effect of MOE (0.5 -2.0 mg/mL) on protein thiol group in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose.

Table 20. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on protein thiol group in the BSA/ MG system.

	BSA	BSA+MG	BSA+MG+AG (1.0 mg/mL)	BSA+MG+MOE (0.5mg/mL)	BSA+MG+MOE (1.0 mg/mL)	BSA+MG+MOE (1.5 mg/mL)	BSA+MG+MOE (2.0 mg/mL)
Day 3	0.4±0.01	0.24±0.01[a]	0.35±0.01[b]	0.28±0.01[b]	0.29±0.01[b]	0.31±0.01[b]	0.34±0.01[b]
Day 7	0.4±0.01	0.15±0.01[a]	0.35±0.01[b]	0.22±0.01[b]	0.23±0.01[b]	0.25±0.01[b]	0.27±0.01[b]
Day 14	0.4±0.01	0.03±0.01[a]	0.33±0.01[b]	0.06±0.01[b]	0.1±0.01[b]	0.16±0.01[b]	0.2±0.01[b]

Data were expressed as mean ± SEM (n = 3). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/MG.

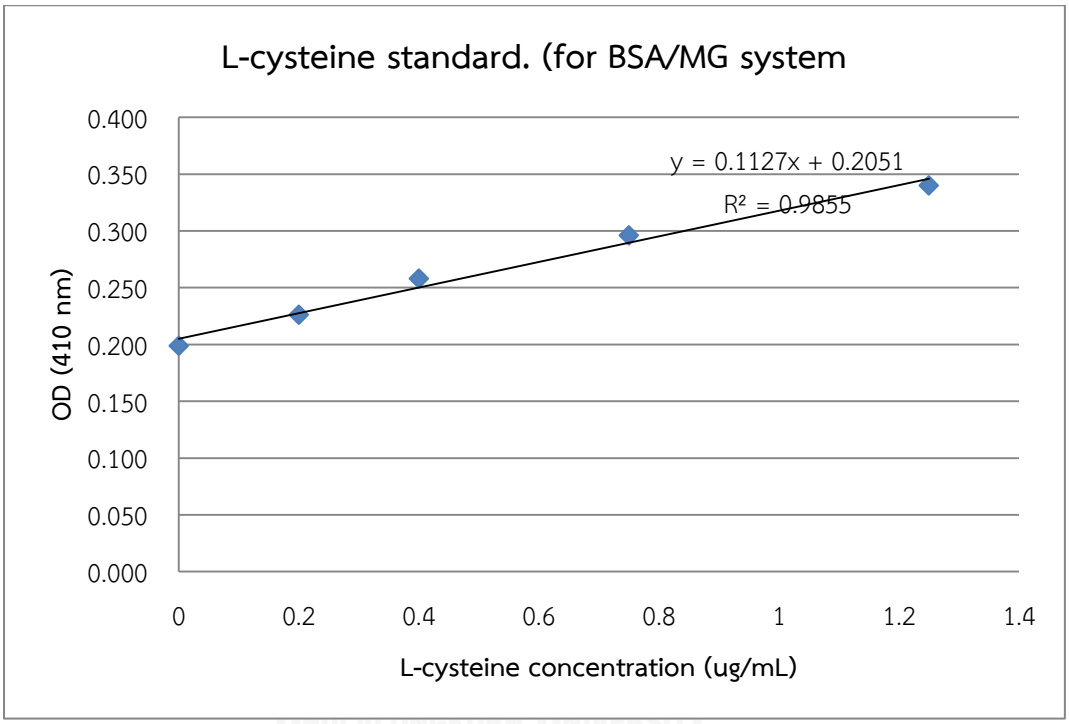


Figure 41. L-cysteine standard curve for BSA/MG system.

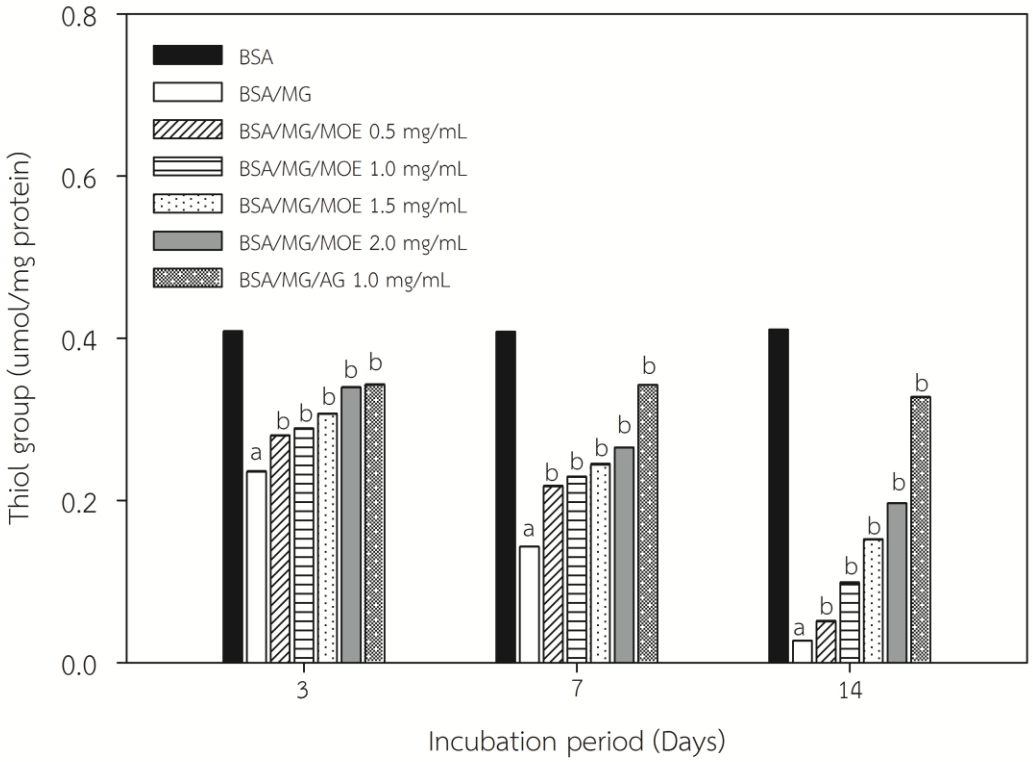


Figure 42. The effect of MOE (0.5 -2.0 mg/mL) on protein thiol group in BSA incubated with MG model. Each value represented the mean±SEM (n=3). $^{a}P< 0.05$ compared to BSA, $^{b}P< 0.05$ compared to BSA/MG.

❖ **The formation of protein carbonyl content**

As shown in figure 41, 43, protein carbonyl content was observed in the BSA/glucose and BSA/fructose system. The experimental period was performed in 7, 14, 21, 28 days. The protein carbonyl content significantly increased 1.71-fold in glucose system and 26.49-folds in fructose system after the incubation period when compared to BSA. The reduction of the protein carbonyl content was found in the dose-dependent manner by showing the percentage of inhibition via 51.12%, 55.36%, 66.86%, 76.31% in glucose system and 58.74%, 62.12%, 77.76%, 88.29% in fructose system when various concentrations of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) were added into the systems. In addition, AG (1mg/mL) also showed a significant decreased the protein carbonyl content by 91.75% in glucose system and 88.29% in fructose system. Moreover, after compared AG with MOE, it was found that AG had a higher potential to protect the elevation of protein carbonyl content than MOE at the same concentration (1mg/mL).

In access to determine the inhibitory effect of MOE on BSA/MG system, MG was incubated with BSA as a negative control and it also increased the level of protein carbonyl formation for 27.73-folds after the end of experimental period. In ordinary, all concentration of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) markedly illustrated a significant lowered in the accumulation of protein carbonyl content by dose-dependent manner which has been demonstrated in percentage of inhibition by 52.41%, 58.89%, 59.57%, 64.14%, respectively. Moreover, after compared the inhibitory effect at the same concentration (1mg/mL) between AG and MOE,

the results demonstrated that AG had a higher potential to protect the elevation of protein carbonyl content than MOE in BSA/MG system.

Accordingly, AG showed the highest ability to reduce protein carbonyl content among in three glycating agent.

Table 21. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on protein carbonyl content in the BSA/glucose system.

	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	87.51 ± 0.01	65.50 ± 0.02	67.99 ± 0.02	71.16 ± 0.01	80.53 ± 0.02
Day 14	90.76 ± 0.04	71.64 ± 0.02	70.71 ± 0.02	74.02 ± 0.02	80.23 ± 0.02
Day 21	91.83 ± 0.02	52.85± 0.03	56.95 ± 0.06	72.54 ± 0.02	78.71 ± 0.02
Day 28	91.76 ± 0.01	51.12 ± 0.02	55.40 ± 0.03	66.86 ± 0.01	76.31 ± 0.01

Data were expressed as mean ± SEM (n = 3).

Table 22. The effect of MOE (0.5-2.0 mg/ml) and Aminoguanidine (1.0 mg/ml) on protein carbonyl content in the BSA/glucose system.

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	0.33 ± 0.03	3.37 ± 0.02[a]	0.43 ± 0.01[b]	1.17 ± 0.02[b]	1.08 ± 0.02[b]	0.98 ± 0.01[b]	0.66 ± 0.02[b]
Day 14	0.37 ± 0.01	4.63 ± 0.04[a]	0.43 ± 0.04[b]	1.32 ± 0.02[b]	1.36 ± 0.02[b]	1.21 ± 0.02[b]	0.92 ± 0.02[b]
Day 21	0.35 ± 0.01	5.30 ± 0.04[a]	0.44 ± 0.02[b]	2.50 ± 0.03[b]	2.28 ± 0.06[b]	1.46 ± 0.02[b]	1.13 ± 0.02[b]
Day 28	0.36 ± 0.01	5.76 ± 0.06[a]	0.48 ± 0.01[b]	2.82 ± 0.02[b]	2.57 ± 0.03[b]	1.91 ± 0.01[b]	1.37 ± 0.01[b]

Data were expressed as mean ± SEM (n = 3).). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/glucose.

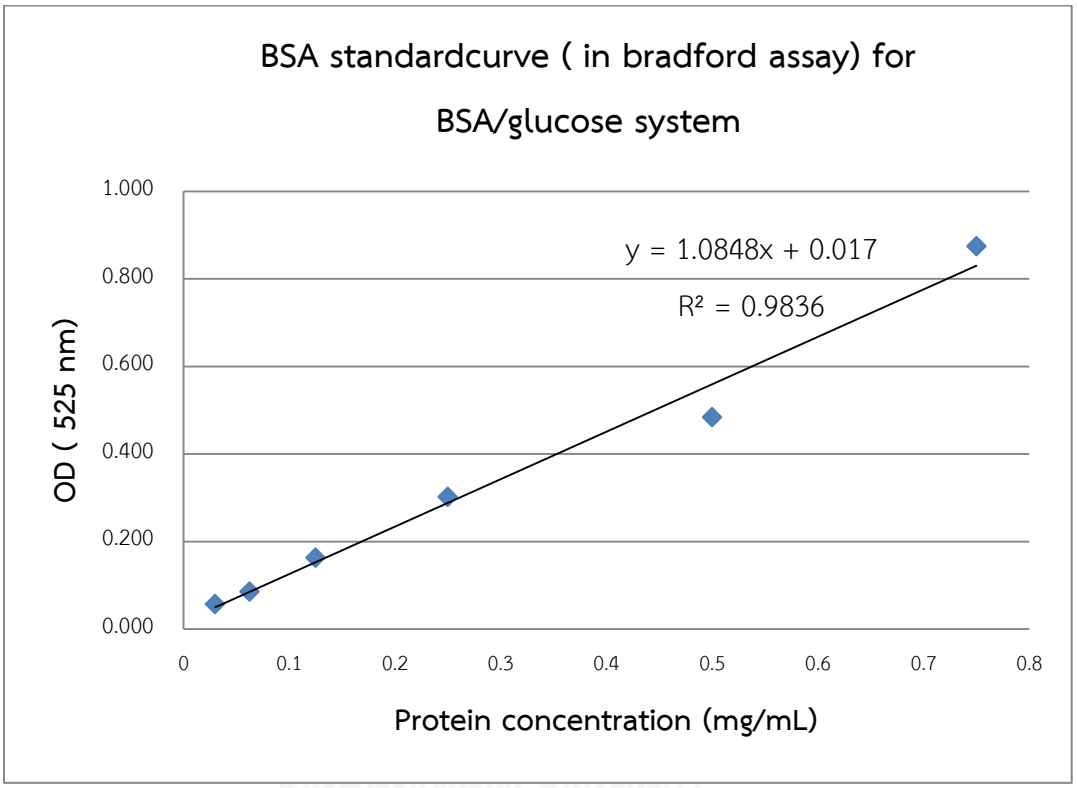


Figure 43. Bradford reagent for BSA/glucose system

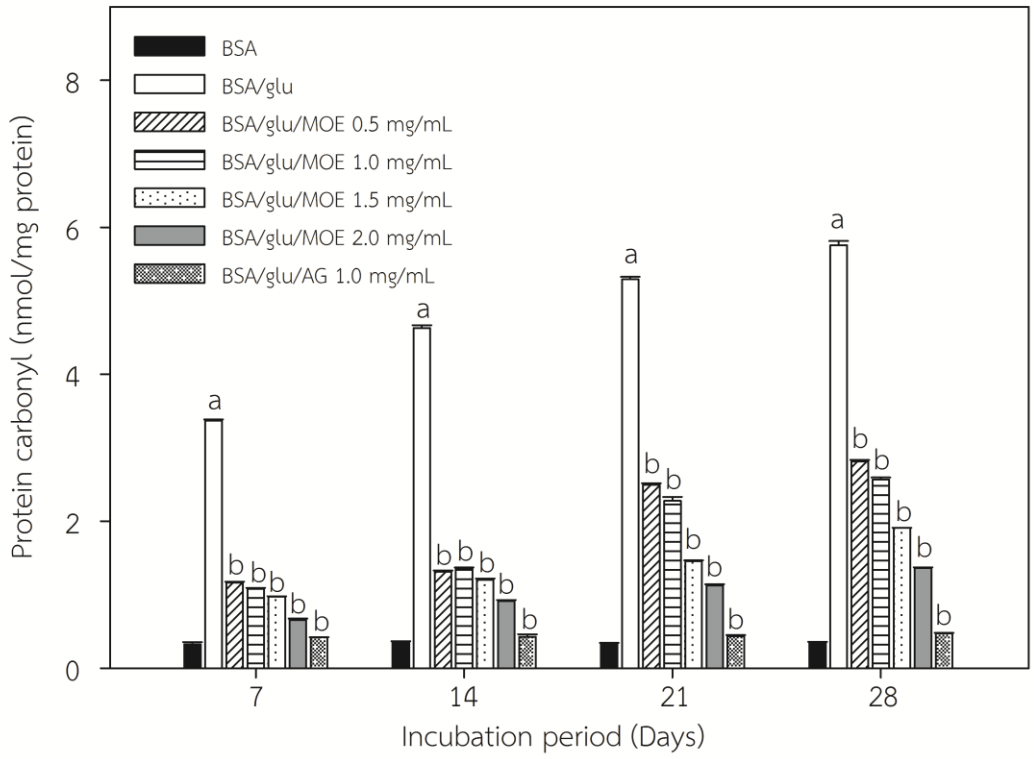


Figure 44. The effect of MOE (0.5 -2.0 mg/mL) on protein thiol group in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ glucose.

Table 23. The percentage inhibition of MOE (0.5-2.0 mg/ml) and Aminoguanidine (1.0 mg/ml) on protein carbonyl content in the BSA/fructose system.

	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	87.51 ± 0.42	65.50 ± 1.16	67.99 ± 1.08	71.16 ± 0.97	80.53 ± 0.66
Day 14	90.76 ± 0.43	71.64 ± 1.31	70.71 ± 1.36	74.02 ± 1.20	80.23 ± 0.92
Day 21	91.83 ± 0.43	52.85 ± 2.50	56.95 ± 2.28	72.54 ± 1.45	78.71 ± 1.13
Day 28	91.76 ± 0.47	51.12 ± 2.81	55.40 ± 2.57	66.86 ± 1.91	76.31 ± 1.36

Data were expressed as mean ± SEM (n = 3).

Table 24. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on protein carbonyl content in the BSA/fructose system.

	BSA	BSA+fr	BSA+fr+AG (1.0 mg/mL)	BSA+fr+MOE (0.5mg/mL)	BSA+fr+MOE (1.0 mg/mL)	BSA+fr+MOE (1.5 mg/mL)	BSA+fr+MOE (2.0 mg/mL)
Day 7	0.30 ± 0.00	5.31 ± 0.04[a]	2.73 ± 0.01[b]	2.19 ± 0.01[b]	2.15± 0.02[b]	1.43 ± 0.09[b]	1.04 ± 0.01[b]
Day 14	0.27 ± 0.01	6.10 ± 0.02[a]	2.92 ± 0.02[b]	2.50 ± 0.03[b]	2.28± 0.02[b]	1.62 ± 0.02[b]	1.07 ± 0.01[b]
Day 21	0.33 ± 0.01	7.46 ± 0.03[a]	3.30 ± 0.00[b]	2.97 ± 0.09[b]	2.70± 0.01[b]	1.73 ± 0.03[b]	0.99 ± 0.01[b]
Day 28	0.30 ±0.01	8.05 ± 0.03[a]	3.32 ± 0.01[b]	3.05 ± 0.03[b]	2.73± 0.03[b]	1.79 ± 0.03[b]	0.94 ± 0.05[b]

Data were expressed as mean ± SEM (n = 3). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/fructose.

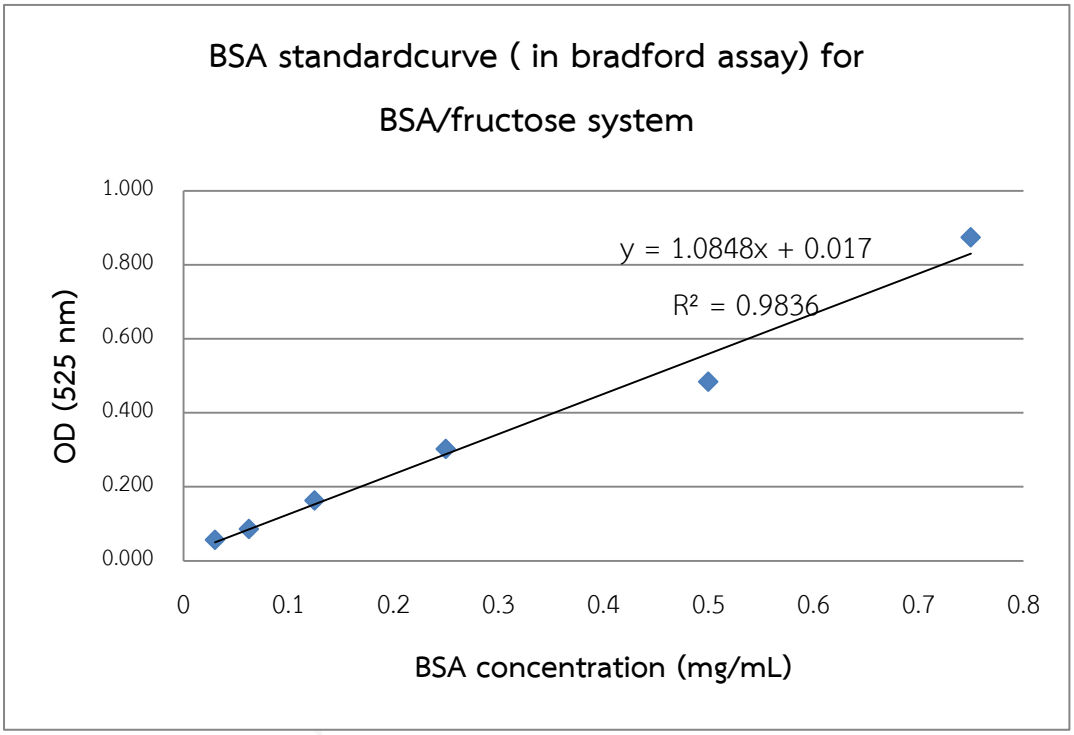


Figure 45. Bradford reagent for BSA/fructose system

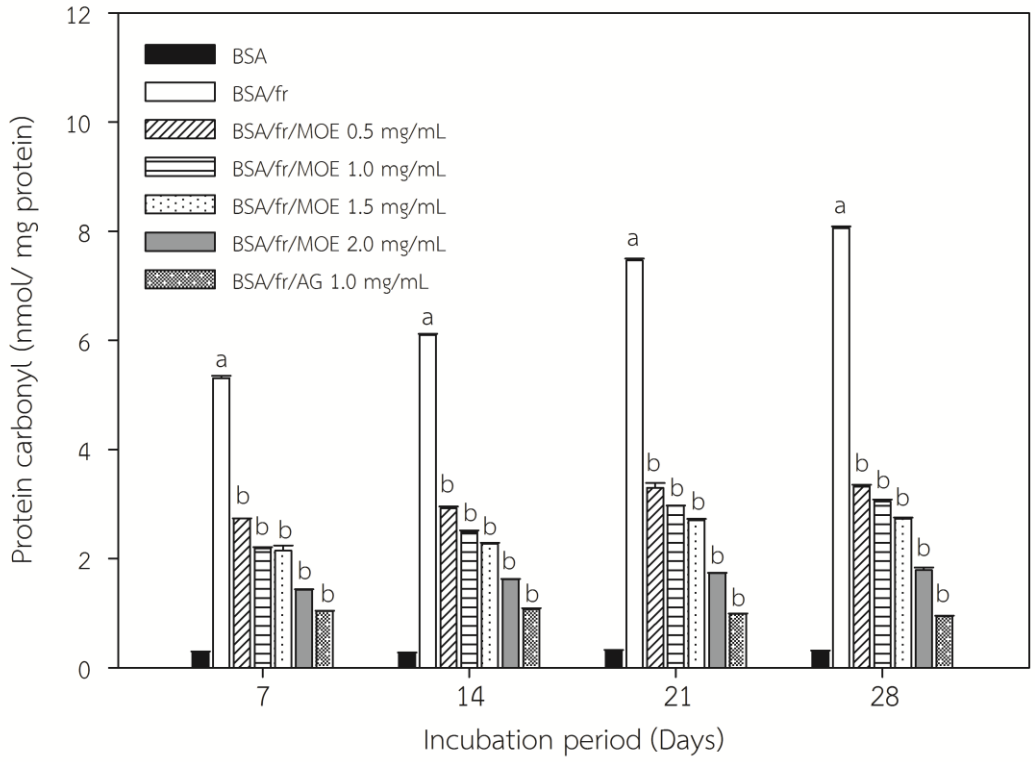


Figure 46. The effect of MOE (0.5 -2.0 mg/mL) on protein thiol group in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA. [b]*P*< 0.05 compared to BSA/ fructose.

Table 25 The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on protein carbonyl content in the BSA/MG system.

	BSA+ MG +AG (1.0 mg/mL)	BSA+ MG +MOE (0.5mg/mL)	BSA+ MG +MOE (1.0 mg/mL)	BSA+ MG +MOE (1.5 mg/mL)	BSA+ MG +MOE (2.0 mg/mL)
Day 3	67.30± 0.01[b]	59.09± 0.02[b]	53.82 ± 0.02[b]	52.63± 0.01[b]	44.19± 0.01[b]
Day 7	71.73± 0.02[b]	57.75± 0.01[b]	53.84 ± 0.01[b]	51.69± 0.02[b]	42.57± 0.02[b]
Day 14	76.22± 0.02[b]	64.14± 0.03[b]	59.58 ± 0.01[b]	58.90± 0.01[b]	52.42± 0.01[b]

Data were expressed as mean ± SEM (n = 3).

Table 26. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/ml) on protein carbonyl content in the BSA/MG system.

	BSA	BSA+MG	BSA+ MG +AG (1.0 mg/mL)	BSA+ MG +MOE (0.5mg/mL)	BSA+ MG +MOE (1.0 mg/mL)	BSA+ MG +MOE (1.5 mg/mL)	BSA+ MG +MOE (2.0 mg/mL)
Day 3	0.30 ± 0.01	5.34 ± 0.02[a]	1.75 ± 0.01[b]	2.98 ± 0.02[b]	2.53 ± 0.02[b]	2.47 ± 0.01[b]	2.19 ± 0.01[b]
Day 7	0.27 ± 0.01	6.44 ± 0.02[a]	1.83 ± 0.02[b]	3.70 ± 0.01[b]	3.12 ± 0.01[b]	2.98 ± 0.02[b]	2.72 ± 0.02[b]
Day 14	0.33 ± 0.01	8.31 ± 0.04[a]	1.98 ± 0.02[b]	3.96 ± 0.03[b]	3.42 ± 0.01[b]	3.36 ± 0.01[b]	2.98 ± 0.01[b]

Data were expressed as mean ± SEM (n = 3). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/MG.

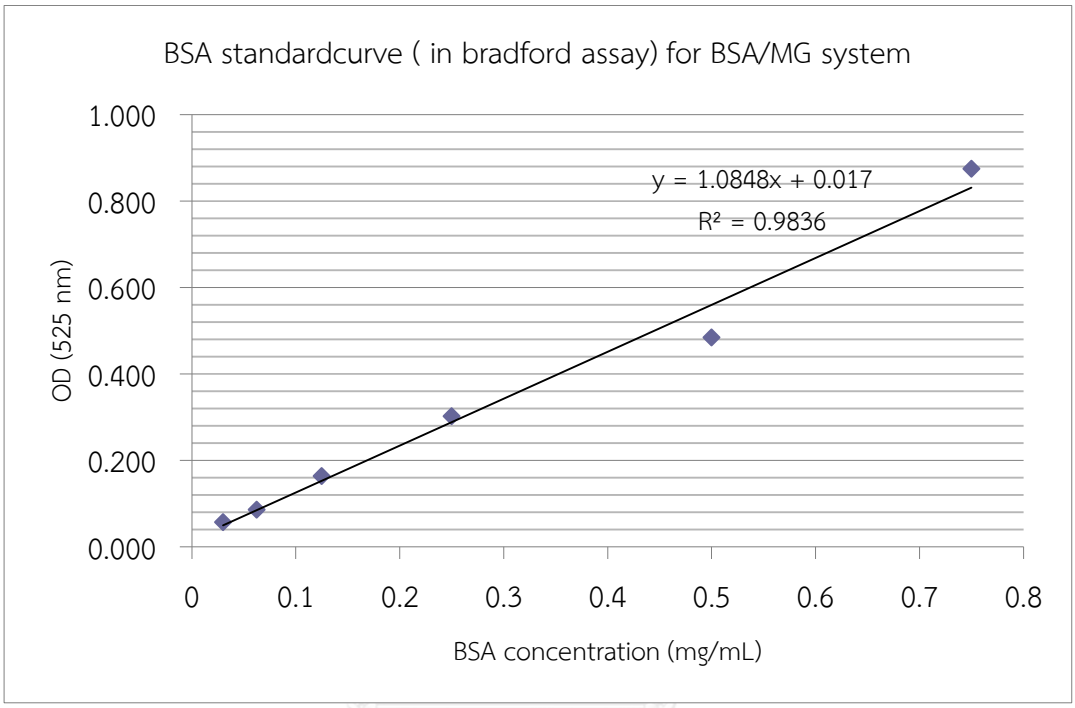


Figure 47. Bradford reagent for BSA/MG system

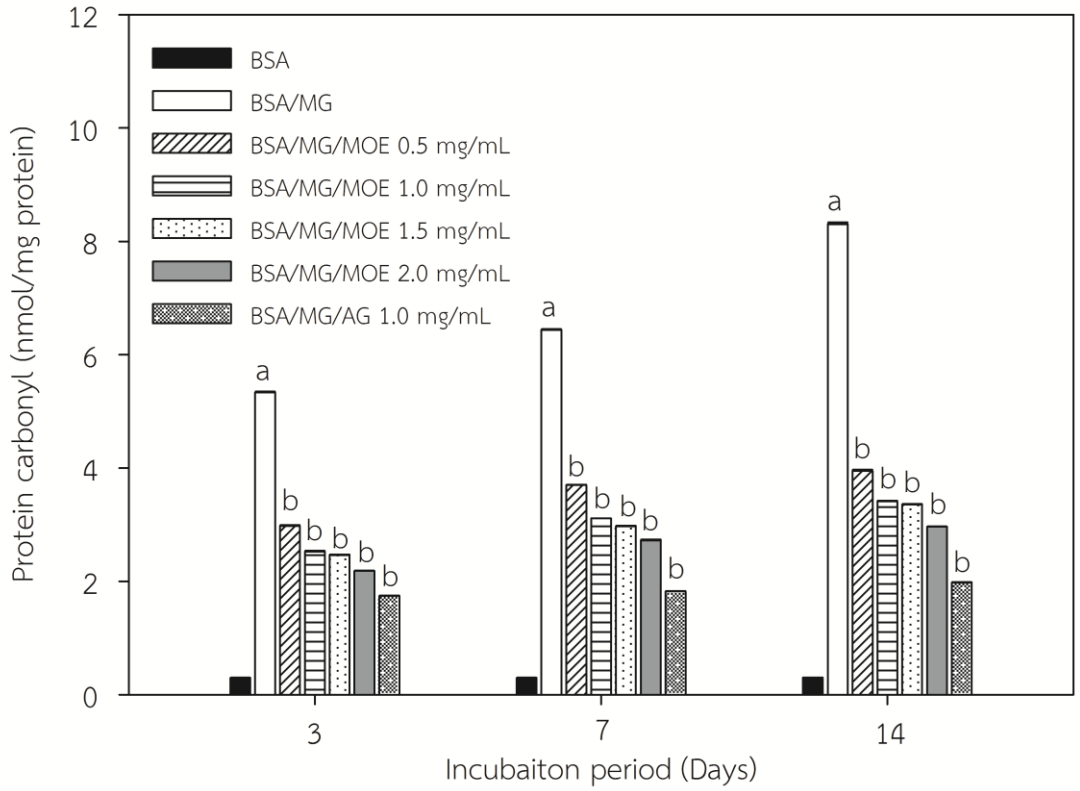


Figure 48. The effect of MOE (0.5 -2.0 mg/mL) on protein carbonyl content in BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ MG.

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4.4 To investigate the protein aggregation

β-amyloid cross structure was the indicator to measure the protein modification resulting in protein aggregation. The experimental setting was incubated BSA with glucose and fructose to illustrate the accumulation of β-amyloid level as shown in the figure 20-21. The system with glucose raised the formation of β-amyloid cross structure for 1.49-fold and also 1.75-fold in fructose system when compared to BSA at the end of incubation period.

Besides, the addition of MOE in various concentrations (0.5, 1.0, 1.5, 2.0 mg/mL) showed a significant decreased β-amyloid level in a dose-dependent manner throughout of the study period by 25.14%, 29.98%, 38.97%, and 39.76%, respectively in glucose system and also 36.06%, 40.93%, 46.66%, 48.47%, respectively in fructose system. To access the effect of AG (1mg/mL), it remarkably decreased the β-amyloid level by 22.82% in glucose system and 34.04% in fructose system. MOE at concentration 1 mg/mL in both systems demonstrated more inhibitory effect on protein cross-linking than AG at the same concentration.

In access to determine the inhibitory effect of MOE on BSA/MG system, MG was incubated with BSA as a negative control and it also increased the level of β-amyloid level for 1.56-folds at day 14 of incubation period. Generally, all concentration of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) markedly manifested a significant lowering in the formation of β-amyloid cross structure by dose-dependent manner which has been demonstrated in percentage of inhibition by 8.15%, 12.03%, 17.79%, 26.35%, respectively.

Moreover, after compared to the same concentration (1mg/mL) between AG and MOE, it has been shown that AG has a higher potential to protect the elevation of protein carbonyl content than MOE in BSA/MG system.

Thus, AG had a higher power lowering effect in β-amyloid level than MOE in BSA/MG system whereas MOE displayed the inhibitory effect more than AG both in glucose and fructose system

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Table 27. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on β-amyloid level in the BSA/glucose system.

	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	4.64 ± 15.38	32.96 ± 7.22	35.11 ± 17.19	38.91 ± 22.01	47.10 ± 28.61
Day 14	17.60 ± 23.12	32.47 ± 18.37	37.26 ± 18.04	41.12 ± 10.34	44.44 ± 7.94
Day 21	22.48 ± 1.16	27.92 ± 11.87	31.89 ± 42.16	37.94 ± 39.73	40.49 ± 33.78
Day 28	22.82 ± 14.84	25.14 ± 25.01	29.98 ± 33.24	38.97 ± 18.53	39.76 ± 4.10

Data were expressed as mean ± SEM (n = 3).

Table 28. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on β-amyloid level in the BSA/glucose system.

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	3032.67± 29.84	3503.67± 8.69[a]	3341.00 ± 15.38[b]	2349± 7.22[b]	2273.67 ± 17.19[b]	2140.34 ± 22.01[b]	1853.34 ± 28.61[b]
Day 14	3084.67± 19.48	4181.00 ± 15.31[a]	3445.00 ± 23.12[b]	2823.34± 18.37[b]	2623.00 ± 18.04[b]	2461.67 ± 10.34[b]	2323.00 ± 7.94[b]
Day 21	3105.34± 36.44	4442.67 ± 7.86[a]	3444.00 ± 1.16[b]	3202.34± 11.87[b]	3026.00 ± 42.16[b]	2757.34 ± 39.73[b]	2644.00 ± 33.78[b]
Day 28	3097.67± 8.52	4632.67± 59.84[a]	3575.34 ± 14.84	3468± 25.01[b]	3243.67± 33.24[b]	2827.34 ± 18.53[b]	2790.67 ± 4.10[b]

Data were expressed as mean ± SEM (n = 3).). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/glucose.

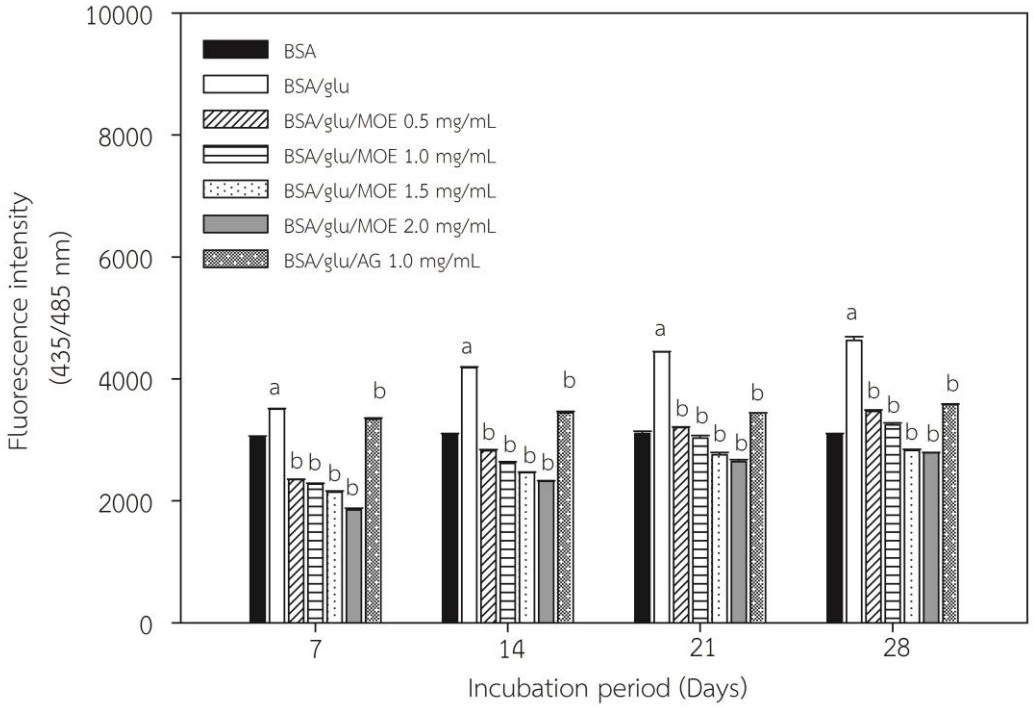

Figure 49. The effect of MOE (0.5 -2.0 mg/mL) on β-amyloid level in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ glucose.

Table 29. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on β-amyloid level in the BSA/fructose system.

	BSA+fr+AG (1.0 mg/mL)	BSA+ fr +MOE (0.5mg/mL)	BSA+ fr +MOE (1.0 mg/mL)	BSA+ fr +MOE (1.5 mg/mL)	BSA+ fr +MOE (2.0 mg/mL)
Day 7	20.85 ± 8.20	41.82 ± 14.00	44.89 ± 32.04	46.32 ± 32.31	52.46 ± 11.60
Day 14	26.20 ± 3.79	39.34 ± 9.53	43.52 ± 16.66	46.83 ± 20.89	51.26 ± 1.21
Day 21	30.56 ± 19.43	35.14 ± 10.79	38.93 ± 5.86	46.59 ± 15.38	52.36 ± 26.83
Day 28	34.05 ± 4.10	36.07 ± 12.92	40.94 ± 11.90	46.67 ± 7.89	48.48 ± 26.84

Data were expressed as mean ± SEM (n = 3).

Table 30. The effect of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on β-amyloid level in the BSA/ fructose system.

	BSA	BSA+glu	BSA+glu+AG (1.0 mg/mL)	BSA+glu+MOE (0.5mg/mL)	BSA+glu+MOE (1.0 mg/mL)	BSA+glu+MOE (1.5 mg/mL)	BSA+glu+MOE (2.0 mg/mL)
Day 7	2982.00 ± 12.29	4360.00 ± 17.25[a]	3451.34 ± 8.20[b]	2537.00 ± 14.00[b]	2403.00 ± 32.04[b]	2340.67 ± 32.31[b]	2073.00 ± 11.60[b]
Day 14	2976.67 ± 2.41	4711.34 ± 7.34[a]	3477.00 ± 3.79[b]	2858.34 ± 9.53[b]	2661.00 ± 16.66[b]	2505.34 ± 20.89[b]	2296.67 ± 1.21[b]
Day 21	3011.34 ± 8.30	4934.67 ± 21.31[a]	3426.67 ± 19.43[b]	3201.00 ± 10.79[b]	3014.00 ± 5.86[b]	2636.00 ± 15.38[b]	2351.34 ± 26.83[b]
Day 28	2991.34 ± 6.77	5256.00 ± 27.69[a]	3466.67 ± 4.10[b]	3360.67 ± 12.92[b]	3104.34 ± 11.90[b]	2803.34 ± 7.89[b]	2708.00 ± 26.84[b]

Data were expressed as mean ± SEM (n = 3).). [a]$P < 0.05$ compared to BSA, [b]$P < 0.05$ compared to BSA/fructose.

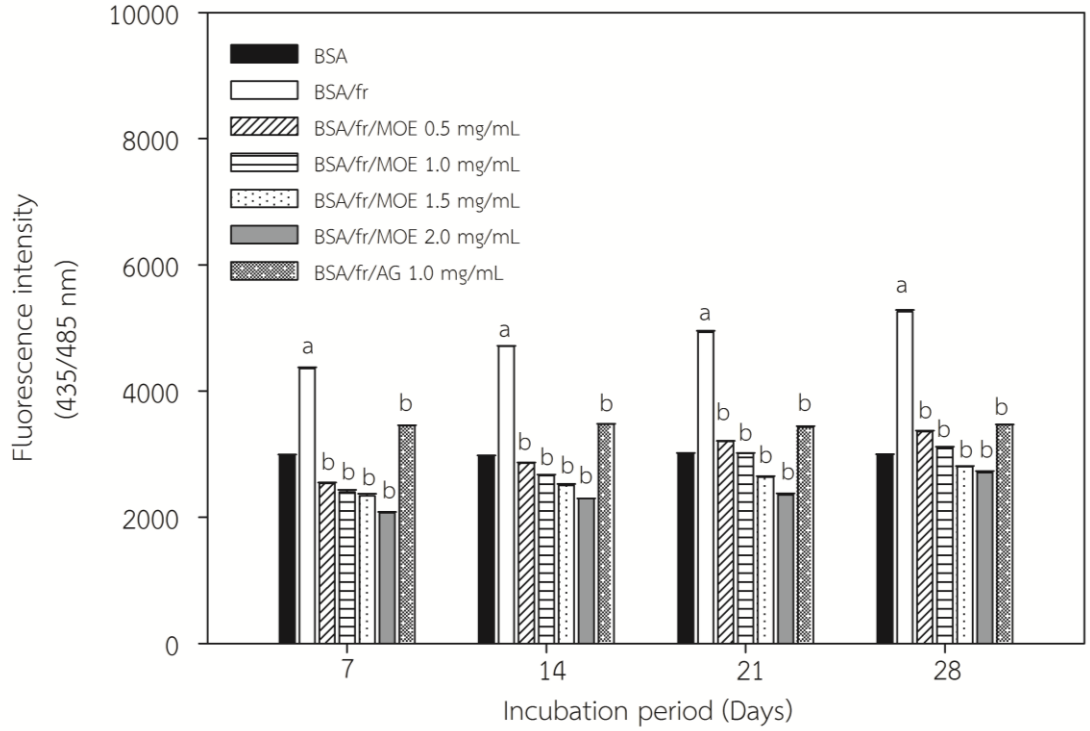

Figure 50. The effect of MOE (0.5 -2.0 mg/mL) on β-amyloid level in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ fructose.

Table 31. The percentage inhibition of MOE (0.5-2.0 mg/mL) and Aminoguanidine (1.0 mg/mL) on β-amyloid level in the BSA/MG system.

	BSA+ MG +AG (1.0 mg/mL)	BSA+ MG +MOE (0.5mg/mL)	BSA+ MG +MOE (1.0 mg/mL)	BSA+ MG +MOE (1.5 mg/mL)	BSA+ MG +MOE (2.0 mg/mL)
Day 3	35.54 ± 59.01	4.06 ± 38.02	7.70 ± 26.78	15.36 ± 22.31	15.43 ± 43.19
Day 7	40.70 ± 75.15	2.35 ± 22.22	13.20± 12.45	16.32 ± 21.98	22.30 ± 28.02
Day 14	43.58 ± 66.22	8.16 ± 34.05	12.04± 8.42	17.80 ± 20.21	26.26 ± 16.45

Data were expressed as mean ± SEM (n = 3).

Table 32. The effect of MOE (0.5-2.0 mg/ml) and Aminoguanidine (1.0 mg/ml) on β-amyloid level in the BSA/ MG system.

	BSA	BSA+ MG	BSA+MG+AG (1.0 mg/mL)	BSA+ MG +MOE (0.5mg/mL)	BSA+ MG +MOE (1.0 mg/mL)	BSA+ MG +MOE (1.5 mg/mL)	BSA+ MG +MOE (2.0 mg/mL)
Day 3	3542.00 ± 59.01	4732.00 ± 38.02[a]	3050.67 ± 26.78[b]	4540.00 ± 22.31[b]	4367.67 ± 43.19[b]	4005.34 ±31.82[b]	4002.00 ± 29.72[b]
Day 7	3743.00 ±75.15	5266.34 ± 22.22[a]	3123.34 ± 12.45[b]	5142.67 ± 21.98[b]	4571.34 ± 28.02[b]	4407.00 ±18.78[b]	4092.00 ± 12.49[b]
Day 14	3639.00 ± 66.22	5679.34 ± 34.05[a]	3204.34 ± 8.42[b]	5216.00 ± 20.21[b]	4996.00 ± 16.45[b]	4668.67 ±13.39[b]	4188.00 ± 1.74[b]

Data were expressed as mean ± SEM (n = 3).). [a]$P< 0.05$ compared to BSA, [b]$P< 0.05$ compared to BSA/MG.

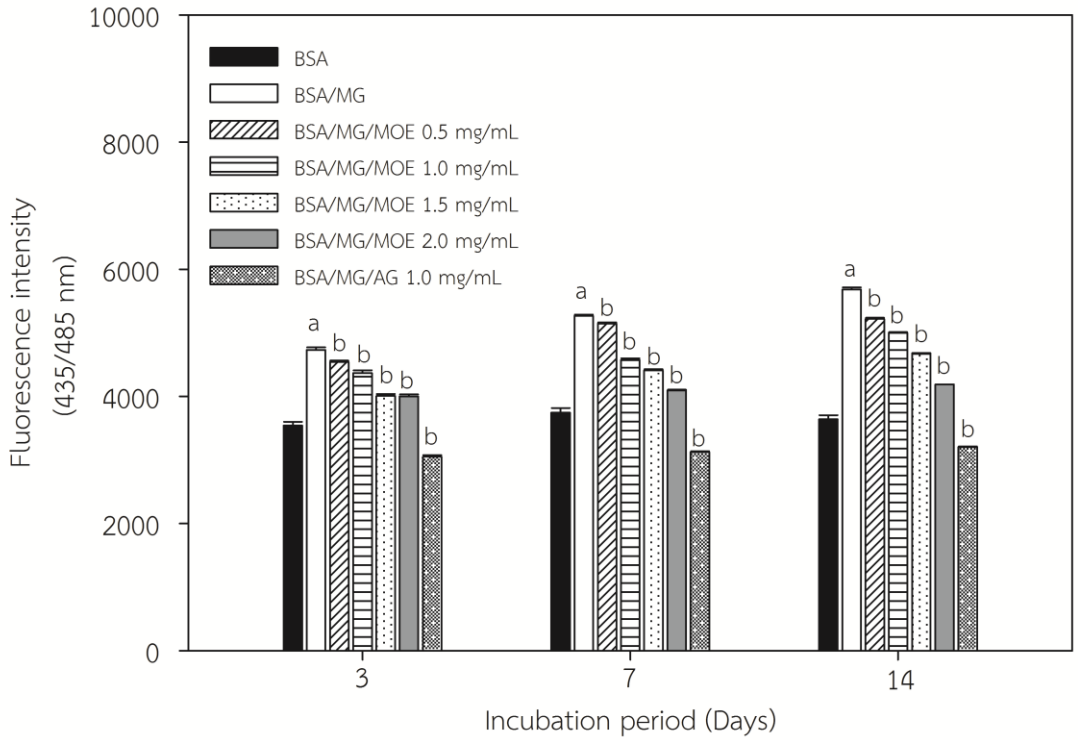

Figure 51. The effect of MOE (0.5 -2.0 mg/mL) on β-amyloid level in BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ MG.

CHAPTER V

DISCUSSION

Up to date, the number of diabetic patients had been expanded to over 500 million people (50.7% from 2011) in 2030 that plays the important role to escalate the number of diabetic complications. Chronic hyperglycemia can cause non-enzymatic protein glycation when carbonyl group of a reducing sugar reacts with an amino group of proteins resulting in AGE formation and also generates reactive oxygen species (ROS) during the reaction which plays a crucial role in the pathogenesis of diabetes complications. ROS cause the alternation of proteins leading to change their characteristics, physiochemical, and biochemical properties resulting in macrovascular and microvascular degenerative diseases as well as cell apoptosis, and DNA damage. Many studies have reported a correlation between polyphenol-rich plants and the ability to reduce the overproduction of free radicals, ROS, and reactive nitrogen species (RNS) (Suwannaphet, Meeprom, Yibchok-Anun, & Adisakwattana, 2010). Additionally, recent data describing the anti-glycation activity of polyphenol-rich plants resulting in decreasing AGEs formation have also been reported (Adisakwattana & Chanathong, 2011; Chumark et al., 2008).

In this study, the results showed that total phenolic and flavonoid content *in Moringa Oleifera* leaf water extract (MOE) were 38.56 mg GAE /g extract and 23.12 mg catechin / g dry extract, respectively. Our results are concomitant with previous studies that also found total phenolic and flavonoid content in the range of 33.82 to 45.21 mg GAE /g extract and 15.39 to 27.20 mg catechin / g dry extract (Adisakwattana & Chanathong, 2011; Verma et al., 2009a). The results from HPLC of

MOE revealed that phenolic content was ferulic acid whereas flavonoid contents were quercetin, rutin and keamferol. Previous study also reported other phenolic compound in MOE by using HPLC such as gallic acid > chlorogenic acid (534.4 μg/g) > ellagic acid (488.5 μg/g) > ferulic acid (189.1 μg/g) and also flavonoids including quercetin (807 μg/g) > keamferol (497.6 μg/g) > rutin (190 μg/g) (Pari, Karamac, Kosinska, Rybarczyk, & Amarowicz, 2007). In the present study, ferulic acid was the highest amount that could be identified in the MOE which is consistent with previous study that found ferulic acid in MOE extracted by methanol: water (80: 20) (Vongsak, Sithisarn, & Gritsanapan, 2012). However, it should be noted that there are some phenolic compounds that cannot be characterized in this study because of instrument limitations. Thus, to confirm the highest amount of MOE and other phenolic compounds, HPLC-MS should be used for identification in the further study. A number of evidences have documented that ferulic acid has more potent on ROS scavenging than other phenolic compounds such as vanillic, coumaric, and cinnamic acid (Ramkissoon, Mahomoodally, Ahmed, & Subratty, 2013). In addition, ferulic acid protects against free radical mediated changes in conformation of membrane proteins (Sompong, Meeprom, Cheng, & Adisakwattana, 2013; Wang, Sun, Cao, & Tian, 2009). Moreover, other phenolic compounds containing in MOE including rutin, quercetin and keamferol are also powerful antioxidant capacity that can protect against ROS production through various mechanisms *in vitro* such as scavenging the free radical, metal chelation, or trapping with dicarbonyl compound (DuPont, Day, Bennett, Mellon, & Kroon, 2004; Nijveldt et al., 2001). Additionally, Burda and Oleszek reported the comparison antioxidant activity among in flavonoid group, they were found that keamferol had the highest ability (65%) on methylation process than

quercetin (63%). In order to DPPH assay, keamferol also had the highest percentage of antiradical activity (93%) than rutin (90%) and quercetin (89%) (Nijveldt et al., 2001).

In this study, reducing sugars including glucose and fructose and dicarbonyl compound, methylglyoxal (MG), can induce AGE formation both fluorescence and non-fluorescence AGE; the late stage product of AGE reaction. MG illustrated the highest ability to induce glycation formation that increased 6.52-folds of fluorescence AGE and 11.76-folds in CML (non-fluorescence formation). Previous study also showed that MG was much more reactive and potent glycating agent than glucose (Suarez et al., 1989). Another study also demonstrated the dicarbonyl compound such as α-oxoaldehyde (MG) that has the most potential to induce glycation when compares to glucose, fructose, ribose and α-oxoaldehyde (P. J. Thornalley, 1996). Moreover, all inducers used in the current study increased level of fructosamine which is a product of an initiation stage of AGE reaction. Fructosamine was commonly used as diabetic indicator which known as glycated hemoglobin or generally referred in term of HbA1c. It was the index of glycemic control in human blood sugar that ameliorated under hyperglycemic condition (Armbruster, 1987). Although many studies reported fructose can react with amino group of protein and undergoes glycation reaction faster than glucose, the results in this study showed that glucose increased the fructosamine level more than fructose 7.6- folds. The contradiction of the findings might be because the Amadori products from glucose and fructose are structurally different. The NBT reagent under alkaline condition can react with Amadori products in the form of ketone produced from glucose more than Amadori products in the form of aldehyde produced from fructose

(Semchyshyn et al., 2014). This may lead to underestimate the determination of frutosamine level in fructose system. These results are similar to previous study that found the rate of protein glycation in Amadori phase upon 10 times of fructation than glucation (Suarez et al., 1989).

The effect of MOE protect against glucose, fructose and methylglyoxal-induced protein glycation and oxidation were shown in the present study. It was found that MOE reduced fluorescence and CML level. This inhibitory effect might be because the active compounds of MOE can reduce fructosamine level which is an early product of glycation reaction. The previous studies demonstrated the ability of phenolic compound such as ferulic acid which was also play a significantly effect on reduction of fructosamine level in monosaccharide-mediated protein glycation (Sompong et al., 2013). In addition, flavonoid potentiality in Thyme (*Thymus valgaris*) leaf extracts referred to cirsilineol and quercetin that has been shown the inhibitory effect on fructosamine formation in BSA/glucose system by blocking the activity at lysine terminal (MoRiivnrsU, Yosrnox, Esaki, & Hirota, 1995). Thus, the protective effect of MOE on protein glycation may be resulted from phenolic compounds. The expected mechanism of two active compounds may be the competition attaching at the functional structure of the reducing sugar, the Amadori product production may be decreased that directly effect to the AGE formation both in fluorescence and non-fluorescence AGE. It is possible that hydroxyl group of beta ring of flavonoid can replace the attachment between the binding group of the protein with aldehyde group of the reducing sugar. Moreover, antioxidant of phenolic compound containing in MOE can scavenge free radical that may react in the dienol group of Schiff base rearrangement during glycation reaction (Suarez et al., 1989).

The efficacy of phenolic and flavonoid were also the main topic on anti-AGE formation. Wu and Yen shown the ranking of flavonoid potentiality showed the inhibitory effect on glucose and MG-mediated fluorescence AGE formation by followed the order of flavone> flavonol > flavanol > flavanone especially in keamferol > quercetin > rutin because of their radical scavenging activities on DPPH radical (Wu & Yen, 2005). In term of ferulic acid, Sompong et al have been shown the inhibitory effect of ferulic acid on both fluorescence AGE and CML formation, the results was proposed that ferulic acid may blocked carbonyl or dicarbonyl groups in monosaccharide, Amadori products can be used as a process to inhibit protein glycation (Sompong et al., 2013). Comparison to this study, both of products significantly diminished in a dose-dependent manner after MOE were added in all systems. The expected mechanism may include possessing free radical scavenging properties toward hydroxyl radicals, and attaching to the protein forming the complexes like an Aminoguanidine (AG) which used as the positive control in glycation assay (Brownlee et al., 1986; Corbett et al., 1992).

In order to excess protein oxidation, ROS production during intermediate AGE reaction stimulated protein oxidation by increase carbonyl group and deplete protein thiol group that was the prominent pathway to motivate AGE-associated diabetic complications (Aronson, 2008). The relationship between protein thiol and protein carbonyl were a little bit correlated because the glycating agents presumably to give hemithioacetal that induced to the depletion of thiol groups. Direct reaction of MG with amino acid binding site such as Lysine (Lys), Histidine (His), or Argenine (Arg) residues has not been clearly, but it is likely to contribute enzyme inactivation (Kalapos, 2008)Protein-bound carbonyls (i.e. proteins preglycated with MG) results in

enzyme inactivation, with concomitant loss of thiol groups Cysteine (Cys) and methionine (Met) are especially tended to react with oxidative compound by attacking free radical species (ROS) such as superoxide and hydroxyl radicals to form protein carbonyl content that was the reason why protein carbonyl related to protein thiol group (Morgan, Dean, & Davies, 2002). Accordingly, the protein carbonyl content is most commonly used as a marker for protein oxidative damage (Kang, 2003). Scavenging activity, chelating metal ion, and trapping dicarbonyl compound were also the mechanism which insisted to block oxidative damage in the recent study. Scavenging activity and chelating metal ion were the screening method. In addition, MG-trapping was the most revealed as a fundamental method to prove that major active compounds can decrease the ROS in the reaction (Matsuda et al., 2003). Lishuang et al and Zheng et al explained that quercetin (flavonols) inhibits AGE formation via reduced protein oxidation by directly trapping MG and Glyoxal (GO) (Fu et al., 2008). Pashikanti et al. point out the proposed mechanism of rutin, a possible mechanism was involve the condensation of the adjacent phenolic hydroxyl moieties with dicarbonyl intermediates, forming a hemiacetal intermediate that can react further to form an acetal. To inhibit MG-mediated protein glycation, Rutin had more effectively trapped and inactivated RCS such as MG and GO with potentiality (Pashikanti, de Alba, Boissonneault, & Cervantes-Laurean, 2010). H.Y. Kim et al also reported that kaempferol 3-O-d-glucopy ranoside (astragalin) and quercetin 3-O--d-glucopyranoside (isoquercitrin) from *Eucommia ulmoides* leaves extract exhibited protein oxidation inhibitory activity by traped reactive dicarbonyl impeding conversion to AGE From this study, the protective effect on protein thiol depletion, concomitant with the lowering of protein carbonyl content may be related to the

previous mechanism that described on scavenging activity, metal chelating activity, and also MG-trapping of among four active compounds such as ferulic acid, quercetin, keamferol and rutin (Jung, Kim, Chung, & Choi, 2002).

Afterward protein oxidation, in case of proteins ROS can introduce several of protein modification among in cysteine, methionine, tryptophan, arginine, lysine, proline, and histidine. β-amyloid cross structure was lowered by the adduction between carbohydrate and protein residue. Irreversible protein crosslinking in heterogeneous protein aggregation was modified in the end stage of AGE reaction (Rojas et al., 2008). The influences of protein aggregation cause neurodegenerative diseases. Alzheimer's disease was long term effect about protein aggregated in artery supplying to the brain. Previous studies have been shown the effect of flavone subgroup such as rutin, quercetin and also keamferol in lettuce, parsley, cranberry, onion, and apples significantly lowered the protein aggregation by scavenging some free radical and inhibit the enzyme xanthine oxidase (Palumbo et al., 1977). Stefani and Rigacci have been shown the effect of ferulic acid on free radical scavenging activity toward hydroxyl radical. The hydroxyl group in the ferulic acid can readily form a resonance stabilized phenoxy radical which was the key to act as an antioxidant property. In addition, pretreatment of ferulic acid protected against primary neuronal cell cultures against hydroxyl and peroxyl radical-mediated oxidative damage (Stefani & Rigacci, 2013). Regarding to the effect of quercetin on protein aggregation, quercetin (like other flavonols) was also shown to heavily quench ThT fluorescence emission. The way of flavonols inhibit amyloid aggregation is still a matter of debate, as well, even considering their activity on the same amyloidogenic peptide (Stefani & Rigacci, 2013). Generally, quercetin protected

against amyloid-induced cytotoxicity acting at multiple levels by metal chelation, and also scavenges ROS Overall, these and other data indicate that, at present, MOE contained ferulic acid, quercetin, keamferol and rutin, that may be reinforced the effect to inhibited the cross-linking of protein by reducing β-amyloid structure formation via many mechanism which mentioned before (Stefani & Rigacci, 2013).

However, the inhibitory effect on protein glycation may relate to other active ingredients that still unknown; LC-MS will be used to confirm the unidentified peak. In the same way, the certain active biological constituents of MOE also remain unknown. To prove the second objective, MG-trapping or antioxidant abilities were used to confirm the mechanism.

CHAPTER VI

CONCLUSION

In this present research indicates that *Moringa oleifera* aqueous leaf extract (MOE) has high amount of polyphenolic and flavonoid contents including ferulic acid, rutin, quercetin, and keamferol. These polyphenolic and flavonoid have ability to inhibit non-enzymatic glycation reaction by reducing fluorescence, non-fluorescence AGEs and fructosamine level. Moreover, phenolic-rich MOE also protects against reducing sugars and dicarbonyl compound-induced protein oxidation and protein cross-linking in glycation reaction. It is possible that antioxidants properties of its polyphenolic and flavonoid contents play an important role in such effects. Thus, MOE may be used as an alternative therapeutic agent to prevent or ameliorate diabetic complications. Moreover, MOE could be emerging as a new nutraceutical food or functional food for applied to lessen the progression of AGE-associated diabetic complications.

Notwithstanding, there are some active compounds that cannot be identified by HPLC. In addition, the anti-glycation properties of MOE were demonstrated only in *in vitro* model. Therefore, further analysis of the unknown compounds should be done by LC/MS. Moreover, to confirm its anti-glycation abilities and toxicity, animal model and clinical trial are needed.

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APPENDIX

VITA

Pornpimon Nunthanawanich was born in January 10th, 1990 in Nakhonratchasima, Thailand. After graduating high school grade12 in 2009 from Suranari Wittaya School, she attended Mahidol University for undergraduate degree in the department of Nutrition and dietetics from Faculty of Public Health. She graduated with her Bachelors of Science (Public Health) with second class honor in July, 2011. After that she really interested to finding the new knowledge and experiences in graduate degree. Thus, she entered to study in the master degree at Faculty of Allied Health Sciences, Chulalongkorn University in the department of Nutrition and dietetics and the major of applied nutrition. Her research focused on anti – glycation properties of Moringa Oleifera leaf extract in vitro and her thesis advisor was Dr. Sathaporn Ngamukote.

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