รายงานวิจัยฉบับสมบูรณ์

โครงการฤทธิ์ของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง

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สัญญาเลขที่ MRG5680120

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รหัสโครงการ **MRG5680120**

ชื่อโครงการ **ฤทธิ์ของสารกสัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง**

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บทคัดย่อ

ภาวะระดับน้ำตาลในเลือดสูงเรื้อรัง เป็นสาเหตุของการเกิดกระบวการไกลเคชั่นโดยไม่อาศัยเอนไซม์จากการจับกันระหว่างน้ำตาลรีดิวซ์ซึ่ง และหมู่อะมิโนของโปรตีน เกิดเป็นแอดวานซ์ ไกลเคชั่น เอ็น โปรดักส์ (advanced glycation end products) ข้อมูลจากงานวิจัยในอดีตยืนยันแน่ชัดว่า แอดวานซ์ ไกลเคชั่น เอ็น โปรดักส์ เป็นปัจจัยสำคัญที่ส่งผลต่อการเกิดภาวะแทรกซ้อนในหลอดเลือดขนาดเล็ก และหลอดเลือดขนาดใหญ่ในผู้ป่วยเบาหวาน มะรุม (*Moringa oleifera*) เป็นหนึ่งในพืชสมุนไพรที่เป็นที่รู้จัก และนิยมใช้อย่างแพร่หลายในประเทศไทย ซึ่งหลักฐานการศึกษาทางวิทยาศาสตร์พบว่า มะรุมมีฤทธิ์ในการป้องกันหรือรักษาโรคเบาหวานได้หลากหลายกลไก อย่างไรก็ตามยังไม่มีการศึกษาใดศึกษาฤทธิ์ของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นที่ถูกเหนี่ยวนำด้วยน้ำตาลกลูโคส น้ำตาลฟรุกโตส และสารเมทิลไกลออกกซอล โดยการบ่มโปรตีนอัลบูมินจากวัว (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) อีกทั้งสารสกัดจากใบมะรุมยังสามารถป้องกันการเกิดโปรตีนออกซิเดชัน โดยลดระดับของการเกิดโปรตีนคาร์บอนิล (protein carbonyl) และป้องกันการลดลงของหมู่ไทออล (thiol group) นอกจากนี้สารสกัดจากใบมะรุมสามารถยังยับยั้งการเกิดการตกตะกอนของโปรตีนโดยลดระดับของการสร้างโครงสร้างเบต้า อะไมลอยด์ (β-amyloid structure) อย่างมีนัยสำคัญตามความเข้มข้นที่เพิ่มขึ้น จากผลการทดลองสามารถสรุปได้ว่า สารสกัดจากใบมะรุมอาจนำไปประยุกต์เพื่อใช้เป็นสมุนไพรทางเลือก เพื่อป้องกันการเกิดภาวะแทรกซ้อนอันเนื่องมาจากกระบวนการไกลเคชั่นในผู้ป่วยเบาหวาน อย่างไรก็ตามควรศึกษาถึงสารสำคัญในสารสกัดจากใบมะรุมด้วยวิธี Liquid chromatography–mass spectrometry ต่อไปในอนาคต

คำสำคัญ: สารสกัดจากใบมะรุม การยับยั้งกระบวนการไกลเคชั่น ภาวะแทรกซ้อนจากโรคเบาหวาน

Project Code **MRG5680120**

Project Title **Antiglycation Properties of *Moringa oleifera* Leaf Extract In Vitro**

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Abstract

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.

Keywords : *Moringa oleifera* / anti-glycation / diabetes complication

Executive Summary

Antiglycation properties of *Moringa oleifera* leaf extract *in vitro*

ฤทธิ์ของสารสกัดใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นในหลอดทดลอง

1. ความสำคัญและที่มาของปัญหาที่ทำการวิจัย

โรคเบาหวานเป็นโรคไม่ติดต่อ (Noncommunicable diseases; NCDs) ที่เป็นสาเหตุสำคัญในการเพิ่มอัตราการเสียชีวิตของประชาชนทั่วโลก และจากการศึกษาขององค์การอนามัยโลก (WHO) พบว่ามากกว่า 80% ของผู้เสียชีวิตด้วยโรคเบาหวาน เป็นประชากรในกลุ่มประเทศที่มีรายได้ต่ำถึงรายได้ปานกลาง โรคเบาหวานอาจเกิดจากความผิดปกติที่ตับอ่อนทำให้หลั่งฮอร์โมนอินซูลินลดลง หรือเกิดจากการที่ฮอร์โมนอินซูลินไม่สามารถออกฤทธิ์ได้หรือที่เรียกว่ามี “ภาวะดื้อต่ออินซูลิน” (Insulin resistance) หรือมีความผิดปกติทั้งสองอย่างร่วมกัน ส่งผลให้ร่างกายมีระดับน้ำตาลในกระแสเลือดสูงกว่าปกติ

การมีภาวะน้ำตาลในเลือดสูงเป็นเวลานาน ทำให้เกิดหมู่คาร์บอนิลของน้ำตาลจับกับหมู่อะมิโนของโปรตีนแบบไม่อาศัยเอนไซม์ (nonenzymatic glycation) ทำให้เกิดสารที่เรียกว่า advanced glycation end products (AGEs) ซึ่ง AGEs นี้ ก่อให้เกิดการอักเสบแก่หลอดเลือด ทำให้เกิดการเปลี่ยนแปลงโครงสร้างของโปรตีน ทำลายโครงสร้าง extracellular matrix ของเซลล์ ส่งผลให้เกิดความผิดปกติของโครงสร้างและการทำงานของเซลล์ นอกจากนี้ AGEs ยังเหนี่ยวนำให้เกิดอนุมูลอิสระในร่างกาย เรียกว่า reactive oxygen species (ROS) อันได้แก่ ซูเปอร์ออกไซด์แอนไอออน (superoxide anion), ไฮโดรเจนเพอร์ออกไซด์ (hydrogen peroxide) และ ไฮดรอกซิลแรดิเคิล (hydroxyl radical) อันนำมาสู่ภาวะ oxidative stress ซึ่งเหนี่ยวนำให้เกิดภาวะแทรกซ้อนของโรคเบาหวาน ได้แก่ ภาวะแทรกซ้อนที่เกิดกับหลอดเลือดขนาดเล็ก เช่น พยาธิสภาพที่ตา ไต และเส้นประสาทตามมือ เท้า รวมทั้งภาวะแทรกซ้อนเกิดที่หลอดเลือดขนาดใหญ่ เช่น หัวใจ สมอง เป็นต้น ดังนั้น การได้รับสารที่มีคุณสมบัติในการต้านการเกิดไกลเคชั่น อาจช่วยชะลอความรุนแรงของโรคเบาหวานและลดการเกิดภาวะแทรกซ้อนได้

โพลีฟีนอล (Polyphenols) เป็นสารในกลุ่มสารประกอบฟีนอลิค (Phenolic Compound) พบได้มากในพืช ผัก ผลไม้และสมุนไพรหลากหลายชนิด มีคุณสมบัติในการเป็นสารต้านอนุมูลอิสระ ช่วยป้องกันการเกิดโรคเรื้อรังต่างๆ เช่น โรคมะเร็ง โรคหัวใจและหลอดเลือด เป็นต้น นอกจากนี้ยังพบว่าสารโพลีฟีนอล สามารถช่วยลดการเกิด advanced glycation end products (AGEs) ได้ จากการศึกษาในอดีตพบว่า สารสกัดจากใบมะรุม (*Moringa oleifera* leaf extract) มี โพลีฟีนอล ในปริมาณสูง และมีคุณสมบัติในการต้านอนุมูลอิสระ นอกจากนี้ยังพบว่า สารสกัดจากใบมะรุมสามารถลดระดับน้ำตาลในกระแสเลือดในสัตว์ทดลองได้ แต่ยังไม่มีการศึกษาใดที่แสดงถึงคุณสมบัติของสารสกัดดังกล่าวในการต้านการเกิดไกลเคชั่น ดังนั้นงานวิจัยนี้ผู้วิจัยจึงสนใจที่จะศึกษากลไกของสารสกัดจากใบมะรุมต่อการต้านการเกิดไกลเคชั่นในหลอดทดลอง

2. วัตถุประสงค์ของโครงการ

1. เพื่อวิเคราะห์สารสำคัญในสารสกัดจากใบมะรุมด้วยเครื่อง **High-Performance Liquid** Chromatography

2. ศึกษาฤทธิ์ของสารสกัดจากใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นและการออกซิเดชั่นของ bovine serum albumin (BSA) จากการเหนี่ยวนำด้วยน้ำตาลกลูโคส ฟรุกโทส และ เมทิลไกลออกซอล

3 สรุปงานวิจัย

จากงานวิจัยพบว่าสารสกัดจากใบมะรุมด้วยน้ำมี ปริมาณสารประกอบฟีนอลิก 38.56±1.50 มิลลิกรัม gallic acid equivalents (GAE) ต่อกรัมของน้ำหนักสารสกัดแห้ง และสารประกอบฟลาโวนอยด์ 23.12±0.80มิลลิกรัม catechin equivalents (CE) ต่อกรัมของน้ำหนักสารสกัดแห้ง และเมื่อทำการวิเคราะห์ชนิดของสารสำคัญพบว่า มีสาร ferulic acid 225.53±1.27 μg/mL) quercetin 0.38±0.04 μg/mL keamferol 0.15±0.04 μg/mL และ rutin 0.09±0.05 μg/mL เมื่อทำการศึกษาถึงฤทธิ์ของสารสกัดจากใบมะรุมต่อการยับยั้งการเกิดไกลเคชั่นพบว่า สารสกัดจากใบมะรุมที่ความเข้มข้น 1 mg/ml สามารถยับยั้งการเกิด fluorescent AGEs จากการเหนี่ยวนำด้วยน้ำตาลกลูโคส ฟรุกโทส และ เมทิลไกลออกซอลได้ 26.22%, 54.06% และ 25.20% นอกจากนี้สารสกัดจากใบมะรุมที่ความเข้มข้น 1 mg/ml สามารถยับยั้งการเกิด Non-fluorescent AGEs (N^{ε}-(carboxymethyl) lysine (CML)) AGEs จากการเหนี่ยวนำด้วยน้ำตาลกลูโคส ฟรุกโทส และ เมทิลไกลออกซอลนาน 28 วัน ได้ 31.01%, 66.81% และ 67.97% ตามลำดับ อย่างไรก็ตามเมื่อเปรียบเทียบสารสกัดจากใบมะรุมที่ความเข้มข้นเดียวกันกับ aminoguanidine (1.0 mg/mL) ซึ่งเป็นกลุ่ม positive control พบว่า aminoguanidine มีฤทธิ์ในการยับยั้งการเกิด fluorescent AGEs และ CML สารสกัดจากใบมะรุม

เมื่อทำการศึกษาถึงฤทธิ์ของสารสกัดจากใบมะรุมต่อการเกิดสาร amadori product (fructosamine) โดยทำการวิเคราะห์การเกิด fructosamine เฉพาะ BSA ที่ถูกเหนี่ยวนำให้เกิดไกลเคชั่นด้วย glucose และ fructose เท่านั้น เนื่องจากน้ำตาลทั้งสองชนิดสามารถเหนี่ยวนำการเกิดปฏิกิริยาไกลเคชั่นตั้งแต่ระยะ early stage ซึ่งทำให้เกิดการสร้าง amadori product แต่การเหนี่ยวนำให้ให้เกิดไกลเคชั่นด้วย methylglyoxal ซึ่งเป็นสารประกอบคาร์บอนิลในระยะ intermediate stage ของปฏิกิริยาไกลเคชั่น จึงไม่มีผลต่อการสร้าง amadori product จากการศึกษาพบว่า สารสกัดจากใบมะรุมสามารถยับยั้งการเกิด fructosamine ได้ 76.76% และ 29.31% จากการเหนี่ยวนำด้วยน้ำตาลกลูโคสและฟรุกโทสตามลำดับ เป็นเวลานาน 28 วัน เมื่อเปรียบเทียบสารสกัดจากใบมะรุมที่ความเข้มข้นเดียวกันกับ aminoguanidine (1.0 mg/mL) พบว่า สารสกัดจากใบมะรุม มีฤทธิ์ในการยับยั้งการเกิด fructosamine ได้ดีกว่า aminoguanidine

เมื่อทำการศึกษาฤทธิ์ของสารสกัดจากใบมะรุมต่อการเกิด protein oxidation พบว่า การลดการสูญเสีย protein thiol groups และลดการสร้าง protein carbonyl compounds ซึ่งในการเกิดกระบวนการไกลเคชั่นมีการเหนี่ยวนำให้เกิดสารอนุมูลอิสระสูง ซึ่งอนุมูลอิสระนี้จะสามารถทำให้เกิด protein oxidation ส่งผลต่อการเปลี่ยนแปลงโครงสร้างของกรดอะมิโนที่สำคัญคือ cysteine มีหมู่ thiol ที่เป็นส่วนสำคัญ หากเกิดการ oxidation ของ หมู่ thiol จะทำให้โครงสร้างของโปรตีนเกิดการ

เปลี่ยนแปลง นอกจากนี้การเกิด protein oxidation ยังสามารถส่งผลให้เกิดการสร้าง protein carbonyl compounds ดังนั้น สารสกัดจากใบมะรุมซึ่งมีสารสำคัญในการต้านอนุมูลอิสระ จึงอาจเป็นกลไกที่สำคัญในการช่วยลดการลดการสูญเสีย protein thiol groups และลดการสร้าง protein carbonyl compounds ของโปรตีนที่ถูกเหนี่ยวนำด้วยน้ำตาลกลูโคส ฟรุกโทส และ เมทิลไกลออกซอลได้

ดังนั้นจากฤทธิ์ของสารสกัดสกัดจากใบมะรุมในการยับยั้งกระบวนการไกลเคชั่นในการศึกษานี้ จึงอาจนำเอาสารสกัดดังกล่าวมาพัฒนาเป็นสารต้านการเกิดไกลเคชั่นในผลิตภัณฑ์อาหารสุขภาพเพื่อลดความเสี่ยงต่อการเกิดภาวะแทรกซ้อนจากโรคเบาหวานได้

INTRODUCTION

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 (American Diabetes 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) (American Diabetes 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) (Fiorentino et al., 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 et al., 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 et al., 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 (Thornalley et al., 1999).

Many studies have shown that AGEs can generate reactive oxygen species (ROS), leading to increase oxidative stress (Tan et al., 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 et al., 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 et al., 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 et al., 2009; Verma et al., 2009a). 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, 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.

MATERIALS AND METHODS

Chemicals

Glucose, Fructose, and 2,4-dinitrophenyl hydrazine (DNPH), Disodium phosphate, Sodium chloride, Sodium carbonates, Sodium hydroxide were purchased from Ajax Finechem (Taren Point, Australia). Methylglyoxal solution, catechin, gallic acid, sodium azide, Nitroblue tetrazolium (NBT), aminoguanidine hydrochloride (AG), guanidine hydrochloride, Thioflavin T (ThT), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and L-cysteine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) was purchased from Merck (Darmstadt, Germany). OxiSelectTM CML ELISA kit was purchased from Cell Biolabs (San Diego, CA, USA). All other reagents used were of analytical grade.

Plant material

Moringa oleifera leaf was collected 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).

Preparation of *Moringa oleifera* aqueous leaf extract

The dried leaves (250 g) were extracted with distilled water twice (3 L) for 3h at 100ºC. The extraction was filtered through Whatman No.1 filter paper under the vacuum. The filtrate was further subjected to a spray dryer SD-100 (Eyela world, Tokyo Rikakikai Co., LTD, Japan) to obtain the extract powder. The spray drying conditions, inlet and outlet air temperature was set at 160ºC and 89-99ºC, respectively.

Determination of total phenolic compound

Total phenolic content of *Moringa oleifera* leaf extract was determined by Folin-Ciocalteu method (Verma et al., 2009b). The extract powder was dissolved in distilled water at a final concentration of 1.25, 2.5, 5 and 10 mg/mL. The freshly prepared Folin-Ciocalteu reagent, diluted 10-fold (75 µL) was gently mixed with 10 µL of sample and kept in the dark. After incubation at room temperature for 5 min, 75 µL of 7.5% Na_2CO_3 was added and allowed to stand for 30 min at room temperature in dark. Then, the mixtures were measured at 725 nm by UV spectrophotometer. To prepare the standard curve, gallic acid (0.025 – 0.4 mg/ml) was used. All determinations were performed in triplicate. The total phenolic content was expressed as mg gallic acid equivalents/g dry extract.

Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride assay through colorimetry as previously described with some modifications. Briefly, the extract was dissolved in distilled water followed by 30 µL of 5% $NaNO_2$ and allowed to stand for 5 min at room temperature. Then, 30 µL of 10% $AlCl_3$ was added to start the reaction. After 6 min of incubation, the reaction mixtures were treated with 200 µL of 1M NaOH and total volume were made up to 1 mL with distilled water. The absorbance was measured at 510 nm immediately using spectrophotometer. Catechin (0-300 mg/mL) was used as the standard for the calibration curve. All determinations were performed in triplicate. The total flavonoid content was expressed as mg catechin equivalents/g dry extract.

Identification of active compounds by using HPLC

The HPLC system Shimadzu LC-10A (Kyoto, Japan) was equipped with dual pump LC-10A binary system, UV detector SPD – 10A. Vertisep, C18 column (4.6 *x* 250 mm, 5µm) was used. The binary mobile phased for chromatographic separation consisted of pump A (0.1% formic acid (v/v) in water) and pump B (0.1% formic acid (v/v) in acetonitile) for following gradient: 0-2 min: 5% B, 32% B in 15 min, 50% B in 30 min, 75% B in 45 min, 50% B in 47 min, and 5% B in 55 min at a flow rate of 1 ml/min. The injection volume was 10 µL and wavelength was set at 254 nm. Phenolic standards including gallic acid, quercetin, rutin, caffeic acid and ferulic acid were used for identification of phenolic constituents of *Moringa oleifera* leaf extract. All Standards (1 mg/mL) were dissolved in absolute methanol and filtered with polyethersulfone membrane (PES).

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

-Preparation of bovine serum albumin glycation *in vitro.*

0.1M phosphate buffer saline (PBS) was used as the solvent in all of sample which mixed from the combination of NaCl, Na_2HPO_4 and NaH_2PO_4 in distilled water containing 0.02% NaN_3 and adjusted pH at 7.4 before used.

Bovine serum albumin (BSA) 10mg/mL, Aminoguanidine (AG) 1mg/mL, 0.5M glucose or 0.5M fructose or 1mM methylglyoxal solution were dissolved in 0.1M PBS and incubated with or without *Moringa oleifera* leaf extract (MOE) in the different concentration starting from 0.5, 1, 1.5, and 2 mg/mL which dissolved in 0.1M PBS at 37ºC for different incubation period that shown in the table 1. On the issue of the samples, the constituents were present in the table 2.

Table1. The incubation period of AGE prepared sample

Inducer	Incubation period
0.5 M Glucose	7, 14, 21, 28 days
0.5 M Fructose	7,14,21,28 days
1 mM MGO	3,7,14 days

Table2. The constituents of the AGE prepared sample.

Sample	Constituents
Blank	BSA
Negative control	BSA + Glucose or fructose or MGO
Treatment	BSA + Glucose or fructose or MGO + MOE (0.5, 1, 1.5, 2 mg/mL)
Positive control	BSA + Glucose or fructose or MGO + AG (1 mg/mL)

-Determination of AGE formation and Amadori product.

Fluorescent AGEs

The fluorescent AGEs is the irreversible product, 50 µL of glycated BSA was determined by spectrofluorometer at excitation and emission wavelengths of 355 nm and 460 nm, respectively (Povichit et al., 2010). All measurements were done in triplicate. The percentage inhibitions of each sample were calculated from:

$$\%Inhibition = \frac{Fluorescence\ negative - Fluorescence\ sample}{Fluorescence\ negative} \boldsymbol{X}\ 100$$

Non - Fluorescent AGEs

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).

To start with diluted 1:100 of sample with 1x PBS then diluted 10-fold dilution in reduced BSA and placed the sample in 96-well plate for 100µl. All samples were incubated at 37°C for 2 hours. After that, 250 µl of 1x PBS was used to remove unbound substances for twice in each well and added 200 µl of assay dilutant in the plate then incubated at 25°C for 2 hours on an orbital shaker. Washed each well with 250 µl of wash buffer for 3 times and 100 µl of diluted anti-CML antibody were added into all wells. Then, the samples were incubated for an hour at 25°C on an orbital shaker. After the incubation period, washed the strip well with 250 µl of wash buffer for 3 times and 100 µl of diluted HRP conjugated antibody was added in the plate to coat the strip well and put them into an orbital shaker for 1 hour at 25°C. The wells were washed again by using 250 µl of wash buffer for 5 times to remove unbound HRP conjugated antibody. The 100 µl of substrate solution was warmed at the room temperature and placed into the wells for 20 minutes on an orbital shaker to trap with bound HRP conjugated antibody and stop the reaction by using 100 µl of stop solution. 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).

Fructosamine level

Fructosamine, an Amadori product of non-enzymatic glycation between reducing sugars and amino groups of proteins, 10 µL of glycated BSA were incubated with 90 µL of 0.5 mM Nitrobluetetrazolium (NBT) reagent in carbonate buffer (pH 10.3) as at 37°C. To compare with the control, the samples were incubated with carbonate buffer (pH 10.3). Interpreting time was divided into 2 times interval. The first was read at 10 minute, the second reading was 15 minute. The absorbance was measured at 590 nm using spectrophotometer. 1-deoxy-1-morpholino-D-fructose (DMF) will be used as the standard for the calibration curve (concentration range 0-10 mM) (Armbruster, 1987).

-Determination of Protein oxidation

Protein carbonyl

Protein carbonyl group is the marker of oxidative damage of BSA. According to a slightly modified method of Levine and colleagues (Levine et al., 1990). 400 µL of 10 mM 2, 4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl was added to 100 µL of glycated BSA and incubated in the dark at room temperature for 60 minutes. Then, 500 µL of 20% (w/v) trichloroacetic acid (TCA) was added and kept on ice. After 5 min, protein precipitation will be centrifuged at 10,000 rpm (4 ºC) for 10 minutes. The protein

pellet were washed 5 times with 500 µL of 1:1 (v/v) ethanol/ethyl acetate and dissolved in 250 µL of 6 M guanidine hydrochloride. The absorbance was determined at 370 nm. 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 using 5, 50-dithiobis-(2-nitrobenzoic acid) (DTNB) with slight modifications. After the end of each incubation periods, 10 µL of glycated BSA were incubated with 90 µL 6mM DTNB in 0.1M PBS, pH 7.4, at room temperature for 15 minutes. The absorbance was determined at 410 nm. L-cysteine (0.3-10 µM) will be used as the standard and the concentration of free thiol group will be expressed as µmol/ mg protein (concentration range 0-10 µM) (Ellman, 1959).

-Determination of Protein aggregation

Protein aggregation was measured by Thioflavin T assay by using (4-(3, 6-dimethyl-1, 3-benzothiadiazol-3-ium-2-yl)-N,N dimethyl aniline chloride, Tht reagent which used to detect amyloid cross structure during AGE reaction (Hudson et al., 2009). Glycated BSA (50 µL) were incubated with 50 µL of 64 µM Tht reagent T in 0.1M PBS (pH 7.4) at room temperature. After 60 min of incubation, fluorescence intensity was measured at excitation wavelength of 435 nm and emission wavelength of 485 nm.

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.

RESULTS

Determination of Phenolic compound and Flavonoid content in *Moringa oleifera* leaf extract (MOE)

Phenolic compound quantification

The extract was measured phenolic compounds by using Folin's ciocalteu method. The results showed that MOE contained polyphenolic compound about 38.56±1.50 mg gallic acid/ g dry extract.

Flavonoid content quantification

The extract was measured total flavonoid content 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.

The identification of active compound in MOE by HPLC

Polyphenolic compound and flavonoid in MOE were identified by HPLC. Rutin, quercetin, and keamferol were used as a standard compound for flavonoid quantification, whereas ferulic acid was used as a standard compound for phenolic quantification. HPLC chromatogram of MOE was shown in figure 1. The concentration of phenolic and flavonoid was compared to the standard curve. 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.

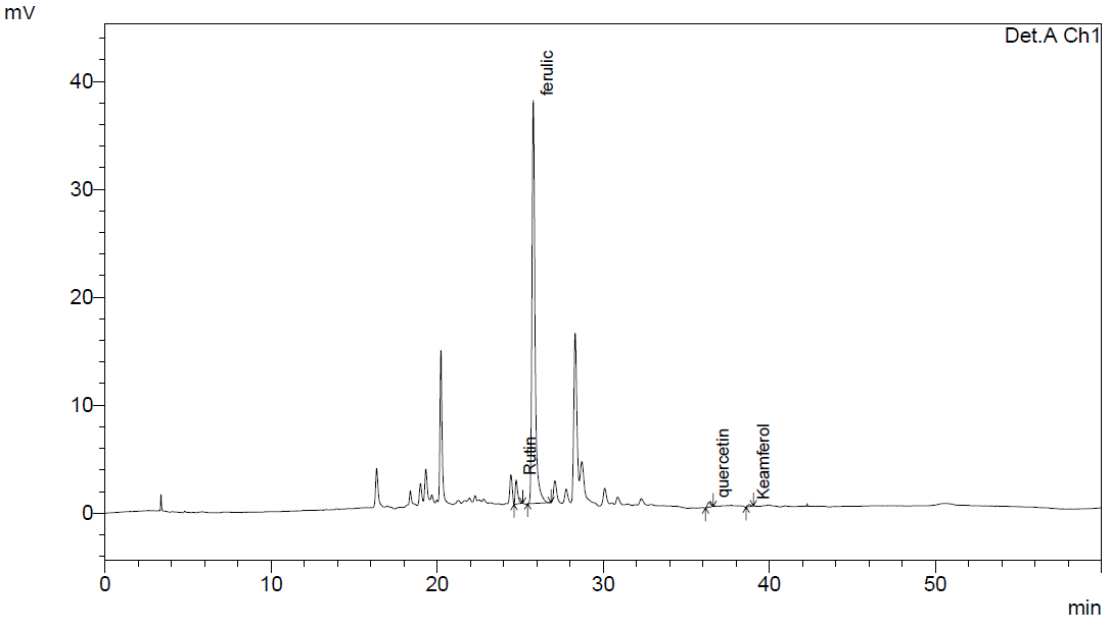

Figure 1. Chromatogram of *Moringa oleifera* leaf extract (MOE) by HPLC

The inhibitory effect of MOE on AGE formation

Fluorescence AGE Formation

As shown in Figure 2-3 fluorescence AGE formation of BSA incubated with glucose and fructose was represented as a negative control for 7, 14, 21, and 28 days. It 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 represented in the BSA/glucose and BSA/fructose system, it has been showed that the addition of MOE in glucose and fructose system had a significantly lowered the fluorescence AGE 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 to the addition of AG (1.0 mg/mL), the inhibitory effect of AG on fluorescence AGE has been shown the percentage of inhibition 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. As shown in Figure 4, 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).

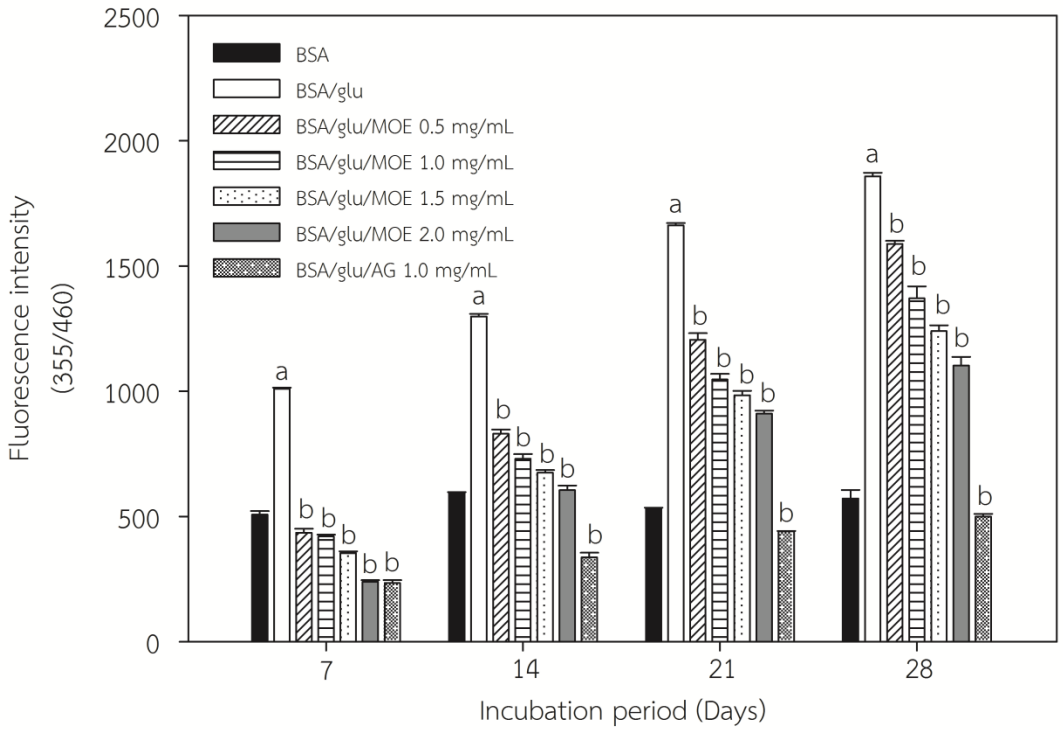

Figure 2. The effect of MOE (0.5 -2.0 mg/mL) on the fluorescence AGE formation in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/glucose.

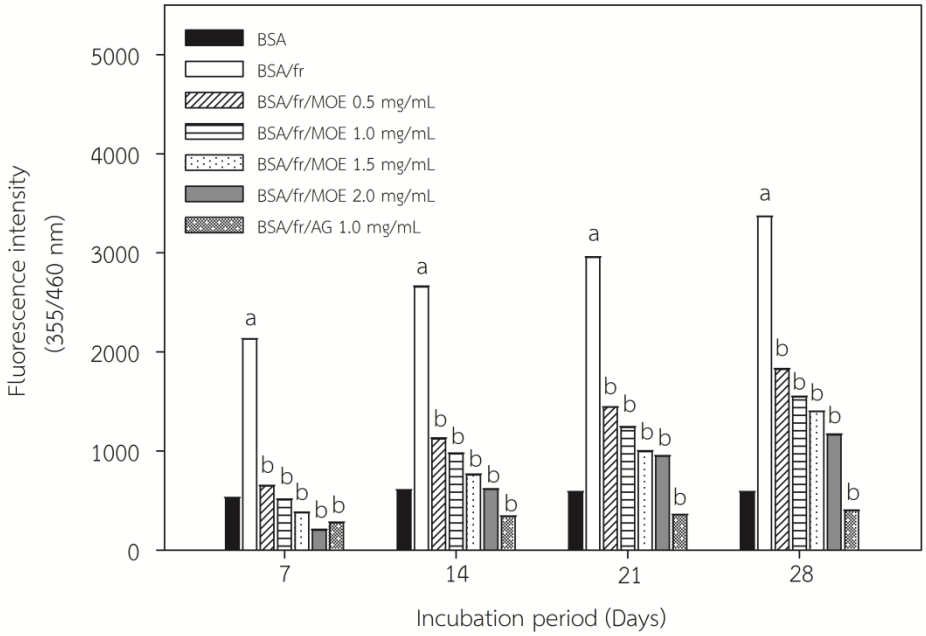

Figure 3. The effect of MOE (0.5 -2.0 mg/mL) on the fluorescence AGE formation in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose

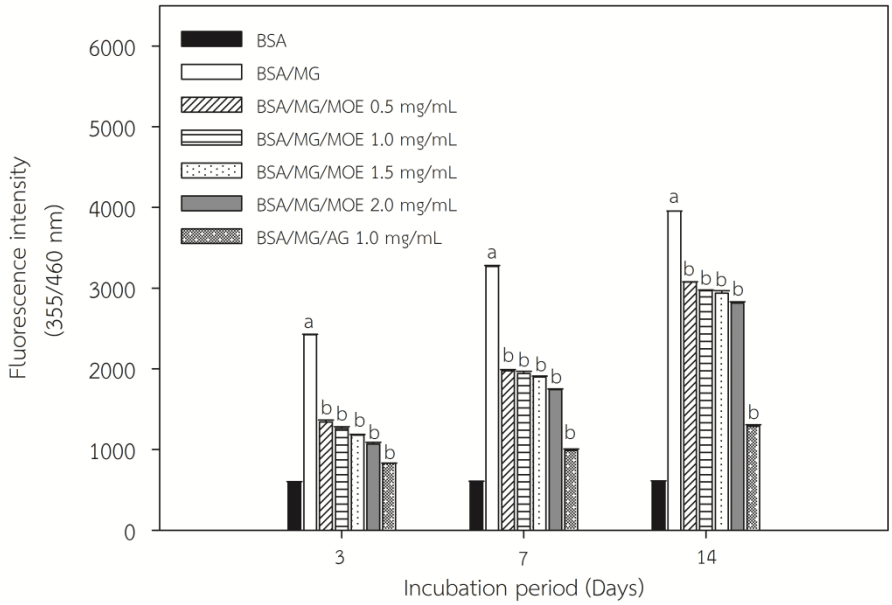

Figure 4. The effect of MOE (0.5 -2.0 mg/mL) on the fluorescence AGE formation in BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/MG

Non-fluorescence AGE Formation

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 Figure 5-6. 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 Figure 7. 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.

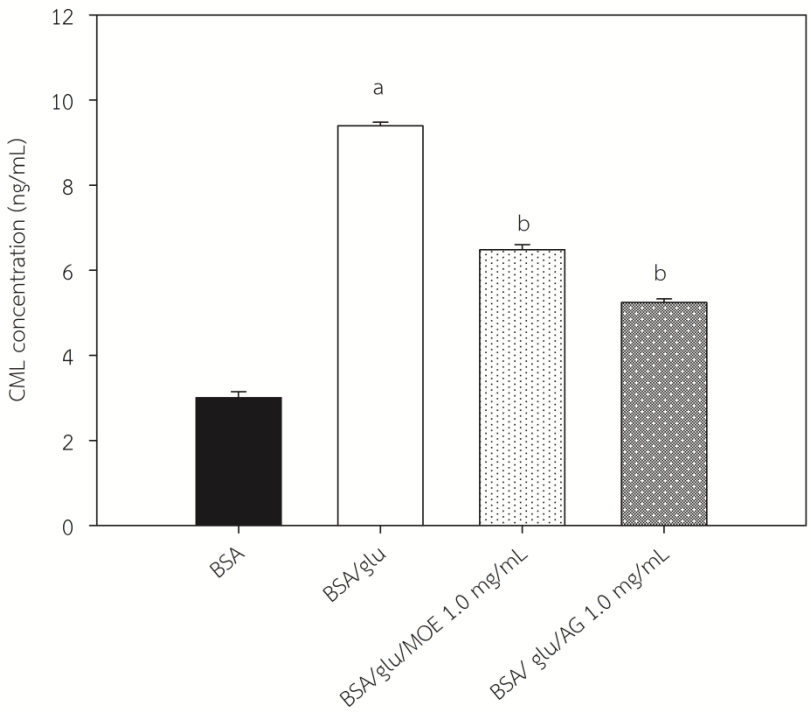

Figure 5. The effect of MOE (0.5 - 2.0 mg/mL) on N^{ε}- carboxymethyl lysine; CML (Non-fluorescence AGE) formation in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/glucose.

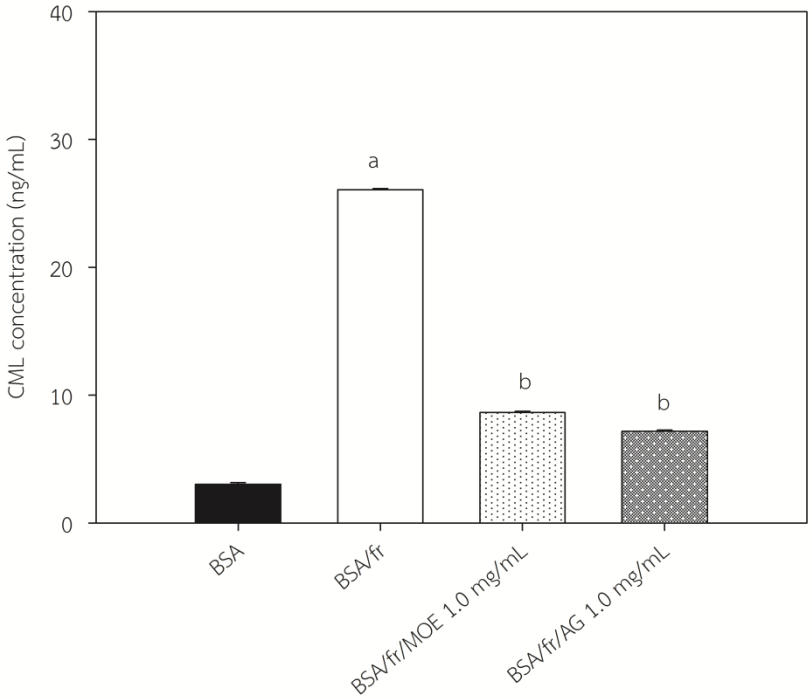

Figure 6. The effect of MOE (0.5 - 2.0 mg/mL) on N^{ε}- carboxymethyl lysine; CML (Non-fluorescence AGE) formation in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose.

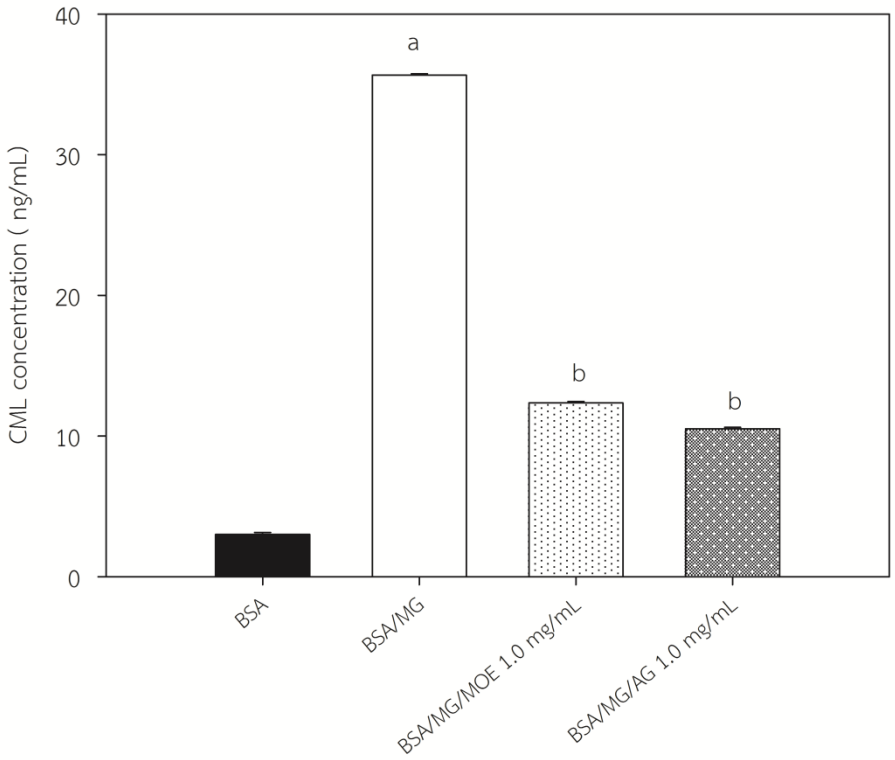

Figure 7. The effect of MOE (0.5 - 2.0 mg/mL) on N^{ε}- carboxymethyl lysine; CML (Non-fluorescence AGE) formation in BSA incubated with MG model. Each value represented the mean±SEM (n=3). $^{a}P<$ 0.05 compared to BSA, $^{b}P<$ 0.05 compared to BSA/fructose.

The inhibitory effect of MOE on Amadori product

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 8-9. 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.

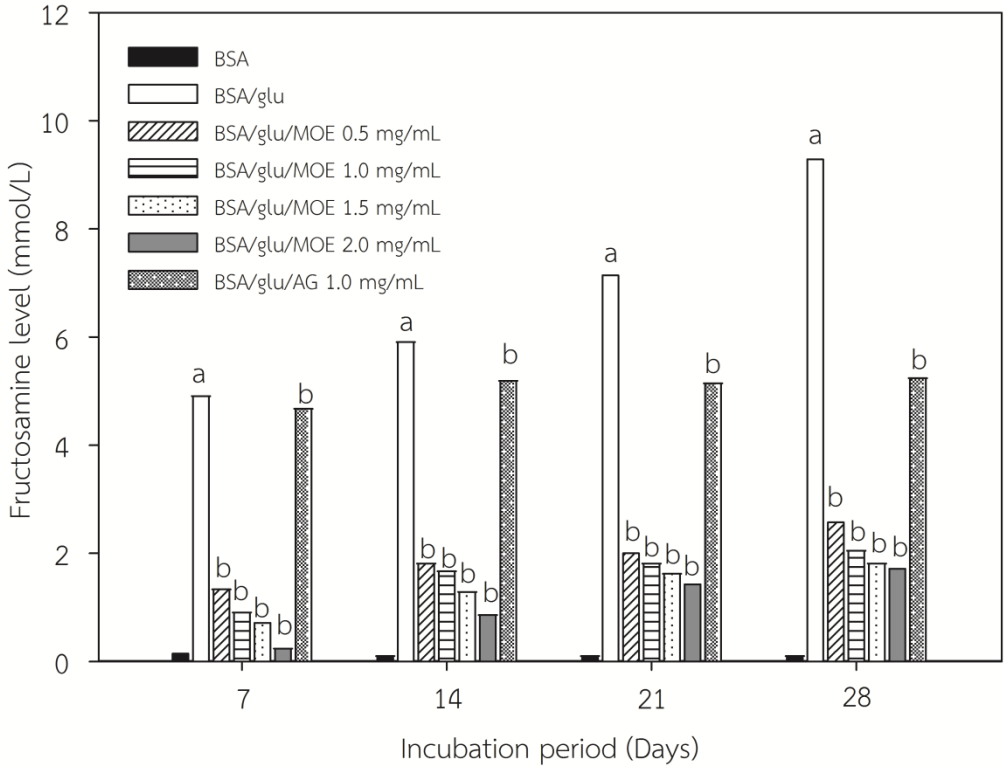

Figure 8. The effect of MOE (0.5 -2.0 mg/mL) on fructosamine level in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). $^{a}P< 0.05$ compared to BSA, $^{b}P< 0.05$ compared to BSA/glucose.

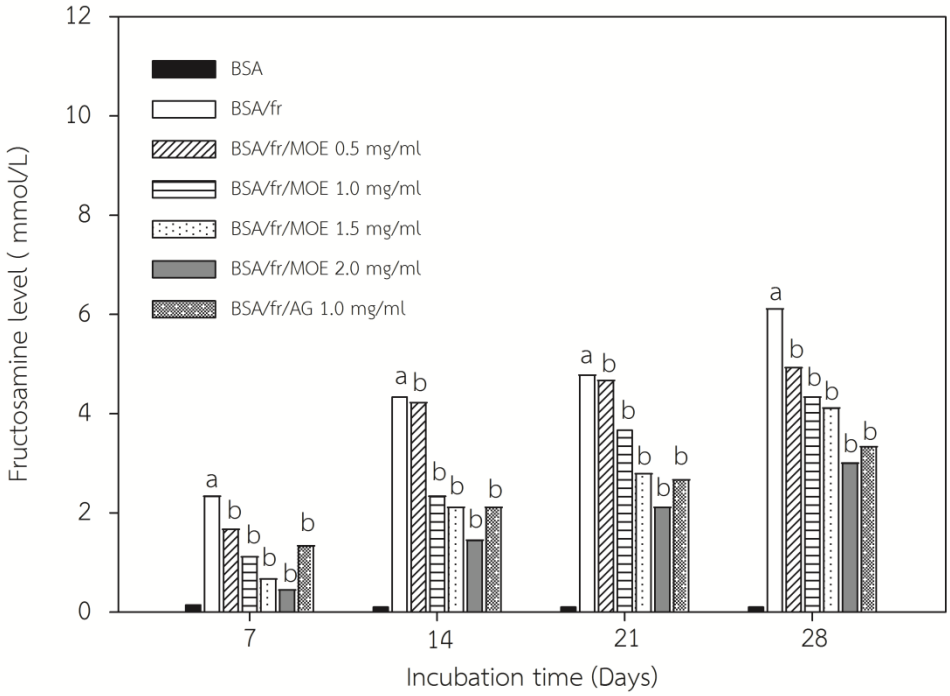

Figure 9. The effect of MOE (0.5 -2.0 mg/mL) on fructosamine level in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). $^{a}P<0.05$ compared to BSA, $^{b}P<0.05$ compared to BSA/fructose.

The inhibitory effect of MOE on 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, BSA/fructose were represented as a negative control. On the other hand, 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.

As shown in figure 10-11, the addition of MOE (0.5, 1.0, 1.5, 2.0 mg/mL) protected the protein thiol group depletion by 1.08, 1.08, 1.09, 1.10-folds in glucose system, 1.05, 1.07, 1.08, 1.10-folds in fructose system. In addition, 1 mg/mL of AG showed a significantly 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 a quietly higher potential effect to elevate protein thiol group than MOE.

To determine the BSA/MG system, the incubation time was 7, 14, 21 days. MG had a significantly 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 12.

Accordingly, AG showed the highest ability to protect the depletion of protein thiol group among in three glycating agent.

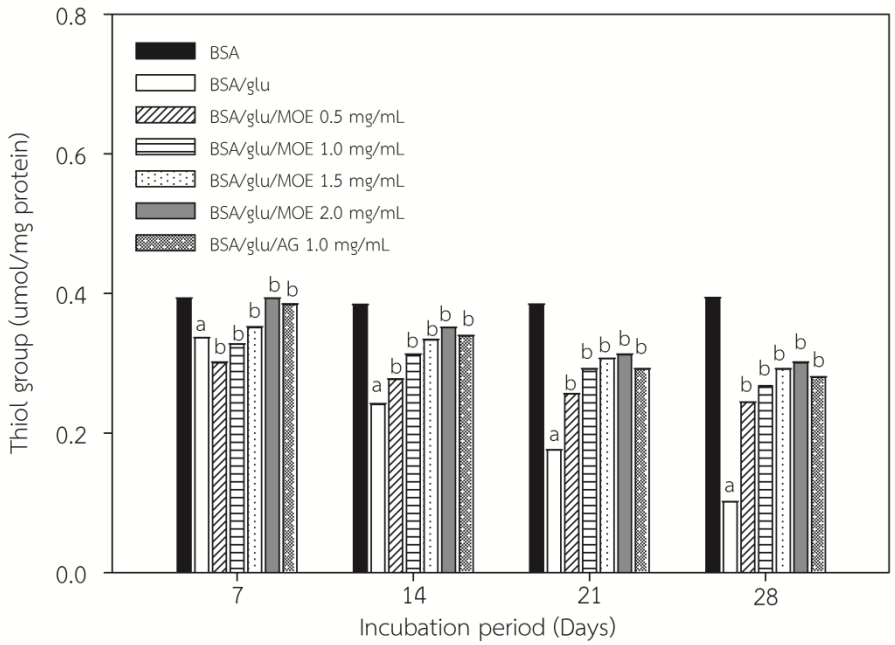

Figure 10. The effect of MOE (0.5 -2.0 mg/mL) on fructosamine level in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/glucose.

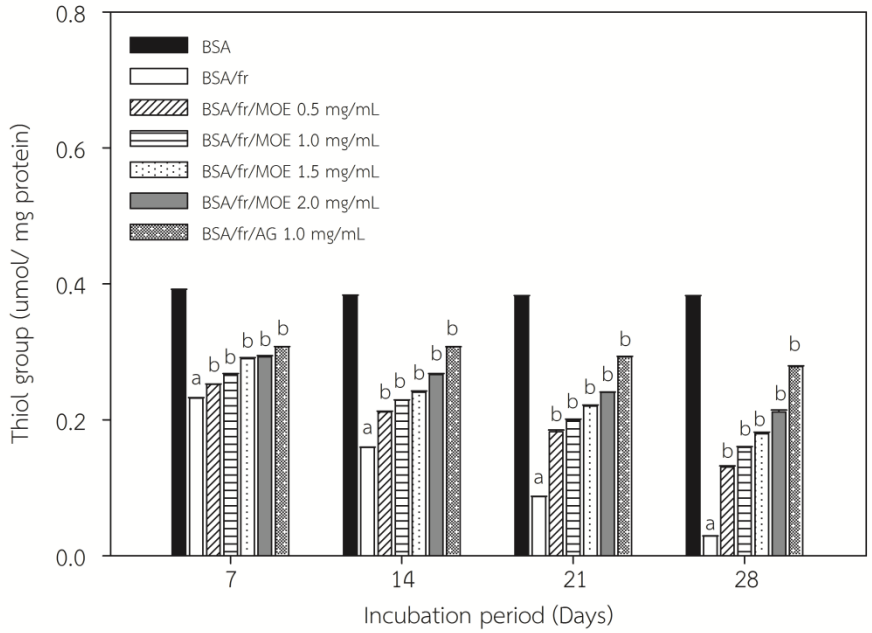

Figure 11. The effect of MOE (0.5 -2.0 mg/mL) on protein thiol group in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/fructose.

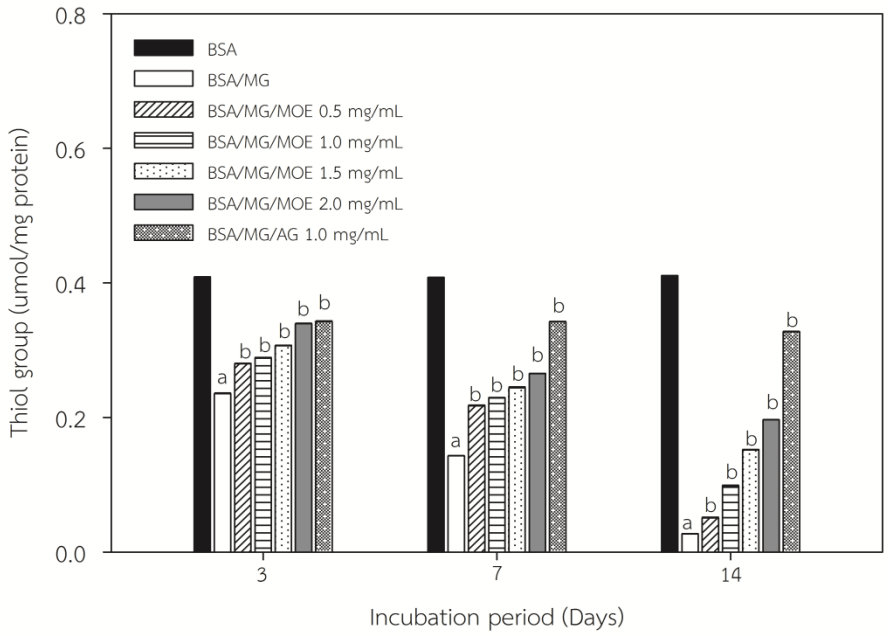

Figure 12. The effect of MOE (0.5 -2.0 mg/mL) on protein thiol group in BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/MG

The formation of protein carbonyl content

As shown in figure 13-14, 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.

Apart from the various concentration of MOE (0.5, 1.0, 1.5, 2.0 mg/mL), the extract was able to reduce the level of protein carbonyl content 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. In term of AG (1mg/mL), it also showed a significant to decrease the protein carbonyl content by 91.75% in glucose system and 88.29% in fructose system. Moreover, after compared AG with MOE, it has been shown that AG has 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, it has been shown that AG has 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.

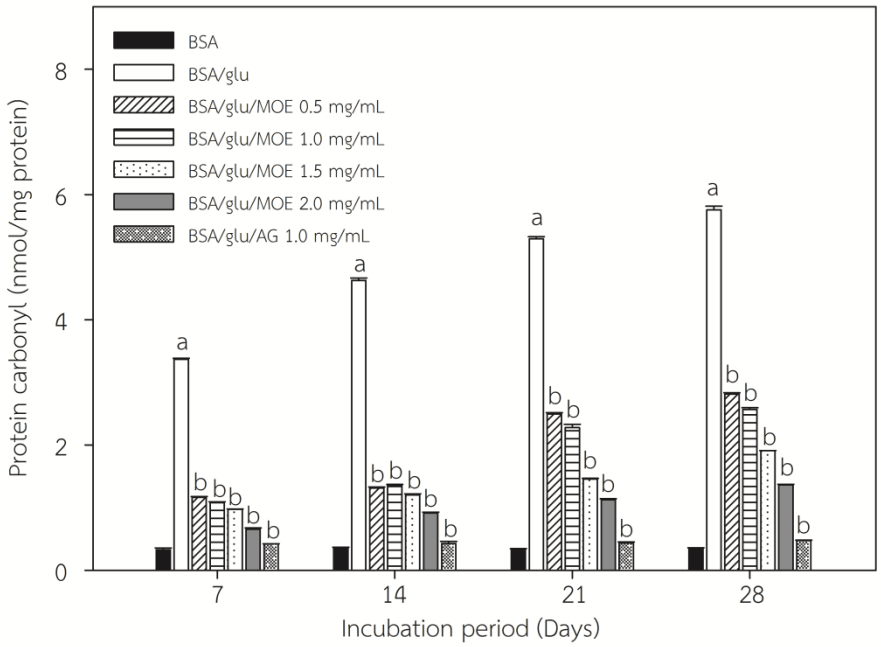

Figure 13. The effect of MOE (0.5 -2.0 mg/mL) on protein carbonyl content in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ glucose.

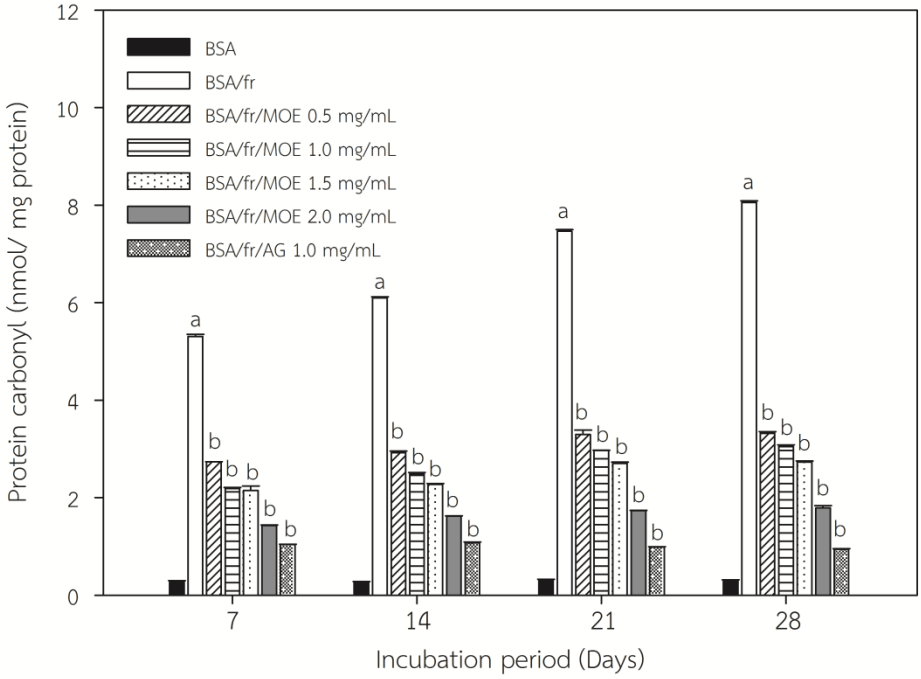

Figure 14. The effect of MOE (0.5 -2.0 mg/mL) on protein carbonyl content in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ fructose.

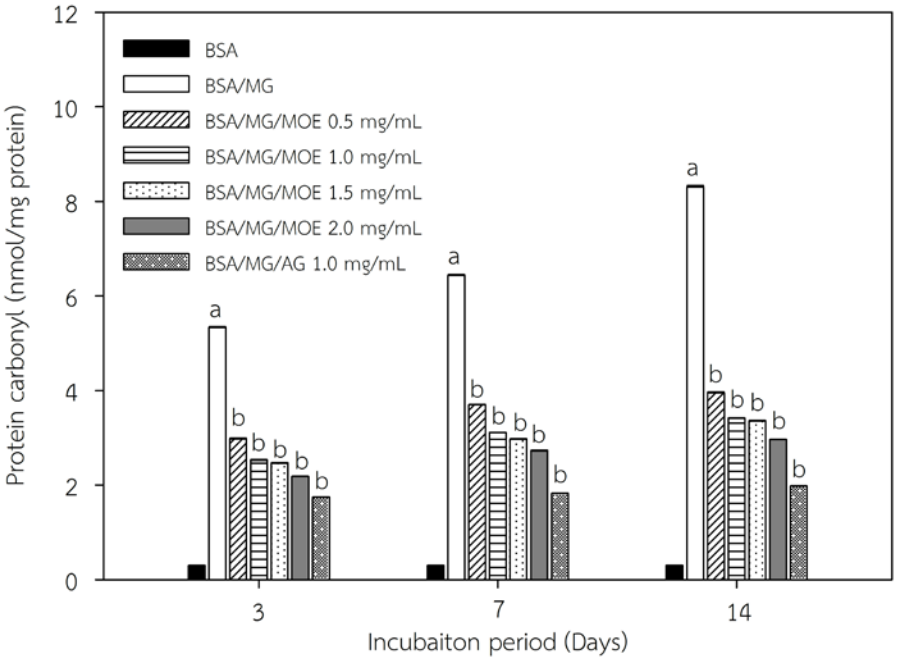

Figure 15. The effect of MOE (0.5 -2.0 mg/mL) on protein carbonyl content in BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ MG.

The inhibitory effect of MOE on 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 see the accumulation of β-amyloid level as shown in the figure 16-17. 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 significantly 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 remarkable decreased the β-amyloid level by 22.82% in glucose system and 34.04% in fructose system. Greatly the overall MOE (1 mg/mL) in both systems were able to manifest a significantly higher lowering power than AG at the same concentration (1mgmL).

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 to lowering β-amyloid level than MOE in BSA/MG system whereas MOE displayed the inhibitory effect more than AG both in glucose and fructose system.

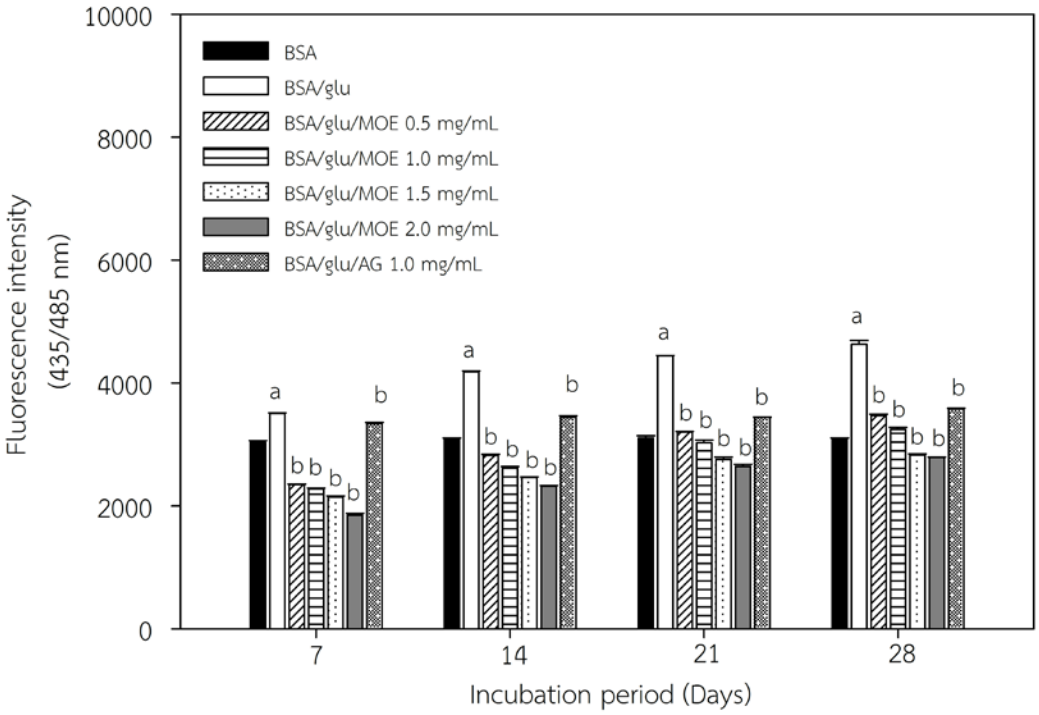

Figure 16. The effect of MOE (0.5 -2.0 mg/mL) on β-amyloid level in BSA incubated with glucose model. Each value represented the mean±SEM (n=3). aP< 0.05 compared to BSA, bP< 0.05 compared to BSA/ glucose.

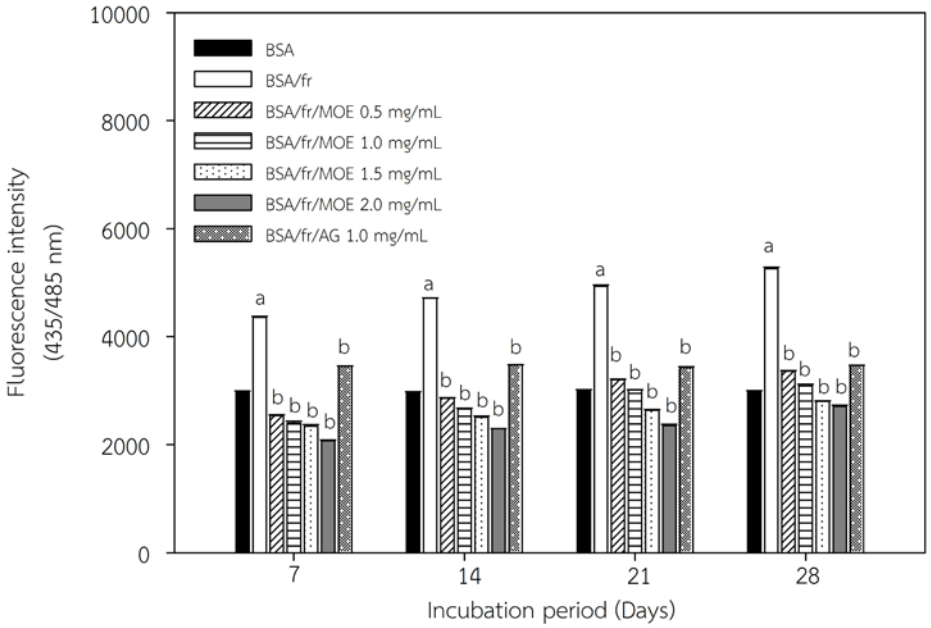

Figure 17. The effect of MOE (0.5 -2.0 mg/mL) on β-amyloid level in BSA incubated with fructose model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ fructose.

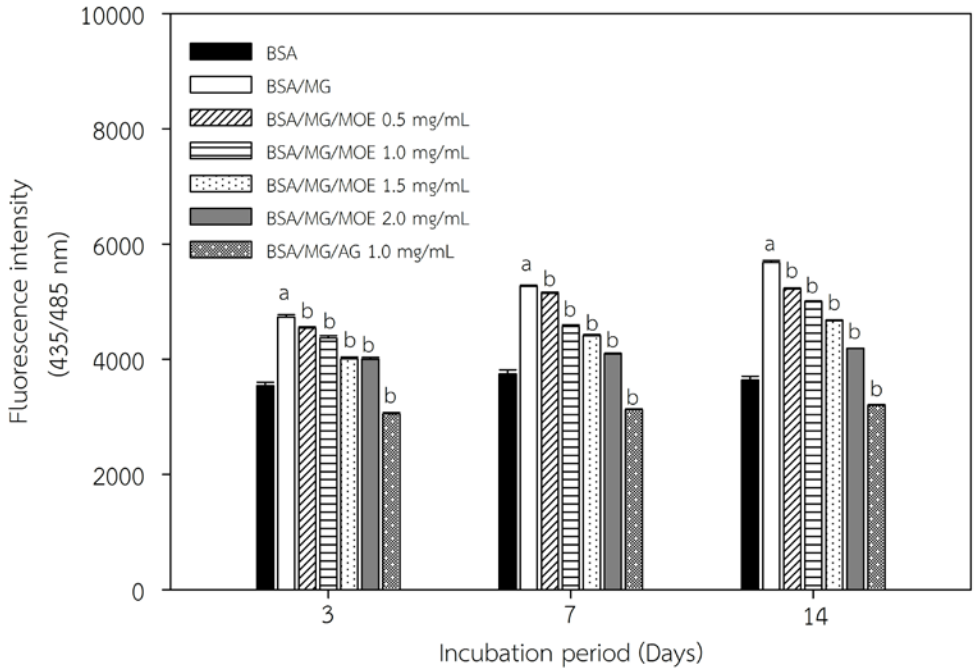

Figure 18. The effect of MOE (0.5 -2.0 mg/mL) on β-amyloid level in BSA incubated with MG model. Each value represented the mean±SEM (n=3). [a]*P*< 0.05 compared to BSA, [b]*P*< 0.05 compared to BSA/ MG.

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 et al., 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 et al., 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 et al., 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 et al., 2013). In addition, ferulic acid protects against free radical mediated changes in conformation of membrane proteins (Sompong et al., 2013; Wang et al., 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 et al., 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 (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 protects 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 et al., 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 et al., 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). To inhibit MG-mediated protein glycation, Rutin had more effectively trapped and inactivated RCS such as MG and GO with potentiality (Pashikanti et al., 2010). Kim et al (2010) 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. In our 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 et al., 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 mechanisms 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.

CONCLUSION

In this present research, we indicated that *Moringa oleifera* aqueous leaf extract (MOE) contained high amount of polyphenolic compound that had an ability to react as antioxidant because of the presented in various flavonoid contents such as cinnamic acid, quercetin as well as keamferol that could be the influential effect on anti-glycation properties.

Notably, MOE effectively inhibited AGE formation, protein oxidation and protein cross-linking in glycation reaction. These findings indicated that MOE could be one of the alternative medicinal plants to use as a therapeutic agent in diabetic patient. Moreover, MOE could be emerging as a new nutraceutical food or functional food for applied to lessen the progression of AGE-associated diabetic complication. However, animinal model and clinical study are needed to confirm the beneficial effects of the extract on anti-glycation properties demonstrated in *in vitro* model.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)

   Nunthanawanich P, Sompong W, Sirikwanpong S, Mäkynen K, Adisakwattana S, Dahlan W, Ngamukote S. *Moringa oleifera* aqueous leaf extract inhibits reducing monosaccharide-induced protein glycation and oxidation of bovine serum albumin. Springerplus. 2016;5(1):1098. doi: 10.1186/s40064-016-2759-3.

2. การนำผลงานวิจัยไปใช้ประโยชน์

   - เชิงสาธารณะ
     - มีการวางแผนนำความรู้ที่ได้จากงานวิจัยเผยแพร่สู่สาธารณะโดยผ่านสถานีวิทยุแห่งจุฬาลงกรณ์มหาวิทยาลัย (CU Radio) FM 101.5 MHz
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
     - เป็นผู้ร่วมวิจัยในโครงการ Special Task Force for Activating Research (Star) จุฬาลงกรณ์มหาวิทยาลัย และผู้ร่วมวิจัยใน Research Group of Herbal Medicine for Prevention and Therapeutic of Metabolic diseases
     - นำงานวิจัยชิ้นนี้ไปใช้เป็นความรู้พื้นฐานในการศึกษาความสามารถของการยับยั้งการเกิดไกลเคชั่นในพืชสมุนไพรอื่นที่มีสารสำคัญคล้ายคลึงกัน และนำไปใช้ในการพัฒนาต่อยอดเป็นผลิตภัณฑ์สุขภาพในอนาคต
     - นำความรู้ที่ได้ไปเป็นส่วนหนึ่งของการเรียนการสอนรายวิชาระดับปริญญาบัณฑิตและบัณฑิตศึกษา

3. อื่นๆ

   -นำเสนอผลงาน หัวข้อ “The Inhibitory Effect Of *Moringa oleifera* Aqueous Leaf Extract On Glucose-Induced Protein Glycation *In Vitro*” ในงานประชุมวิชาการนานาชาติ 1st Joint ACS AGFD-ACS ICSCT Symposium on Agricultural and Food Chemistry วันที่ 4-5 มีนาคม 2014 ประเทศไทย และตีพิมพ์ในลักษณะ proceeding

   - นำเสนอผลงานหัวข้อ “The protective effect of *Moringa oleifera* leaf extract on fructose-mediated protein glycation *in vitro*” ในงานประชุมวิชาการนานาชาติ Experimental Biology 2014 วันที่ 26-30 เมษายน 2014 ณ เมือง San Diego ประเทศสหรัฐอเมริกา

   - Nunthanawanich P, Sirikwanpong S, Mäkynen K, Adisakwattana S, Dahlan W, **Ngamukote S**. The protective effect of *Moringa oleifera* aqueous leaf extract on methylglyoxal-induced protein glycation and oxidation. The 8th Thailand Congress of Nutrition (8th TCN) at Bangkok International Trade & Exhibition Centre: BITEC, Bangkok, Thailand. 6th – 8th October 2014. (Oral presentation award)

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RESEARCH Open Access

Moringa oleifera aqueous leaf extract inhibits reducing monosaccharide-induced protein glycation and oxidation of bovine serum albumin

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Abstract

Advanced glycation end products (AGEs) play an important factor for pathophysiology of diabetes and its complications. *Moringa oleifera* is one of the medicinal plants that have anti-hyperglycemic activity. However, anti-glycation property of *Moringa oleifera* leaf extract on the different types of reducing monosaccharides-induced protein glycation has not been investigated. Therefore, the aim of this study was to examine the protective effect of *Moringa oleifera* aqueous leaf extract (MOE) on reducing sugars-induced protein glycation and protein oxidation. Total phenolic content of MOE was measured using the Folin–Ciocalteu method. Bovine serum albumin was incubated with 0.5 M of reducing sugars (glucose or fructose) with or without MOE (0.5–2.0 mg/mL) for 1, 2, 3 and 4 weeks. The results found that total phenolic content was 38.56 ± 1.50 mg gallic acid equivalents/g dry extract. The formation of fluorescent and non-fluorescent AGEs [N^{ε}-(carboxymethyl) lysine (CML)] and the level of fructosamine were determined to indicate protein glycation, whereas the level of protein carbonyl content and thiol group were examined for protein oxidation. MOE (0.5–2.0 mg/mL) significantly inhibited the formation of fluorescent, N^{ε}-CML and markedly decreased fructosamine level ($P < 0.05$). Moreover, MOE significantly prevented protein oxidation manifested by reducing protein carbonyl and the depletion of protein thiol in a dose-dependent manner ($P < 0.05$). Thus, the findings indicated that polyphenols containing in MOE have high potential for decreasing protein glycation and protein oxidation that may delay or prevent AGE-related diabetic complications.

Keywords: *Moringa oleifera*, Polyphenol, Glycation, Glucose, Fructose

Background

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 (Kaneko et al. 2005). The accumulation of AGEs in various types of tissues causes the alteration of proteins leading to change their characteristics, physiochemical, and biochemical properties (Vanessa et al. 2013). The interaction of AGEs with the receptor of advanced glycation end products evokes oxidative stress and subsequently elicits vascular inflammation and thrombosis (Kang 2003). Studies have also shown that reactive oxygen species (ROS) formed by AGEs cause DNA damage and induction of cell apoptosis (Kang 2003). Glucose and fructose, the most common reducing sugar found in blood circulation react spontaneously with amino groups of proteins to AGEs. Although glucose plays a vital role in the formation of AGEs, it is now known that fructose undergoes protein glycation much faster than glucose (Semchyshyn et al. 2014). Endogenous fructose production by the sorbitol pathway

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is also considered to contribute the formation of AGEs and consequently accumulate in the tissues (Suarez et al. 1989; Vinson and Howard 1996). There are several clinical studies which demonstrate the link between the long-term consumption of fructose and the development of aging process (Levi and Werman 1998). Therefore, there has been seriously concern regarding the critical role of fructose in the glycation process. Studies on antiglycating agents have recently emerged as the new therapeutic approaches in preventing AGE-related diseases (Adisakwattana et al. 2010). Aminoguanidine (AG) is one of therapeutic agents for use in the prevention of AGE formation by cleavage of AGE-induced chemical cross-links (Brownlee et al. 1986). However, it has shown serious side effects including vascularitis, gastrointestinal disturbances, and anemia (Brownlee et al. 1986). For this reason, the search for alternative prevention of AGE formation has been focused on the natural products.

Polyphenolic compounds are commonly found in vegetables, fruits, spices, and medicinal herbs. Previously, it has been shown that polyphenols play an important role in human health, including reduced risk of chronic and degenerative diseases (Vauzour et al. 2010; Ngamukote et al. 2011; Adisakwattana and Chanathong 2011). *Moringa oleifera* (Ma-rum) is the most widely cultivated species of a monogeneric family, the Moringaceae which is commonly found in tropical countries such as India, Afghanistan, as well as Thailand. In addition, 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 (Fahey 2005; Anwar et al. 2007). A number of previous studies have reported pharmacological properties of *Moringa oleifera* particular in antioxidant property and antidiabetic activity that may provide benefits for diabetic patients (Jaiswal et al. 2009; Chumark et al. 2008; Adisakwattana and Chanathong 2011). However, there are no reports in the literature showing that MOE can inhibit protein glycation induced by different types of reducing monosaccharaides in vitro models. Thus, the aim of present study was to evaluate the inhibitory effects of MOE on glucose- and fructose-induced protein glycation, about which no previous reports exist. Furthermore, the inhibitory effects of MOE on oxidation-dependent damages to bovine serum albumin mediated by glycation were also determined.

Methods

Chemicals

Glucose, fructose, and 2,4-dinitrophenyl hydrazine (DNPH) were purchased from Ajax Finechem (Taren Point, Australia). Catechin, gallic acid, sodium azide, Nitroblue tetrazolium (NBT), aminoguanidine hydrochloride (AG), guanidine hydrochloride, Thioflavin T (ThT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and L-cysteine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) was purchased from Merck (Darmstadt, Germany). OxiSelectTM CML ELISA kit was purchased from Cell Biolabs (San Diego, CA, USA). All other reagents used were of analytical grade.

Preparation of *Moringa oleifera* aqueous leaf extract

The leaves of *Moringa oleifera* were obtained from local areas of Bangkok in Nongkhame district, Thailand. The herbarium number of A014172 (BCU) was authenticated by a Taxonomist at Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The dried leaves (250 g) were extracted with distilled water twice (3 L) for 3 h at 100 °C. The extraction was filtered through Whatman No. 1 filter paper under the vacuum. The filtrate was further subjected to a spray dryer SD-100 (Eyela world, Tokyo Rikakikai Co., LTD, Japan) to obtain the extract powder. The spray drying conditions, inlet and outlet air temperature was set at 160 and 89–99 °C, respectively.

Determination of total phenolic content

Total phenolic content of *Moringa oleifera* leaf extract (MOE) was determined by the Folin-Ciocalteu method (Verma et al. 2009). The extract powder was dissolved in distilled water (1.25 mg/mL). The freshly prepared Folin-Ciocalteu reagent was gently mixed with 10 µL of sample. Then, 75 µL of 7.5 % sodium carbonate (Na_2CO_3) was added and allowed to stand for 30 min at room temperature in the dark. The mixture was measured at 725 nm by a spectrophotometer. Gallic acid (0.025–0.4 mg/mL) was used as a standard and the content of total phenolics was expressed as mg gallic acid equivalents/g dried extract.

Preparation of glycated bovine serum albumin (BSA)

Glycated BSA was performed according to a previously described method (Povichit et al. 2010) with slight modifications. Briefly, BSA (10 mg/mL) was incubated with glucose or fructose (0.5 M) in 0.1 M phosphate buffer (pH 7.4) containing 0.02 % sodium azide (NaN_3) with or without (MOE) (0.5–2.0 mg/mL) and aminoguanidine (AG, 1.0 mg/mL) at 37 °C for 4 weeks. Samples were kept at −20 °C until analysis.

Determination of advanced glycation end product (AGE) formation

The fluorescent AGEs, the irreversible products at the end stage of non-enzymatic glycation, were determined by a spectrofluorometer (Wallac 1420 Victor3 V, PerkinElmer, Santa Clara, CA, USA) at excitation and emission

wavelengths of 355 nm and 460 nm, respectively (Povichit et al. 2010).

Determination of N^{ε}-(carboxymethyl) lysine (CML)

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

Determination of fructosamine

The levels of fructosamine was analyzed by nitrobluetetrazolium (NBT) assay with minor modification (Armbruster 1987). Briefly, 90 µL of 2.5 mM nitrobluetetrazolium (NBT) reagent was added to 10 µL of glycated BSA in carbonate buffer (pH 10.3). After 10 and 15 min of incubation, the mixture was measured at 590 nm. The concentration of fructosamine was calculated by using the different absorption at 10 and 15 min time points compared with the standard 1-deoxy-1-morpholino-fructose (1-DMF) curve.

Determination of protein carbonyl

Protein carbonyl content were determined according to a previously published method with minor modifications (Levine et al. 1990). In brief, 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl (400 µL) was added to 100 µL of glycated BSA and incubated in the dark at room temperature for 60 min. Then, 20 % (w/v) trichloroacetic acid (500 µL) was added and kept on ice for 5 min. Protein precipitation was centrifuged and the protein pellet was then washed with 1:1 (v/v) ethanol/ethyl acetate and dissolved in 6 M guanidine hydrochloride. The absorbance was determined at 370 nm. The concentration of protein carbonyl content was calculated using an absorption coefficient of 22,000 $M^{-1}cm^{-1}$. The results were expressed as nmol carbonyl/mg protein.

Determination of protein thiol groups

The determination of free thiol groups were performed according to Ellman's assay using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman 1959). Glycated BSA (10 µL) was incubated with 6 mM DTNB in 0.1 M PBS (pH 7.4) for 15 min at room temperature. The absorbance was determined at 410 nm. The free thiol concentration was calculated for the standard curve of L-cysteine (0.3–10 µM) and expressed as nmol/mg protein.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) of triplicate determination (n = 3). Differences among groups were analyzed for statistical significance by one-way ANOVA followed by Duncan as post hoc comparison. P value < 0.05 was considered statistically significant.

Results

Phytochemical analysis

In the present study, the content of total phenolic compounds in *Moringa oleifera* leaf extract (MOE) was 38.56 ± 1.50 mg gallic acid equivalents/g extract.

Effect of *Moringa oleifera* leaf extract on the different types of reducing monosaccharide-induced fluorescent AGE formation

The formation of fluorescent AGEs in different monosaccharide-induced protein glycation was monitored during 4 weeks of incubation. As shown in Fig. 1, the significant increase in fluorescent intensity in BSA incubated with glucose and fructose was seen during 4 weeks of the incubation. The results demonstrated that the fluorescent AGE formation was increased 3.24-fold in glucose model (Fig. 1A) and 5.76-fold in fructose model (Fig. 1B) whereas MOE (0.5–2.0 mg/mL) inhibited the formation of AGEs in a dose-dependent manner during experimental periods both glucose and fructose models at week 4. The percentage inhibition of AGE formation by MOE (0.5–2.0 mg/mL) ranged from 14.52–40.65 % in glucose-glycated BSA and 45.82–65.43 % in fructose-glycated BSA. However, MOE has less potent in the inhibition of AGE formation when compared with AG at the same concentration (1 mg/mL).

Effect of *Moringa oleifera* leaf extract on the level of N^{ε}-(carboxymethyl) lysine (CML)

In order to examine the formation of non-fluorescent AGEs, the level of N^{ε}-CML was measured at week 4 of incubation. As shown in Fig. 2, it was found that the level of N^{ε}-CML dramatically increased in glucose-glycated BSA (3.10-fold) and fructose-glycated BSA (8.60-fold). Conversely, the addition of MOE to the solution (1.0 mg/mL) inhibited N^{ε}-CML formation about 31.02 % in glucose-glycated BSA and about 66.82 % in fructose-glycated BSA, whereas AG (1.0 mg/mL) inhibited N^{ε}-CML formation about 44.17 % in glucose-glycated BSA and about 72.45 % in fructose-glycated BSA.

Effect of *Moringa oleifera* leaf extract on the level of fructosamine

The effects of MOE on the level of fructosamine are shown in Fig. 3. The level of fructosamine in glucose-glycated BSA and fructose-glycated BSA markedly increased throughout 4 weeks of the experiment. In contrast, the increasing level of fructosamine was attenuated by MOE (0.5–2.0 mg/mL) during 4 weeks of the study. At week 4 of incubation, MOE (0.5–2.0 mg/mL) reduced the level

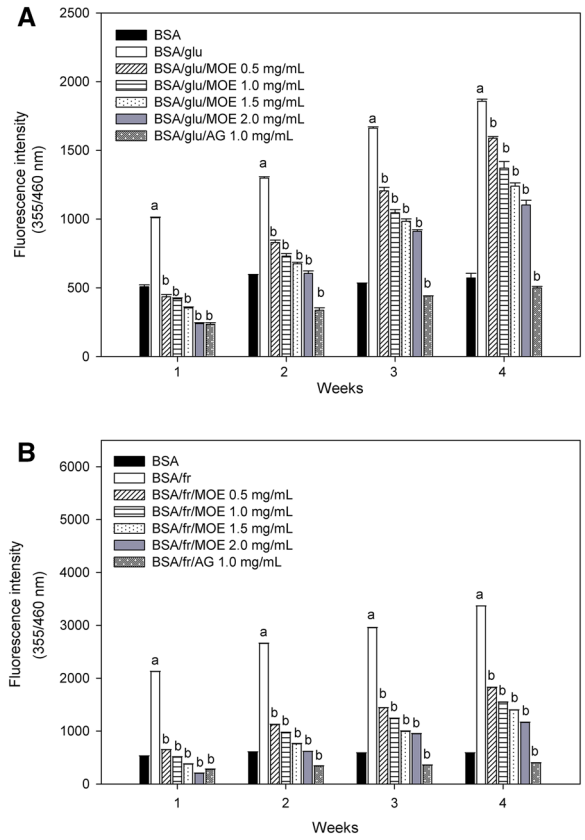

Fig. 1 The effect of *Moringa oleifera* leaf extract (MOE, 0.5–2.0 mg/mL) and aminoguanidine (AG, 1.0 mg/mL) on the formation of fluorescent AGEs in **A** bovine serum albumin (BSA) incubated with 0.5 M glucose (glu) and **B** bovine serum albumin (BSA) incubated with 0.5 M fructose (fr) at week 1, 2, 3 and 4 of incubation. The results are expressed as mean ± SEM (n = 3). $^{a}P < 0.05$ when compared to BSA alone, $^{b}P < 0.05$ when compared to BSA with glucose (BSA/glu) or BSA with fructose (BSA/fr) at the same week of incubation

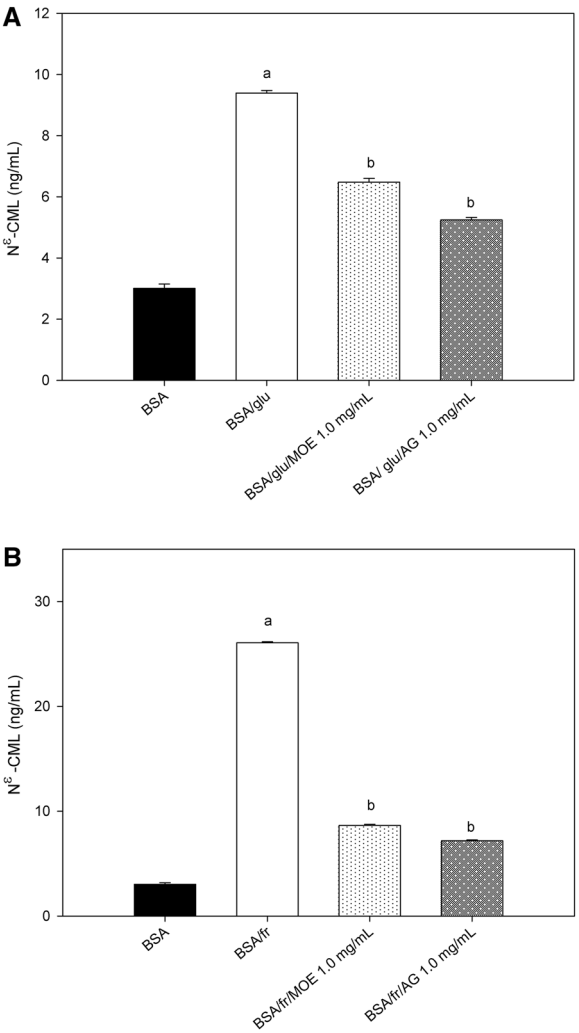

Fig. 2 The effect of *Moringa oleifera* leaf extract (MOE, 1.0 mg/mL) and aminoguanidine (AG, 1.0 mg/mL) on the level of N^{ε}-(carboxymethyl) lysine (CML) in **A** bovine serum albumin (BSA) incubated with 0.5 M glucose (glu) and **B** bovine serum albumin (BSA) incubated with 0.5 M fructose (fr) at week 4 of incubation. The results are expressed as mean ± SEM (n = 3). $^{a}P < 0.05$ when compared to BSA alone, $^{b}P < 0.05$ when compared to BSA with glucose (BSA/glu) or BSA with fructose (BSA/fr) at the same week of incubation

of fructosamine in a concentration-dependent manner in glucose-glycated BSA (71.21–80.30 %) and fructose-glycated BSA (18.96–49.56 %). In addition, the percentage reduction of fructosamine by AG (1 mg/mL) was 42.92 % for glucose-glycated BSA and 45.45 % for fructose-glycated BSA.

Effect of *Moringa oleifera* leaf extract on the level of protein carbonyl content and protein thiol groups

The level of carbonyl content and thiol groups were used for indication of the protein oxidation mediated by glycation process. As shown in Fig. 4, the carbonyl content of glucose-glycated BSA and fructose-glycated BSA significantly increased during the experimental period, whereas MOE (0.5–2.0 mg/mL) significantly suppressed an increase in protein carbonyl content of glucose-glycated BSA and fructose-glycated BSA. When comparing with glucose-glycated BSA and fructose-glycated BSA at week 4, the percentage reduction of carbonyl content by MOE (0.5–2.0 mg/mL) ranged from 51.12 to 76.31 % in glucose-glycated BSA and 58.79 to 77.76 % in fructose-glycated BSA. A significant reduction of protein carbonyl content (91.59 % for glucose-glycated BSA and 88.29 % for fructose-glycated BSA) was observed in the presence of AG (1 mg/mL) at the same week. The effects of MOE on the oxidation of protein thiols are presented in Fig. 5. When BSA was incubated with glucose or fructose, the level of thiol groups had continuously declined throughout the experimental periods.

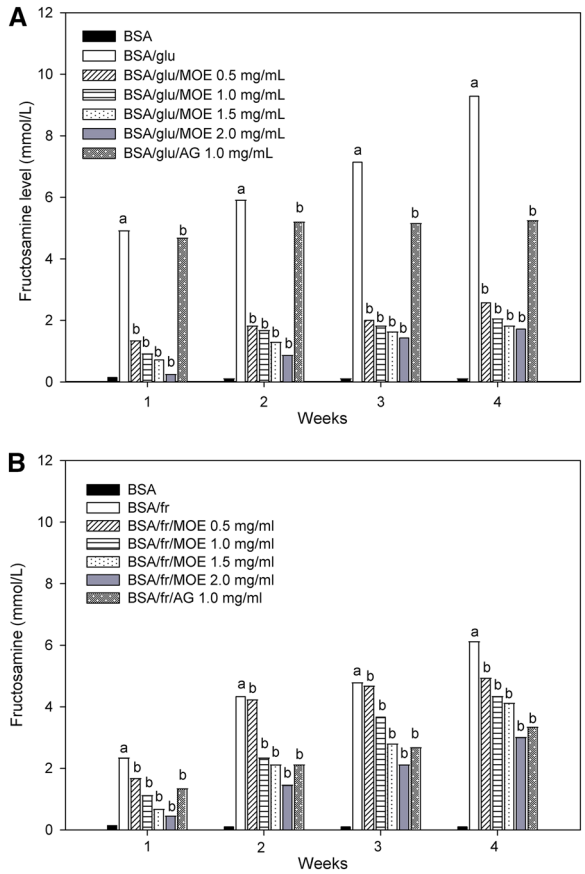

Fig. 3 The effect of *Moringa oleifera* leaf extract (MOE, 0.5–2.0 mg/mL) and aminoguanidine (AG, 1.0 mg/mL) on fructosamine level in **A** bovine serum albumin (BSA) incubated with 0.5 M glucose (glu) and **B** bovine serum albumin (BSA) incubated with 0.5 M fructose (fr) at week 1, 2, 3 and 4 of incubation. The results are expressed as mean ± SEM (n = 3). $^{a}P < 0.05$ when compared to BSA alone, $^{b}P < 0.05$ when compared to BSA with glucose (BSA/glu) or BSA with fructose (BSA/fr) at the same week of incubation

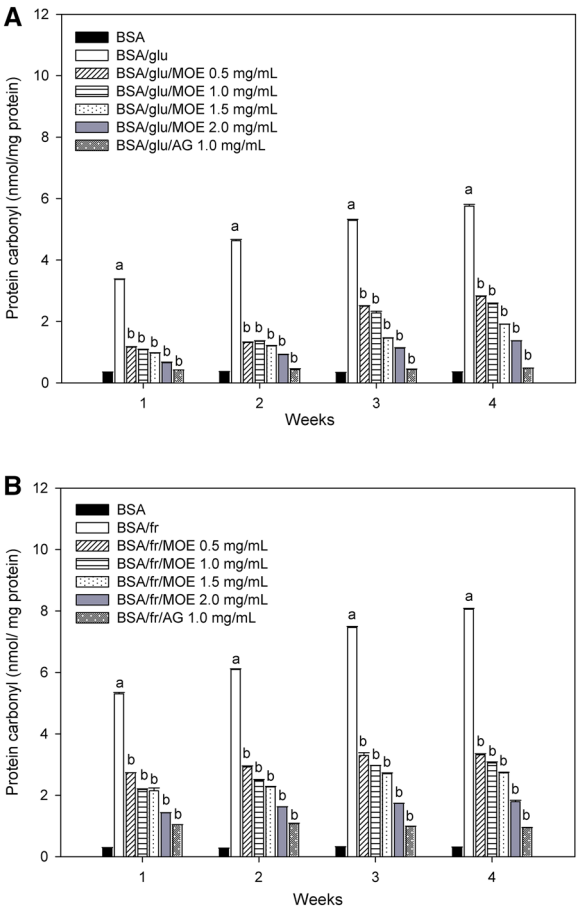

Fig. 4 The effect of *Moringa oleifera* leaf extract (MOE, 0.5–2.0 mg/mL) and aminoguanidine (AG, 1.0 mg/mL) on protein carbonyl content in **A** bovine serum albumin (BSA) incubated with 0.5 M glucose (glu) and **B** bovine serum albumin (BSA) incubated with 0.5 M fructose (fr) at week 1, 2, 3 and 4 of incubation. The results are expressed as mean ± SEM (n = 3). $^{a}P < 0.05$ compared to BSA alone, $^{b}P < 0.05$ when compared to BSA with glucose (BSA/glu) or BSA with fructose (BSA/fr) at the same week of incubation

Interestingly, there was a significant increase in the level of thiol groups after addition of MOE (0.5–2.0 mg/mL) as well as AG (1.0 mg/mL). The findings showed that the percentage prevention of depleting thiol group by MOE ranged from 7.57 to 9.77 % in glucose-glycated BSA and 5.73 to 10.32 % in fructose-glycated BSA, whereas AG (1.0 mg/mL) significantly prevented the depletion of protein thiol groups around 9.46 % and 14.09 % in glucose-glycated BSA and fructose-glycated BSA, respectively at the week 4.

Discussion

In a present study, MOE was investigated the effect on glucose- and fructose-induced florescent and non-fluorescent AGE formation. The results showed that MOE efficiently inhibited fluorescent and non-fluorescent AGE formation. MOE also reduced the level of fructosamine associated with the reduction of AGE formation in glucose-glycated BSA and fructose-glycated BSA. A significant decrease of protein carbonyl content and oxidation of thiols in BSA were seen when MOE was added to the systems, it markedly suppressed these processes. The blockage of the carbonyl group in reducing sugars, the trapping of reactive oxygen species (ROS) and carbonyls during glycation, and breaking the crosslinking structure in the formed AGEs have recently been revealed as the underlying mechanisms of antiglycating agents (Price et al. 2001). The scavenging free radical generation during glycation process may highlight other mechanisms for the prevention of AGE formation (Rout and Banerjee 2007). The Amadori products can be fragmented and consequently generate superoxide anion to form

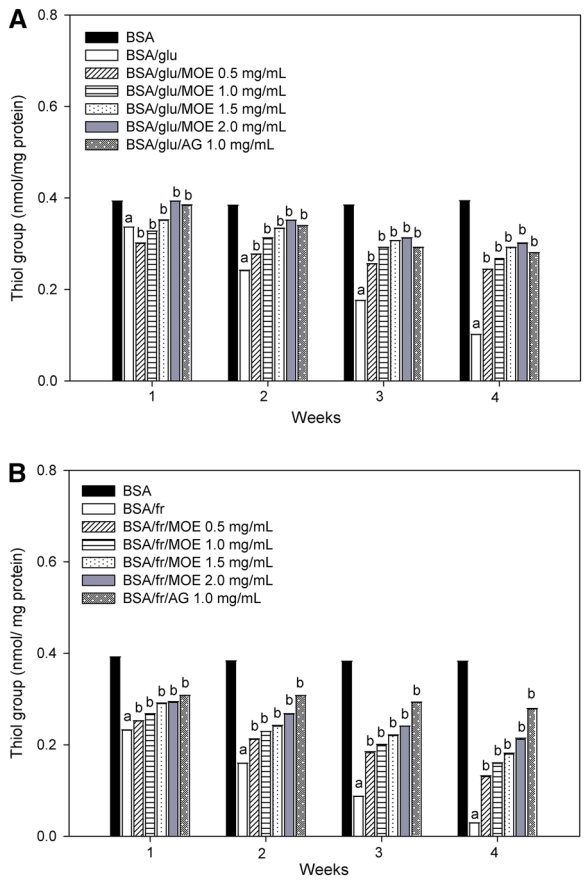

Fig. 5 The effects of *Moringa oleifera* leaf extract (MOE, 0.5–2.0 mg/mL) and aminoguanidine (AG, 1.0 mg/mL) on thiol group in **A** bovine serum albumin (BSA) incubated with 0.5 M glucose (glu) and **B** bovine serum albumin (BSA) incubated with 0.5 M fructose (fr) at week 1, 2, 3 and 4 of incubation. The results are expressed as mean ± SEM (n = 3). $^{a}P < 0.05$ compared to BSA alone, $^{b}P < 0.05$ when compared to BSA with glucose (BSA/glu) or BSA with fructose (BSA/fr) at the same week of incubation

fluorescent and non-fluorescent AGEs at the early stage of glycation (Peyroux and Sternberg 2006). An excess production of ROS causes oxidative damage to proteins which lead to introduce carbonyl groups on the side chain of proteins and deplete thiol group of protein (Dalle-Donne et al. 2003).

Recent studies have shown that polyphenolic compounds from the edible plants play a vital role to protect monosaccharide-induced protein glycation and oxidation (Adisakwattana et al. 2010). Additionally, there is a strong link between the polyphenolic content in the tested plant extracts and the ability to inhibit AGE formation (Peng et al. 2011; Wu and Yen 2005). Evidence also supports that the inhibitory effect of polyphenols against protein glycation is strongly related to their ability of scavenging free radical derived from the glycoxidation process (Povichit et al. 2010). Our findings indicate that MOE has high content of polyphenolic compounds. The content of total phenolic compounds in *Moringa oleifera* leaf extract (MOE) was consistent with previous studies that the content of total phenolic compounds in MOE ranged 33.82–45.21 mg gallic acid equivalents/g extract. According to the results obtained, we addressed the hypothesis that polyphenolic compounds in MOE may be a major contributor to inhibit the formation of AGEs. Previous studies have demonstrated that MOE has antioxidant activity against free radicals including DPPH free radical, superoxide, hydroxyl and nitric oxide radical (Siddhuraju and Becker 2003). Therefore, the inhibitory effect of MOE on glycation-induced protein oxidation may be due to its antioxidant properties. However, certain active biological constituents of MOE remain unknown. To prove this hypothesis, separation and characterization of polyphenolic compound in MOE using HPLC–MS are required for further study.

Conclusion

Moringa oleifera aqueous leaf extract effectively inhibits reducing monosaccharide-induced AGE formation, protein oxidation and protein cross-linking in glycation reaction. This finding could be suggested that *Moringa oleifera* aqueous leaf extract may be used as an anti-glycation agent to prevent the progression of diabetic complications.

Abbreviations

AGEs: advanced glycation end products; N^{ε}-CML: N^{ε}-(carboxymethyl) lysine; BSA: bovine serum albumin; AG: aminoguanidine; MOE: *Moringa oleifera* leaf extract.

Authors' contributions

SN, WS and SA was responsible for conception and design, drafted the manuscript and revised it critically for important intellectual content. WD, KM, and SS contributed to the data analysis and interpretation of the findings. PN conducted the experiments, organized the data analysis, and interpretation of data. All authors contributed to the drafting of the manuscript. In addition, all authors also read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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