INHIBITORY EFFECT OF *NIGELLA SATIVA* (BLACK CUMIN) SEED EXTRACT ON THE GLYCATION OF PHOSPHATIDYLETHANOLAMINE

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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

การศึกษาผลของสารสกัดเมล็ดเทียนดำต่อการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีน

นางสาวมาริสา มารแพ้

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาอาหารและโภชนาการ ภาควิชาโภชนาการและการกำหนดอาหาร
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	INHIBITORY EFFECT OF *NIGELLA SATIVA* (BLACK CUMIN) SEED EXTRACT ON THE GLYCATION OF PHOSPHATIDYLETHANOLAMINE
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มาริสา มารแพ้ : การศึกษาผลของสารสกัดเมล็ดเทียนดำต่อการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีน (INHIBITORY EFFECT OF *NIGELLA SATIVA* (BLACK CUMIN) SEED EXTRACT ON THE GLYCATION OF PHOSPHATIDYLETHANOLAMINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. สุกฤต ศิริขวัญพงศ์ , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ทิพยเนตร อริยปิติพันธ์, หน้า.

ภาวะระดับน้ำตาลในเลือดสูงเรื้อรังส่งผลให้เกิดปฏิกิริยาไกลเคชั่นของไขมันชนิดที่มีหมู่อะมิโนเป็นองค์ประกอบ เช่น ฟอสฟาทิดิลเอทาโนลามีน ซึ่งปฏิกิริยาดังกล่าวทำให้เกิดการสร้างไกลเคทฟอสฟาทิดิลเอทาโนลามีน (glycated-PE) และ แอดวานซ์ ไกลเคชั่น เอ็น โปรดักส์ของฟอสฟาทิดิลเอทาโนลามีน (PE- linked advanced glycation end products; AGE-PE) ซึ่งเป็นปัจจัยสาเหตุของการเกิดภาวะแทรกซ้อนในผู้ป่วยโรคเบาหวาน จากการศึกษาก่อนหน้าพบว่า ไทโมควิโนน (Thymoquinone) ซึ่งเป็นสารสำคัญหลักในเมล็ดเทียนดำ (*Nigella Sativa* L.) มีฤทธิ์ต้านปฏิกิริยาไกลเคชั่นของโปรตีน อย่างไรก็ตาม ฤทธิ์ของสารสกัดจากเมล็ดเทียนดำในการต้านไกลเคชั่นของไขมันนั้นยังคงไม่เป็นที่ปรากฏแน่ชัด ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดเอทานอลของเมล็ดเทียนดำต่อการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีน โดยใช้สารมาตรฐานไขมันกับเซลล์เม็ดเลือดแดงเป็นต้นแบบ และวิเคราะห์ผลิตภัณฑ์ด้วยเทคนิคแมสสเปกโทรเมตรี การทดลองใช้ไขมันชนิดฟอสฟาทิดิลเอทาโนลามีนบ่มกับน้ำตาลกลูโคสในสภาวะที่มีสารสกัดเมล็ดเทียนดำหรือไทโมควิโนนและวิเคราะห์ผลิตภัณฑ์จากปฏิกิริยาไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีนซึ่งประกอบด้วย ไกลเคทฟอสฟาทิดิลเอทาโนลามีน และ AGE-PE ได้แก่ คาร์บอกซี่เมททิล-ฟอสฟาทิดิลเอทาโนลามีน (CM-PE) และ และคาร์บอกซี่เอททิล-ฟอสฟาทิดิลเอทาโนลามีน (CE-PE) ผลการทดลองพบว่า สารสกัดสามารถยับยั้งการเกิดผลิตภัณฑ์เหล่านี้ได้โดยมีความสัมพันธ์กับความเข้มข้น ซึ่งสารสกัดที่ความเข้มข้น 40 มก./มล. ยับยั้งการเกิดได้ทั้งไกลเคทฟอสฟาทิดิลเอทาโนลามีน และ AGE-PE อย่างมีนัยสำคัญ โดยที่ความเข้มข้นเดียวกัน สารสกัดสามารถยับยั้ง CM-PE ได้ดีกว่าอะมิโนกัวนิดีน ซึ่งเป็นยาต้านไกลเคชั่น นอกจากนี้พบว่า ไทโมควิโนนสามารถยับยั้งการเกิดไกลเคทฟอสฟาทิดิลเอทาโนลามีน และ CM-PE ได้ อย่างไรก็ตาม จากการทดลองในเซลล์เม็ดเลือดแดง ไม่พบการเปลี่ยนแปลงอย่างมีนัยสำคัญของผลิตภัณฑ์เหล่านี้ ภายหลังการบ่มกับสารสกัดเมล็ดเทียนดำ ดังนั้นจากผลการทดลองสามารถสรุปได้ว่า สารสกัดเมล็ดเทียนดำอาจเป็นทางเลือกหนึ่งเพื่อใช้ในการป้องกันการเกิดภาวะแทรกซ้อนของโรคเบาหวานที่เหนี่ยวนำโดยปฏิกิริยาไกลเคชั่นของไขมัน

ภาควิชา	โภชนาการและการกำหนดอาหาร	ลายมือชื่อนิสิต .....
สาขาวิชา	อาหารและโภชนาการ	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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5576858637 : MAJOR FOOD AND NUTRITION

KEYWORDS: NIGELLA SATIVA / LIPID GLYCATION / PHOSPHATIDYLETHANOLAMINE / THYMOQUINONE

MARISA MARNPAE: INHIBITORY EFFECT OF *NIGELLA SATIVA* (BLACK CUMIN) SEED EXTRACT ON THE GLYCATION OF PHOSPHATIDYLETHANOLAMINE. ADVISOR: SUKRIT SIRIKWANPONG, Ph.D., CO-ADVISOR: ASST. PROF. TIPAYANATE ARIYAPITIPUN, Ph.D., pp.

Chronic hyperglycemia promotes the non-enzymatic glycation of the amino-containing phospholipids such as phosphatidylethanolamine (PE). This reaction yields the glycated-PE and PE-linked advanced glycation end products (AGE-PE) which are the contributing factors for diabetic complications. Thymoquinone (TQ), the main active compound in *Nigella Sativa* L. (Black cumin) seed, has been reported to have anti-protein glycation properties. However, the inhibitory effect of *Nigella Sativa* seed extract (NSE) on lipid glycation was still remained unknown. Therefore, this study aimed to investigate the inhibitory effect of ethanolic extract of NSE on the glycation of PE in commercial standard and erythrocyte model by using mass spectrometric technique. PE was incubated with glucose in the presence of NSE or TQ. The products of PE glycation including glycated-PE and AGE-PE (carboxymethyl-PE; CM-PE and carboxyethyl-PE; CE-PE) were analyzed. The results demonstrated that NSE inhibited products from PE glycation in a dose-dependent manner. The 40 mg/ml of NSE significantly reduced both glycated-PE and AGE-PE formation ($p < 0.05$). Moreover, NSE inhibited CM-PE to a greater extent than aminoguanidine (AG), a common anti-glycation agent, at the same concentration. TQ also showed the significant reduction in both glycated-PE and CM-PE. In erythrocyte experiment, however, no significant changes of glycated-PE and AGE-PE were observed in NSE treatment as compared to those in negative control. From the present study, it can be concluded that NSE might be other therapeutic option for the prevention of lipid glycation-induced pathogenesis of diabetic complications.

Department:	Nutrition and Dietetics	Student's Signature .....
Field of Study:	Food and Nutrition	Advisor's Signature .....
Academic Year:	2014	Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest appreciation to my advisor, Dr. Sukrit Sirikwanpong for useful advice, technical knowledge, meticulous revision of thesis in a limited time, and encouragement providing me the possibility to complete this thesis and also would like to thank my co-advisor, Assist. Prof. Dr. Tipayanate Ariyapitipun, for her kind advice and supports thoroughly.

I would like to express my appreciation to Assoc. Prof. Dr. Winai Dahlan, the founding director of The Halal Science Center, Chulalongkorn University, for giving me the opportunities to study in this program and also supporting me the analytical and laboratory equipments used in my research.

My sincere gratitude is also extended to my thesis committee: Assist. Prof. Dr. Suwimol Sabwarobol, the chairman, and Dr. Cherdsak Maneeruttanarungroj for their useful guidance, valuable comments and suggestions.

I wish to express my cordial thanks to my lecturers in department of Nutrition and Dietetics for giving me the knowledge and advices during my post-graduated study.

Special thanks are also gone to my colleagues in Master in Food and Nutrition program and staffs of The Halal Science Center for their assistance, encouragement and fruitful discussion.

My acknowledgements are also extended to National Research Council of Thailand, The Halal Science Center, and faculty of Allied Health Science, Chulalongkorn University for the financial support.

Last but not least, I would like to express my gratefulness to my parent and family members for their love, understanding, moral support and prayer for me.

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CHAPTER I
INTRODUCTION

1.1 Background and significant of the study

Diabetes mellitus is one of major cause of death which caused 4.6 million deaths in 2011 (International Diabetes Federation). The number of people with type 2 diabetes is increasing worldwide. The total number of people with diabetes is 366 million in 2011 and it is projected to rise to 552 million in 2030. In Thailand, diabetic patient is accounted for 4 millions in 2011, and it is increasing to 5.4 millions in 2030 (Whiting *et al.*, 2011). Diabetes is characterized by hyperglycemia resulting from quantitative or qualitative deficiency in insulin secretion or from insulin resistance. Chronic hyperglycemia is a cause of long-term complications in diabetic patients which include macrovascular complications leading to atherosclerosis, coronary heart and peripheral arterial disease, and microvascular complications such as neuropathy, nephropathy, retinopathy and also impaired wound healing (Negre-Salvayre *et al.*, 2009).

A major pathway involving in the development of diabetic complications causing from hyperglycemia is glycation (Ahmed, 2005). Glycation is a non-enzymatic reaction between carbonyl group of a reducing sugar and free amino group of proteins, lipids or nucleic acids (Ahmed, 2005; Negre-Salvayre *et al.*, 2009). The reaction between glucose and amino group of protein results in a reversible formation of a structure called a Schiff's base which rearranges to more stable structures called Amadori product. Amadori product undergo further oxidation, generating dicarbonyl compounds to yield advanced glycation end products (AGEs) (Peyroux and Sternberg, 2006). The AGEs are markers and also important causal factors for the pathogenesis of diabetes. AGEs accumulating in vascular wall tissues

and on plasma lipoproteins bind to AGE receptors (RAGEs) on smooth muscle cells, resulting in the increased cellular proliferation, oxidative stress, and production of proinflammatory cytokines leading to diabetic complications (Ahmed, 2005; Forbes *et al.*, 2004; Peyroux and Sternberg, 2006; Yamamoto *et al.*, 2005).

Apart from protein glycation, some studies have found that the glycation reaction also occurred between lipids and glucose called lipid glycation (Bucala *et al.*, 1993; Lertsiri *et al.*, 1998; Ravandi *et al.*, 1996). Generally, the membrane phospholipids are composed of choline- and amino-containing phospholipids. Phosphatidylethanolamine (PE) which was one of amino-containing phospholipids, has been found to be able to react with glucose and can convert to glycated species under hyperglycemic conditions (Bucala *et al.*, 1993). The glycation of PE involved Schiff- PE formation and further rearrangement to a PE-linked Amadori product (deoxy-D-fructosyl PE or Amadori-PE) (Bucala *et al.*, 1993; Lertsiri *et al.*, 1998). The Amadori-PEcan further undergo complex reactions to form PE- linked advanced glycation end products (AGE-PE) such as carboxymethyl-phosphatidylethanolamine (CM-PE) and carboxyethyl-phosphatidylethanolamine (CE-PE) (Utzmann and Lederer, 2000).

In diabetic patient, the concentration of Amadori-PE was found to be higher in plasma and also in erythrocyte as compared to those in healthy subjects (Teruo Miyazawa *et al.*, 2005; Nakagawa *et al.*, 2005; Shoji *et al.*, 2010). CM-PE also has been reported to be found in human erythrocyte and mitochondrial membrane of the mammalian cells (Pamplona *et al.*, 1998; Requena *et al.*, 1997). Products from lipid glycation stimulated inflammatory cytokines production, lipid peroxidation and angiogenesis, and also induced changes in physical and biological properties of the cell membrane. These can contribute to the increased risk of the diabetic

complications (Naudi *et al.*, 2013; J.-H. Oak *et al.*, 2003; J. Oak *et al.*, 2000; T. Obšil *et al.*, 1999; T. A. Obšil, Evžen; Pavlíček, Zdeněk, 1998; Simões *et al.*, 2013).

To prevent the diabetic complications caused by AGEs, the inhibition of AGEs formation is the best therapeutic approach (Yamagishi *et al.*, 2008). Generally, synthetic compounds have been commonly used as anti-glycation agent; however, many of those were withdrawn from clinical trials because of low efficacies, poor pharmacokinetics, and inadequate safety (Kawanishi *et al.*, 2003; Manzanaro *et al.*, 2006). Aminoguanidine, the famous inhibitor against AGEs formation both *in vitro* and in vivo, have been found to have some toxicity and was also withdrawn from the crucial phase III of clinical trials because of safety concerns and lack of efficacy (Bolton *et al.*, 2004; Freedman *et al.*, 1999; Giardino *et al.*, 1998; Ihm *et al.*, 1999). In contrast, some natural products have been proven relatively safe for human consumption and many phytochemical compounds and extracts from dietary plants and herbal medicines have been studied for their ability to prevent glycation (Ardestani and Yazdanparast, 2007a; Losso *et al.*, 2011; Meeprom *et al.*, 2013; Tupe and Agte, 2010; Vinson and Howard Iii, 1996)

Nigella sativa L. (Black cumin or black seed) is a herbal medicine commonly found in Mediterranean region, Pakistan and India (Gali-Muhtasib *et al.*, 2006). It has been traditionally used in Middle East and India for thousands of years as a spice and a protective and health therapy in traditional folk medicine for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema, as a diuretic and carminative, lactagogue and vermifuge (Chopra *et al.*, 1956; Gali-Muhtasib *et al.*, 2006). The pharmacological properties of *Nigella sativa* are related to a major active compound named thymoquinone which found mainly in seed oil (Ghosheh *et al.*, 1999). Several biological and pharmacological activities of *Nigella Sativa* seed have been reported, including antioxidant, antimicrobial, anti-

inflammation and immunomodulation, anti-cancer as well as beneficial effects on metabolic syndromes including hyperglycemia, dyslipidemia, and cardiovascular disease (Al-Ghamdi, 2001; Burits and Bucar, 2000; Dilshad *et al.*, 2012; Ferdous *et al.*, 1992; Gali-Muhtasib *et al.*, 2006; Hanafy and Hatem, 1991; Houghton *et al.*, 1995; Khan *et al.*, 2011; M. F. Ramadan *et al.*, 2003; Sabzghabaee *et al.*, 2012; Suboh *et al.*, 2004; Woo *et al.*, 2012),

Beneficial effects of *Nigella Sativa* seed extract, seed volatile oil and thymoquinone on diabetes mellitus have been reported *in vitro* and vivo including a decrease in blood glucose and glycated hemoglobin (Fararh *et al.*, 2005) a decrease in hepatic gluconeogenesis (Fararh *et al.*, 2005; L. Pari and C. Sankaranarayanan, 2009), an improvement of glucose tolerant through an inhibition of glucose absorption (Meddah *et al.*, 2009), a decreased oxidative stress and an increase in insulin level (Abdelmeguid *et al.*, 2010). For metabolic syndromes, black cumin has been reported to be safe and efficacy. Supplementation with 2.5 ml of *Nigella Sativa* oil twice daily addition to normal medication for 6 weeks led to decrease in fasting blood glucose, cholesterol and LDL (A. Najmi *et al.*, 2008). The recommended dose of 500 mg in addition to daily drugs for 8 weeks can decrease fasting blood glucose, postprandial blood glucose and glycated hemoglobin (Ahmad Najmi *et al.*, 2012). Thymoquinone, a major quinone from black cumin seed, has been reported to be able to inhibit the formation of advanced glycation end products (AGEs) *in vitro*. Twenty μM of thymoquinone can inhibit 39% of hemoglobin glycation and 82% of post-amadori glycation products, reduce methyglyoxal-mediated human serum albumin glycation by 68% and inhibit 78% of late glycation end products (Losso *et al.*, 2011). However, little is known about the effects of *Nigella Sativa* seed extract on the inhibition of glycation, especially on lipid glycation which is now recognized as the new target for the glycation reaction.

Hence, this study aims to investigate the inhibitory effects of *Nigella Sativa* seed extract on glucose-mediated glycation of phosphatidylethanolamine (PE). The commercial PE standard, Dioleoyl-phosphatidylethanolamine (DOPE), and PE in human erythrocyte were used as the model for studying the formation of lipid glycation and the efficacy of the black cumin extract on the inhibition of lipid glycation. The hybrid tandem mass spectrometer (ESI-QTRAP MS) was used to detect and semi-quantify of glycated PE species. Moreover, the inhibitory effect of thymoquinone on lipid glycation was also investigated.

1.2 Objective of the study

1.2.1 To investigate the efficacy of ethanolic extract of *Nigella Sativa* seed on the inhibition of glycated-PE and advance glycation end product of PE (AGE-PE) in the commercial PE standard

1.2.2 To investigate the inhibitory effect of thymoquinone on formation of glycated-PE and AGE-PE in commercial PE standard

1.2.3 To investigate the efficacy of ethanolic extract of *Nigella Sativa* seed on the inhibition of glycated-PE and AGE-PE in human erythrocyte model

1.3 Hypothesis

Since thymoquinone has been reported to have anti-glycation property, therefore, the ethanoic extract of *Nigella Sativa* seed rich in thymoquinone might inhibit the formation of glycated-PE and PE-linked advanced glycation end products (AGE-PE) in both commercial standard and human erythrocyte model

1.4 Benefits of the study

1.4.1 Anti-lipid glycation properties of the *Nigella Sativa* seed extract and/or thymoquinone will be explored.

1.4.2 *Nigella Sativa* (Black cumin) might be the medicinal herb for preventing and reducing the risk of the diabetic complication induced by lipid glycation.

1.4.3 This study provides a model for the study of lipid glycation inhibitors, which can be further used for screening of anti-lipid glycation properties of the other medicinal herbs

CHAPTER II

REVIEW OF LITERATURE

2.1 Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes results in long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2014).

2.1.1 Classification of diabetes mellitus

Diabetes can be classified into four clinical categories (American Diabetes Association, 2014)

i *Type 1 diabetes:* diabetes resulting from β -cell destruction, leading to absolute insulin deficiency. This type is divided into “Immune-mediated diabetes”, induced by a cellular-mediated autoimmune destruction of the β-cells of the pancreas and commonly occurring in childhood and adolescence, and “Idiopathic diabetes”, no evidence of autoimmunity.

ii *Type 2 diabetes:* diabetes ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance. This type is often associated with a strong genetic predisposition, more so than is the autoimmune form of type1 diabetes. However, the genetics of this form of diabetes are complex and not fully defined. Age, obesity, and lack of physical activity may increase the risk of developing this form of diabetes.

iii *Other specific types of diabetes:* diabetes due to other causes, e.g., genetic defects in β –cell function, genetic defects in insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced (such as in the treatment of HIV/ AIDS or after organ transplantation).

iv *Gestational diabetes mellitus (GDM):* diabetes diagnosed during pregnancy that is not clearly overt diabetes

2.1.2 Criteria for the diagnosis of diabetes

Table 1 Criteria for the diagnosis of diabetes

(American Diabetes Association, 2014)

A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.
OR
FPG≥126mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.
OR
Two-hour plasma glucose ≥ 200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L).

2.2 Hyperglycemia and Diabetic complications

People with diabetes have an increased risk of serious health problems. Hyperglycemia is the major risk factor for the development of diabetic complications including macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Fowler, 2008).

Hyperglycemia (high blood glucose) can cause long term lethal effects on various tissues and organs through the activation of several cellular pathways, including increased oxidative stress (Baynes, 1991; Michael Brownlee, 2001; Fatehi-Hassanabad *et al.*, 2010), increased flux of polyol pathway and hexosamine pathways (Michael Brownlee, 2001), activation of protein kinase C (Ishii *et al.*, 1998), and increased formation of advanced glycation end products (AGEs) (Ahmed, 2005; Kim *et al.*, 2005). These are described in figure1.

Consequences of hyperglycemia-induced activation of many involved mechanisms, leading to pathological alterations, have been reviewed by (Aronson, 2008; Michael Brownlee, 2001) including:

i Nonenzymatic glycation of proteins and lipids, producing AGEs, can damages and interfere normal function of target cells by disrupting molecular conformation, alter enzymatic activity, reduce degradative capacity, and interfere with receptor recognition. Moreover, binding of AGEs to AGE receptors on cell, such as endothelial cells, macrophages and smooth muscle cells, results in the production of reactive oxygen species and pro-inflammatory responses.

ii Protein kinase C activation decrease endothelial nitric oxide synthetase, thereby decrease nitric oxide, increase endothelin-1-stimulated MAP-kinase activity, and induce the expression of the vascular endothelial growth factor, transforming growth factor-ß (TGF- ß), and plasminogen activator inhibitor-1 (PAI-1), leading to abnormalities of blood flow, vascular permeability, angiogenesis, and vascular occlusion. In addition, hyperglycemia-induced activation of protein kinase C activates NF-kB and NAD (P) H oxidases, resulting in pro-inflammatory gene expression, and generation of reactive oxygen species.

iii The hexosamine pathway causes O-linked glycosylation thereby increase the production of factor as PAI-1 and TGF- ß, leading to many changes in both gene expression and various enzymes or protein function.

iv The polyol pathway causes the increase of sorbitol, resulting in osmotic damage to microvascular cells. The oxidation of sorbitol to fructose by the reduction of NAD^+ affects the consequence pathway, resulting in the increase of both methylglyoxal, a precursor of AGEs, and diacylglycerol (DAG). Moreover, the decrease in NADPH, used in reduction of glucose to sorbitol, leads to the decrease in level of GSH which is an antioxidant enzyme.

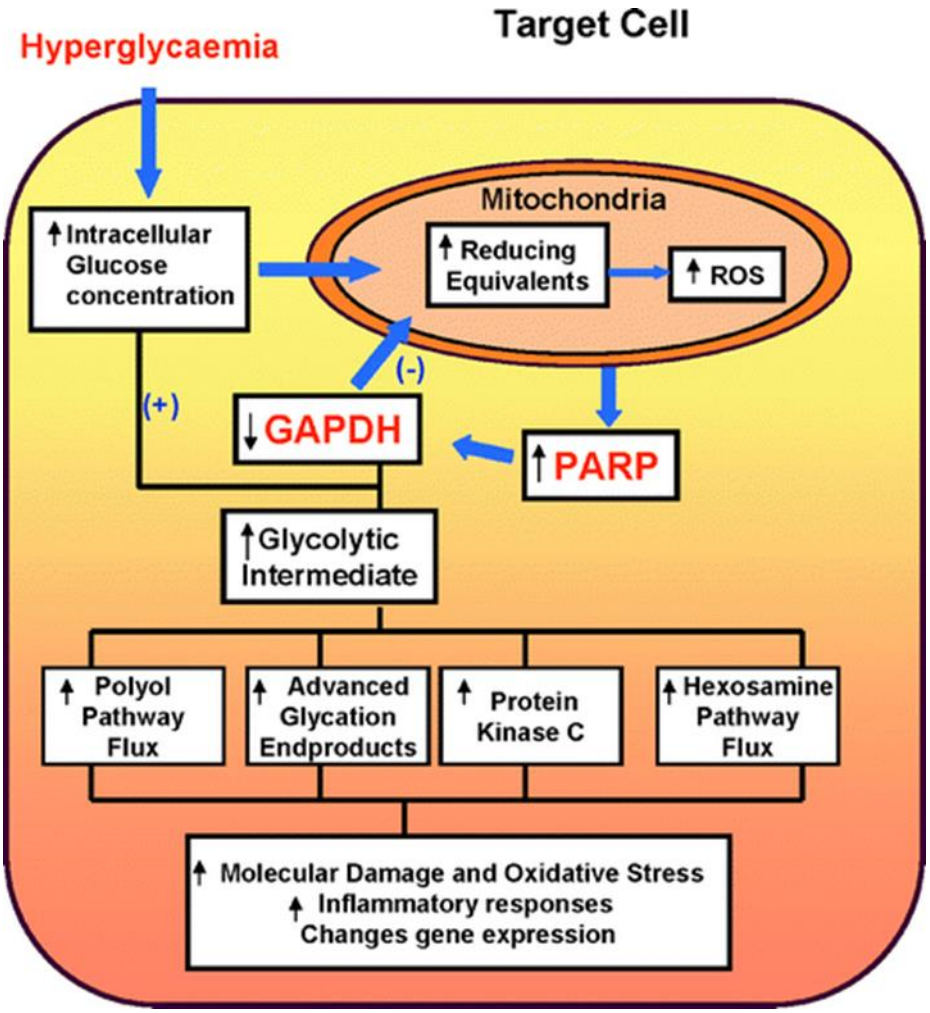

Figure 1 Mechanisms involving hyperglycemia-induced damage.

An increase of intracellular glucose concentration in target cells resulting in increased mitochondrial production of ROS and activation of PARP. PARP then modifies GAPDH, thereby reducing its activity. The decreased GAPDH activity increases glycolytic intermediates which activates the polyol pathway, increases intracellular AGEs formation, activates PKC and subsequently NF-kB, and activates hexosamine pathway flux. All these metabolic pathways cause alterations in gene expression, inflammatory responses, and structural and functional changes in cellular constituents that represent the molecular basis of the diabetic process. (Negre-Salvayre *et al.*, 2009)

2.3 Glycation and advanced glycation end-products (AGEs)

Glycation or non-enzymatic glycocylation is a non-enzymatic reaction between carbonyl group of a reducing sugar and free amino group of proteins, lipids or nucleic acids (Ahmed, 2005; Negre-Salvayre *et al.*, 2009). Glycation can occurs exogenously (called as maillard reaction), from food during food preparation and heating, producing flavorful and brownish compounds. In the same way, endogenous glycation also occurs during hyperglycemia condition, as be reported the presenting of HbA1c, a glycated hemoglobin, in diabetic patients (Rahbar *et al.*, 1969).

Protein glycation is a spontaneous reaction depending in vivo on the degree and duration of hyperglycemia. Protein glycation is initiated by a nucleophilic addition reaction between a free amino group from a protein and a carbonyl group from a reducing sugar such as glucose to form a freely reversible schiff base, occurring over a period of hours. The schiff base rearranges to more stable structure called Amadori product (over a period of days) (Figure 2). The Amadori product can be converted (in a period of weeks) into reactive dicarbonyl compounds such as glucosones to yield glycation end products (AGEs) (Peyroux and Sternberg, 2006) (Figure 2 and 3). The Amadori product can also be fragmented by oxidation (glycoxidation) to yield AGEs such as carboxymethyllysine (CML) or pentosidine as shown in Figure 3. In addition, AGEs formation can come from autoxidation of glucose in the presence of catalytic metals and $O_{2,}$ as shown in Figure 3 (Ahmed, 2005; Monnier, 2003; Peyroux and Sternberg, 2006).

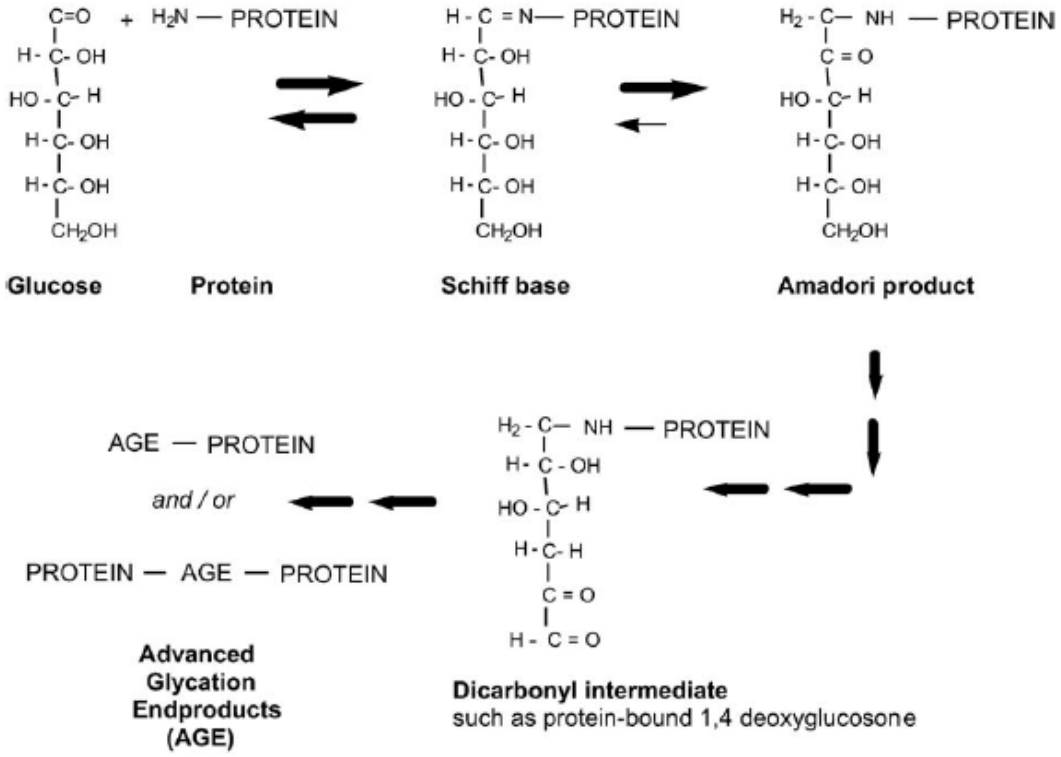

Figure 2 Example of the classical pathway of protein glycation by glucose leading to Advance Glycation End Products (AGEs).

The initial reaction between glucose and protein amino group forms a reversible Schiff base which rearranges to a fructosamine group or Amadori product. With time Amadori products may form AGEs via dicarbonyl intermediates such as protein-bound 1,4 deoxyglucosone. (Peyroux and Sternberg, 2006)

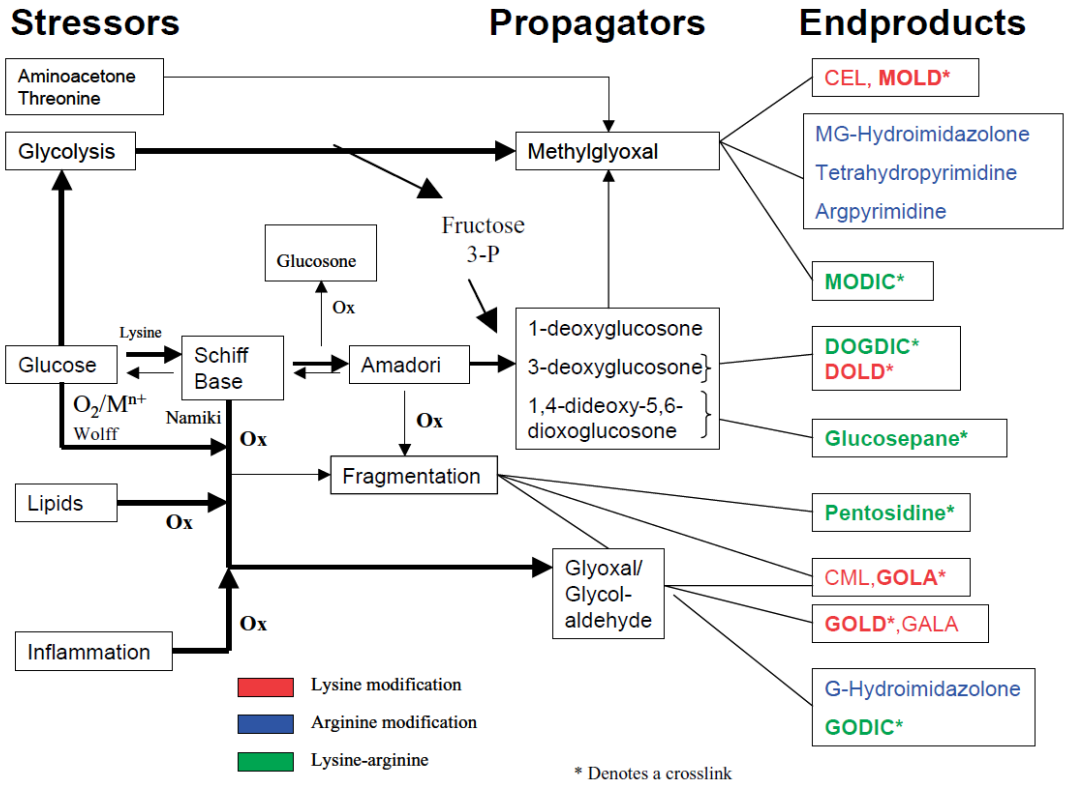

Figure 3 Main chemical pathways leading to AGE formation and selected AGEs of relevance to the Maillard reaction *in vivo*.

(Monnier, 2003)

The AGEs are markers and also important causal factors for the pathogenesis of diabetes (Ahmed, 2005), atherosclerosis (Forbes *et al.*, 2004), cataracts (Stitt, 2005), diabetic nephropathy (Yamamoto *et al.*, 2005), and neuropathy (Jono *et al.*, 2002) . AGEs can damages and interfere normal function of target cells by disrupting molecular conformation, alter enzymatic activity, reduce degradative capacity, and alter functional properties of several important matrix molecules (Charonis *et al.*, 1990; Giardino *et al.*, 1994; Tanaka *et al.*, 1988). Moreover, binding of AGEs to AGE receptors (RAGEs) on smooth muscle cells results in the increased cellular proliferation, oxidative stress, and production of proinflammatory cytokines leading

to diabetic complications (Ahmed, 2005; Forbes *et al.*, 2004; Peyroux and Sternberg, 2006; Yamamoto *et al.*, 2005). Circulating AGE level has been report to relate with RAGE m-RNA expression which contributes to oxidative stress markers in diabetic patients having vascular complications (Chawla *et al.*, 2014).

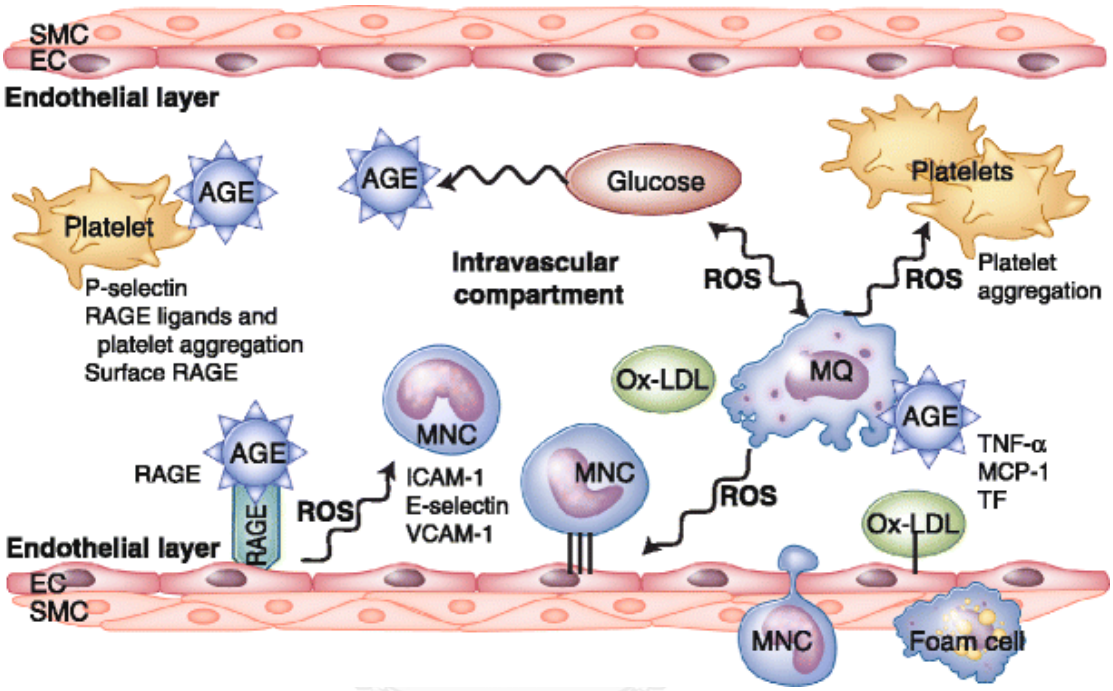

Figure 4 Schematic representation of the effects of diabetes on vascular inflammation and atherosclerosis.

Hyperglycemia induces vascular injury via formation of advanced glycation end products (AGEs) and generation of reactive oxygen species (ROS). AGEs generate ROS directly or through AGE receptor (RAGE), resulting in increasing of oxidized low-density lipoprotein (ox-LDL). RAGE is present on endothelial cells (ECs), fibroblasts, smooth muscle cells (SMCs), monocyte (MNC), and macrophage (MQ), which are involved in the genesis or development of vascular disease, such as producing of inflammatory cytokines which further promote foam cell formation and the progressive of atherosclerosis. (Onat *et al.*, 2011)

While glycation of protein has been thoroughly investigated, there is few studies focus on phospholipids, which is likewise target for glycation. Apart from protein glycation, AGEs could also form on phospholipids by a reaction between glucose and amino groups on phospholipids such as phosphatidylethanolamine and phosphatidylserine, which are important for structure and functionality of biological membrane, called as lipid glycation (Bucala *et al.*, 1993; Lertsiri *et al.*, 1998; Shoji *et al.*, 2010). Products from glycation of phosphatidylethanolamine not only have been found *in vitro* model, but also in diabetic patient, and have been reported their effects relating to pathogenesis of diabetic complications. There for lipid glycation occurred on phosphatidylethanolamine was being interested in this study.

2.4 Phosphatidylethanolamine

2.4.1 General information

Phospholipids are important components of biological membranes and thus essential for structural integrity and functionality of cells. Generally, membrane phospholipid consists of choline-containing phospholipids (i.e. phosphatidlycholine (PC) and sphingomyelin (SM)) and amino-containing phospholipids (i.e. phosphatidylserine (PS) and Phosphatidylethanolamine (PE)). Phosphatidylethanolamine (PE) is an aminophospholipid presenting in membranes of all eukaryotic and prokaryotic cells (Jean E. Vance, 2008). PE is the second most abundant in mammalian, plant and yeast cells and founded mostly in inner monolayer of the membrane bilayer. Regarding to total lipid, phosphatidylethanolamine is accounted for 18% in red blood cell plasma membrane. Moreover, it is also found in cell membrane of liver, mitochondria, endoplasmic reticulum, and myelin as shown in figure 6.

Phosphatidylethanolamine is a neutral or zwitterionic phospholipid at physiological pH. It contains ethanolamine, an amino acid, binding with phosphate group and contains principally palmitic or stearic acid (16:0 or 18:0) esterified at carbon 1 (sn1 position), and a long chain unsaturated fatty acid (e.g. 18:2, 20:4 and 22:6) esterified carbon 2 (Christie, 2013).

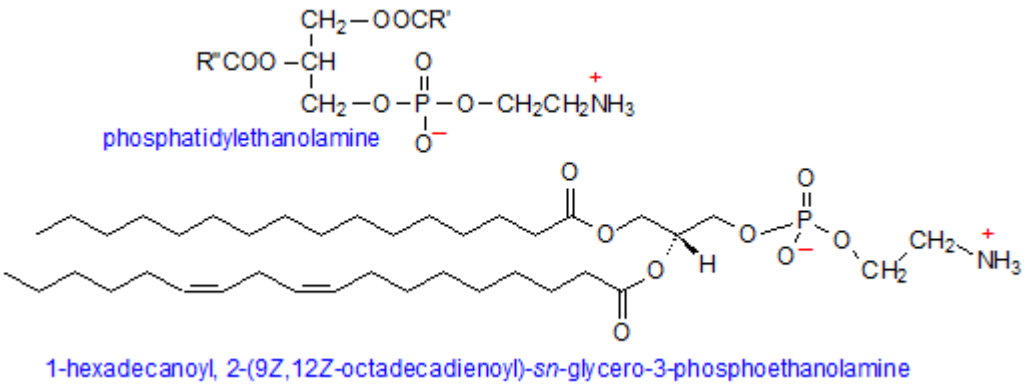

Figure 5 Structure of phosphatidylethanolamine

(source: lipidlibrary.aocs.org)

Table 10–1 **Approximate Lipid Compositions of Different Cell Membranes**

LIPID	PERCENTAGE OF TOTAL LIPID BY WEIGHT					
	LIVER CELL PLASMA MEMBRANE	RED BLOOD CELL PLASMA MEMBRANE	MYELIN	MITOCHONDRION (INNER AND OUTER MEMBRANES)	ENDOPLASMIC RETICULUM	*E. COLI* BACTERIUM
Cholesterol	17	23	22	3	6	0
Phosphatidylethanolamine	7	18	15	28	17	70
Phosphatidylserine	4	7	9	2	5	trace
Phosphatidylcholine	24	17	10	44	40	0
Sphingomyelin	19	18	8	0	5	0
Glycolipids	7	3	28	trace	trace	0
Others	22	14	8	23	27	30

Figure 6 Approximate lipid compositions of different cell membranes

(Bruce Alberts, 2007)

2.4.2 Biosynthesis of PE

In mammalian cells, PE can be synthesized by four biosynthetic pathways. The two major pathways are (i) the Kennedy pathway (i.e. the CDP-ethanolamine pathway), which occurs on endoplasmic reticulum membrane (Figure 8) and (ii) phosphatidlyserine (PS) decarboxylation pathway, which occurs on mitochondrial inner membranes. Moreover, other PE biosynthetic pathways are the acylation of lyso-PE and a calcium-dependent, base-exchange reaction between PS and ethanolamine.

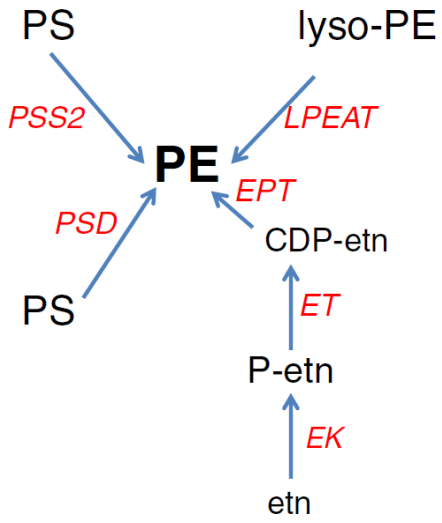

Figure 7 Biosynthetic pathways for PE in mammalian cells.

The two quantitatively major pathways are the PS decarboxylase (PSD) pathway and the CDP-ethanolamine pathway. In the latter, ethanolamine (etn) is phosphorylated by ethanolamine kinase (EK) to produce phosphoethanolamine which is converted into CDPethanolamine by CTP:phosphoethanolamine cytidylyltransferase (ET). Finally, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) combines diacylglycerol with CDP-ethanolamine to produce PE. Small amounts of PE are synthesized by acylation of lyso-PE with lyso-PE acyltransferase (LPEAT), and also by a base-exchange reaction in which PS is

converted to PE by the exchange of serine in PS for ethanolamine by the action of PS synthase-2 (PSS2). (J. E. Vance and Tasseva, 2013)

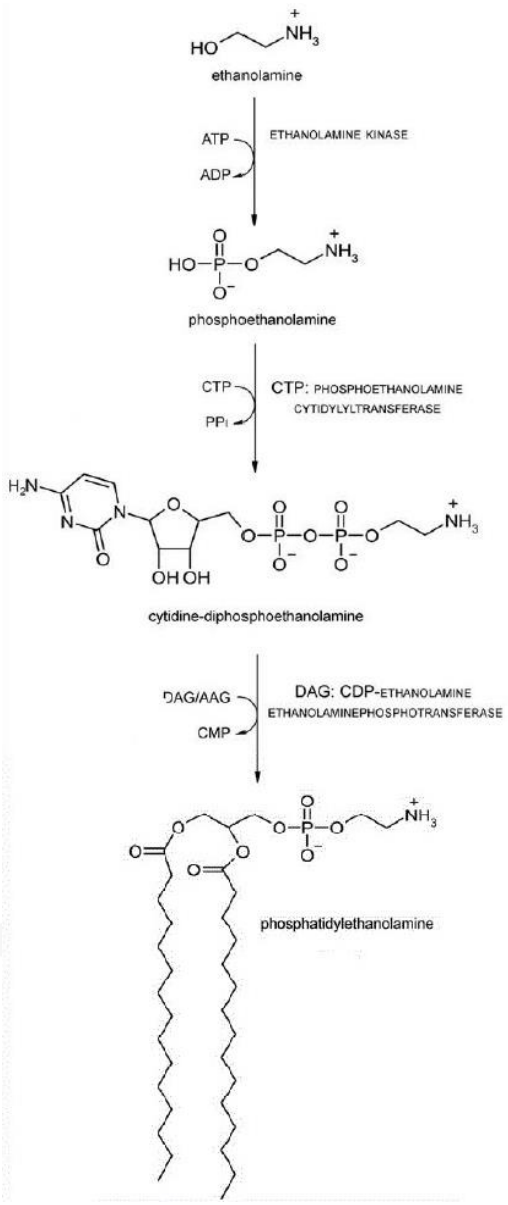

Figure 8 The CDP-ethanolamine pathways.

Ethanolamine kinase catalyzes the ATP-dependent phosphorylation of Ethanolamine, forming Phospho-Ethanolamine. CTP: Phospho-Ethanolamine Cytidylyltransferase uses Phospho-Ethanolamine and CTP to form the high-energy donor **CDP-Ethanolamine** under release of

Pyrophosphate. CDP-Ethanolamine:1,2-Diacylglycerol Ethanolamine-Phosphotransferase catalyzes the formation of Phosphatidyl-Ethanolamine and CMP, using **CDP-Ethanolamine** and a lipid anchor such as Diacylglycerol (DAG) or Alkyl-Acylglycerol (AAG). (Gibellini and Smith, 2010)

2.4.3 Function of PE

PE is evidently a key building block of membrane bilayers. Functions of PE in mammals including:

i Being a substrate for methylation to phosphatidylcholine in the liver (Walkey *et al.*, 1997).

ii Being a precursor for the synthesis of anandamide (N-arachidonoylethanolamine), the ligand for cannabinoid receptors in the brain (Jin *et al.*, 2007).

iii Supplying ethanolamine that covalently modifies several proteins. PE is a donor of the ethanolamine residue for the synthesis of the glycosylphosphatidylinositol anchors that attach many signaling proteins to the surface of the plasma membrane (Menon and Stevens, 1992).

iv Playing an important role in the heart, a decrease in PE content of cardiacmyocytes causes cell damage after ischemia (Post *et al.*, 1995).

v Playing a role in membrane fusion. PE, a cone-shaped lipid has the tendency to form non-lamellar membrane structures and modulates membrane curvature (Birner *et al.*, 2001; Furt and Moreau, 2009).

vi Playing an important role in contractile ring disassembly at the cleavage furrow during cytokinesis of mammalian cells (Emoto *et al.*, 1996; Emoto and Umeda, 2000).

2.5 Glycation of aminophospholipid (Lipid glycation)

Lipid glycation refers to a non-enzymatic reaction between amino group of phospholipids and reducing sugar under hyperglycemic conditions (Bucala *et al.*, 1993; Lertsiri *et al.*, 1998; Ravandi *et al.*, 1996). Target amino-containing phospholipids in glycation reactions are phosphatidylethanolamine (PE) and phosphatidylserine (PS). Phosphatidylethanolamine (PE) has been found to be able to react with glucose and can convert to glycated species under hyperglycemic conditions (Bucala *et al.*, 1993). The glycation of PE initiate by the nucleophilic addition of an amine to the carbonyl of a reducing sugar, forming glucoslyamines or Schiff- PE. Schiff- PE then rearrange to a more stable aminoketose or PE-linked Amadori product (deoxy-D-fructosyl PE or Amadori-PE) (Bucala *et al.*, 1993; Lertsiri *et al.*, 1998). The Amadori-PE can further undergo complex reactions to form PE- linked advanced glycation end products (AGE-PE) such as carboxymethyl-phosphatidylethanolamine (CM-PE) and carboxyethyl-phosphatidylethanolamine (CE-PE) as show in figure 9 (Utzmann and Lederer, 2000). AGE-PE also can be formed by the reaction between PE and dicarbonyl compound such as glyoxal and methylglyoxal. CM-PE not only can be formed by the oxidation of Amadori-PE, but also by the reaction between PE and glyoxal derived from glucose or polyunsaturated fatty acids (PUFAs) oxidation under aerobic condition (figure10) (Naudi *et al.*, 2013; Requena *et al.*, 1997).

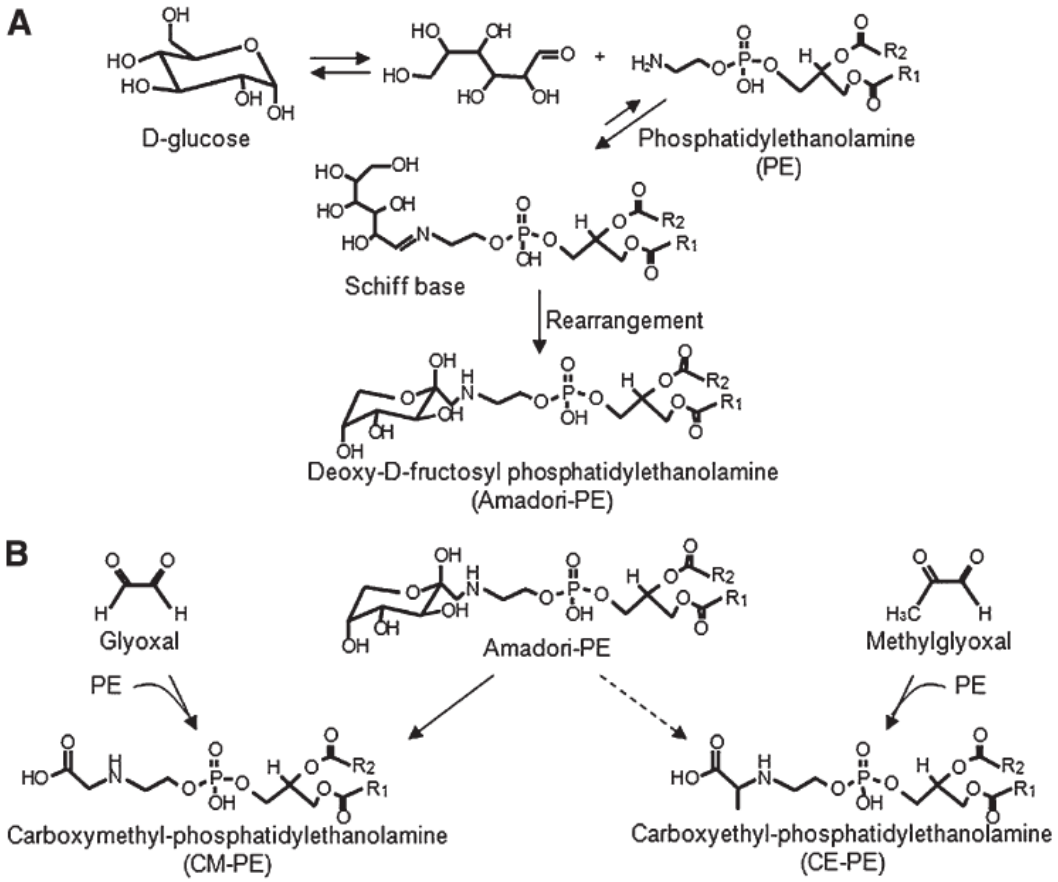

Figure 9 Scheme for glycation of phosphatidylethanolamine.

A: Glucose reacts with the amino group of PE to form a Schiff base, which undergoes an Amadori rearrangement to yield the Amadori-PE (deoxy-D-fructosyl PE). B: Possible routes to formation of AGE-PE (CM-PE and CE-PE) in the late stage of glycation. (Shoji *et al.*, 2010)

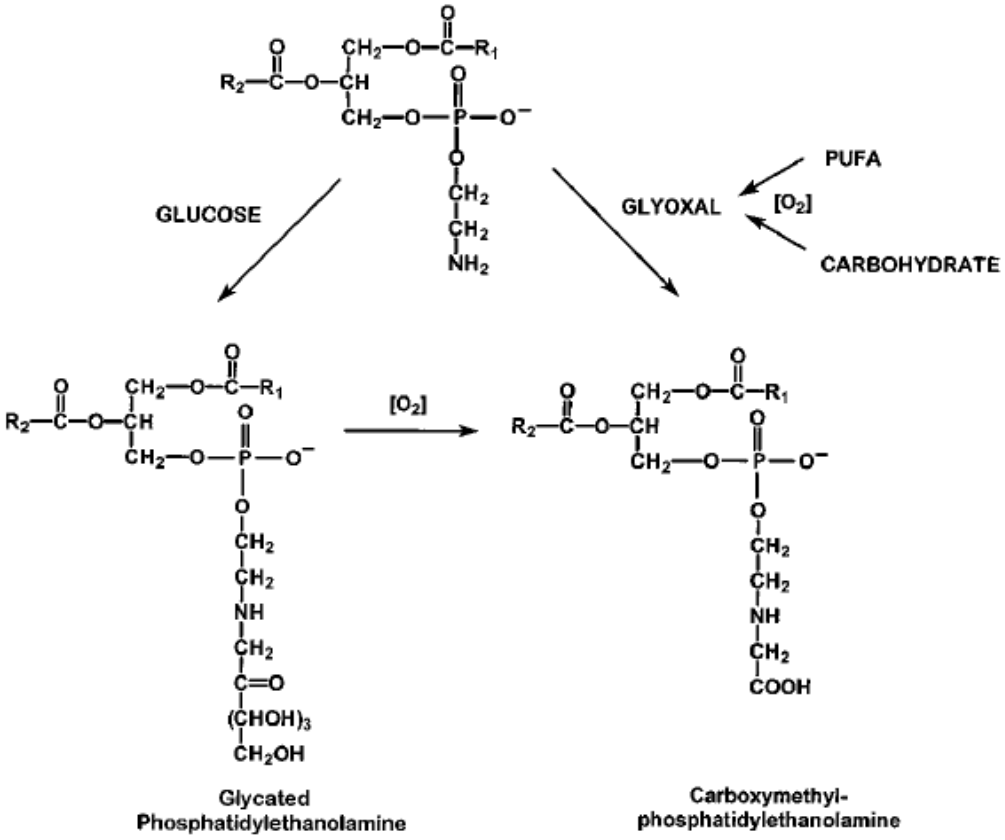

Figure 10 Possible routes to formation of Carboxymethyl-phosphatidyl ethanolamine in glycoxidation and/or lipoxidation reactions.
(Requena *et al.*, 1997)

Shiff-PE and Amadori-PE were found and identified in human erythrocyte; the study demonstrated a higher amount of Shiff-PE and Amadori-PE from diabetic patient (0.18-34.2 mol% and 0.047-0.375 mol%, respectively) than from healthy people (Utzmann and Lederer, 2000). The abnormal quantity of Amadori-PE was reported in diabetic plasma (Teruo Miyazawa *et al.*, 2005; Nakagawa *et al.*, 2005). Amount of Amadori-PE in plasma of diabetic patients (0.15 mol% of PE), diabetic patients with chronic hemodialysis (0.29 mol% of PE), and nondiabetic patients with chronic hemodialysis (0.13 mol% of PE) were higher than those in the healthy subjects (0.08 mol% of PE) (Teruo Miyazawa *et al.*, 2005). It was also reported that

levels of Amadori-PE in red blood cell and plasma of diabetic patient were significant higher than those of healthy subject (Amadori-PE in diabetic patient and healthy subjects in red blood cell were 3.38 and 0.91mmol/mol of native PE respectively). In contrast, there was no significant difference in AGE-PE in red blood cell and plasma of diabetic patients compared to healthy subjects (Shoji *et al.*, 2010). The high level of Amadori-PE was also found in blood and organs that were affected by hyperglycemic condition such as liver, kidney, cerebrum, and pancreas in streptozotocin–induced diabetic rat and its accumulation was faster than that of CML, a protein glycation product (Sookwong *et al.*, 2011). Therefore, the Amadori-PE was suggested to be the helpful predictive marker for early state of diabetes.

CM-PE was detected in human erythrocyte membrane which was accounted for approximately 0.14 mmol of CME/mol of ethanolamine and can be also detected in fasting urine at the concentration of 2-3 mmol/mg of creatinine in healthy and diabetic patient (Requena *et al.*, 1997). Moreover, CM-PE was also found in mitochondrial membranes of mammalian (Pamplona *et al.*, 1998).

Products from PE glycation have been reported the consequent effects. Amadori-PE can trigger lipid peroxidation through generation of superoxide radicals, which may cause membrane lipid peroxidation relating to the pathogenesis of diabetic complications (J. Oak *et al.*, 2000). Glycated-PE stimulated the cytokine producing-cell (monocytes and myeloid dendritic cells), observed by the increased frequently of the cell and increased cytokine production (TNF-α, MIP-1β, and IL-8 in monocytes and TNF-α, IL-6, IL-8 and IL-1β in myeloid dendritic cells) (Simões *et al.*, 2013). The study on glycation of low density lipoprotein (LDL) demonstrated that glycated-PE, a major LDL lipid glycation product, promotes LDL uptake and accumulation of cholesteryl ester (CE) and triacylglycerol (TG) by THP-1 macrophages

(Ravandi *et al.*, 1999). In addition, Amadori-PE stimulated the proliferation, migration and tube formation of cultured human umbilical vein endothelial cells (HUVEC) showing that Amadori-PE may be one of the important inducers of angiogenesis of endothelial cells (J.-H. Oak *et al.*, 2003). Hence, it might be implied that the lipid glycation products may contribute to the development of atherosclerosis.

Glycation of PE also induced changes in physical and biological properties of the cell membrane. The aminophospholipid glycation caused alteration in lipid bilayer structure (an increased in head-group hydration and lipid order in both regions of the membrane) and inhibition of membrane-bound Na^{+}, K^{+}-ATase (T. Obšil *et al.*, 1999; T. A. Obšil, Evžen; Pavlíček, Zdeněk, 1998). Moreover, lipid oxidation accompanying the lipid glycation also affected the lipid order and hydration. For the effects on the interactions between membrane proteins and lipids, phosphospholipid glycation decreases affinity between plasma-membrane Ca^{2+}-ATPase and the surrounding phospholipids, while the protein glycation did not show that effect. PE-glycation decreases about 30% the stability of plasma-membrane Ca^{2+}-ATPase against thermal denaturation (Levi *et al.*, 2008). It can be stated that biological processes involving aminophospholipids could be potentially affected by non-enzymatic glycation and oxidation (figure 11).

Regarding effects of lipid glycation which have been reported relating to generation of free radical, development of atherosclerosis, and alterations of membrane. Therefore, lipid glycation is an important contributing factor of the development of diabetic complication.

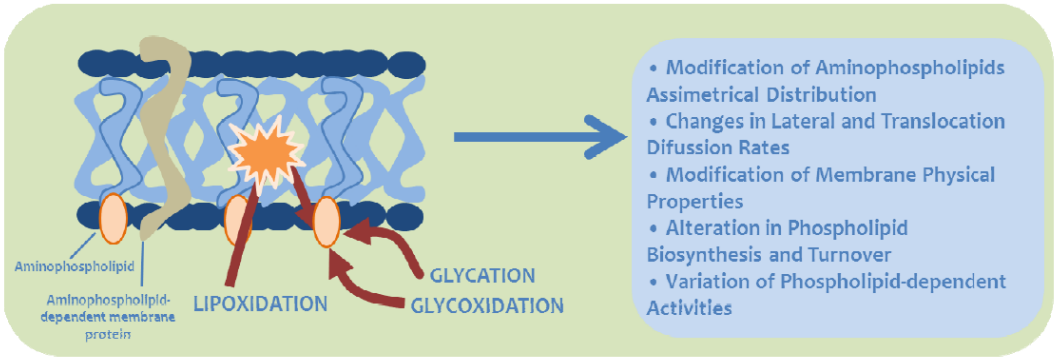

Figure 11 Potential effects of aminophospholipid modification by carbonyl-amine reactions in biological membrane.
(Naudi *et al.*, 2013)

2.6 Anti-glycation agent

To prevent the diabetic complications caused by AGEs, the inhibition of AGEs formation is the best therapeutic approach (Yamagishi *et al.*, 2008). There are several potential sites for inhibition of AGE formation (figure 12) such as using compound that have a reactive dicarbonyl scavenging or antioxidant activity as an anti-glycation agent. In this case, both synthetic compounds and natural products have been evaluated as inhibitors against the AGE formation.

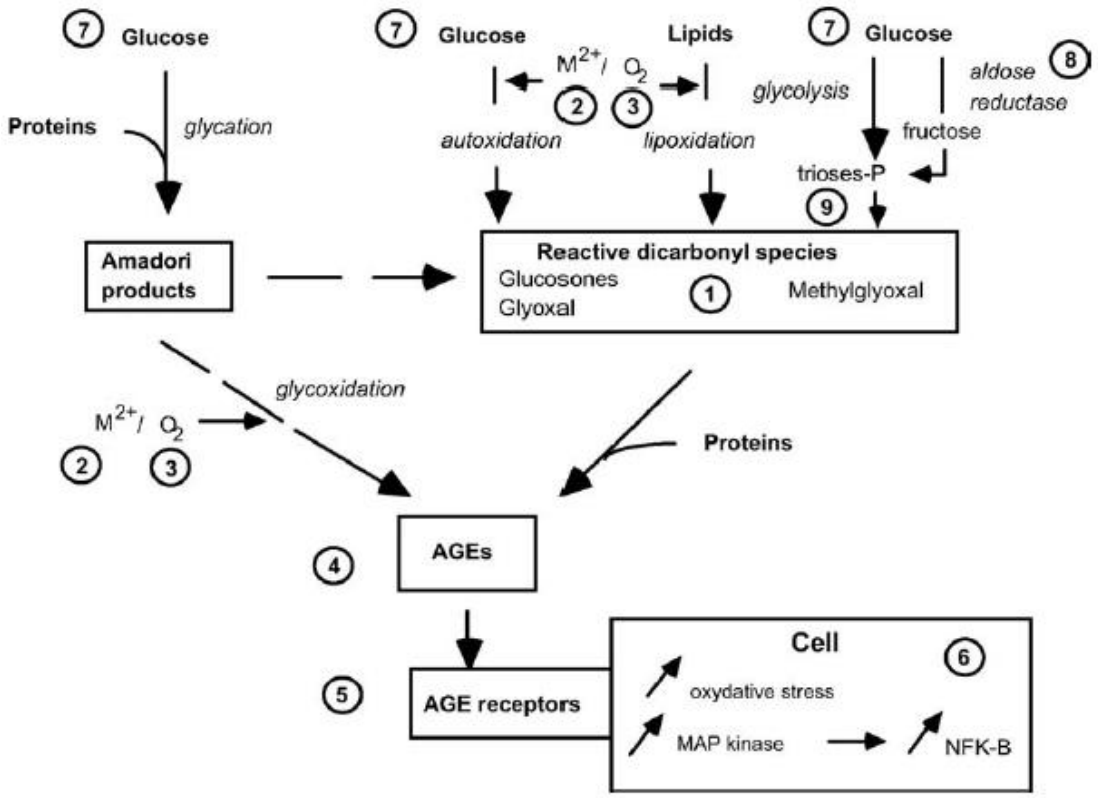

Figure 12 Potential sites of inhibition of AGE formation and AGE-mediated damage.

(1) trapping of reactive dicarbonyl species; (2) antioxidant activity by transition metal (M^{2+}) chelation; (3) free radical scavenging activity by antioxidant; (4) AGE cross-link cleavage; (5) blocking of AGE receptor (RAGE); (6) blocking of AGE receptor signaling; (7) reduction of glycemia condition by anti-diabetic therapy; (8) aldose reductase inhibition; (9) shunting of trioses-P towards the pentose-P pathway by transketolase activation. (Peyroux and Sternberg, 2006)

2.6.1 Synthetic compound/drug

Synthetic compounds have been commonly used as anti-glycation agent. Figure 13 demonstrated inhibitors for advanced glycation end product (AGE) and their kind of action. Aminoguanidine (AG) or known as Pimagedine is the famous inhibitor against AGEs formation both *in vitro* and animal model which has been received the most interest from a clinical trials view (M. Brownlee *et al.*, 1986; Hammes *et al.*, 1994; Soulis-Liparota *et al.*, 1991). AG inhibit AGEs formation by act as a dicarbonyl scavenger, scavenging dicarbonyl glycating agents such as methylglyoxal, glyoxal, and 3-deoxyglucosone (3-DG) (Thornalley *et al.*, 2000). In contrast, AG have been found to have some adverse effects in diabetic patients, such as gastrointestinal symptoms, abnormalities in liver function tests, ANA-associated lupus-like illness and flu-like syndromes (Bolton *et al.*, 2004; Freedman *et al.*, 1999). Therefore, AG was withdrawn from the crucial phase III of clinical trials because of safety concern and low efficacies (Freedman *et al.*, 1999).

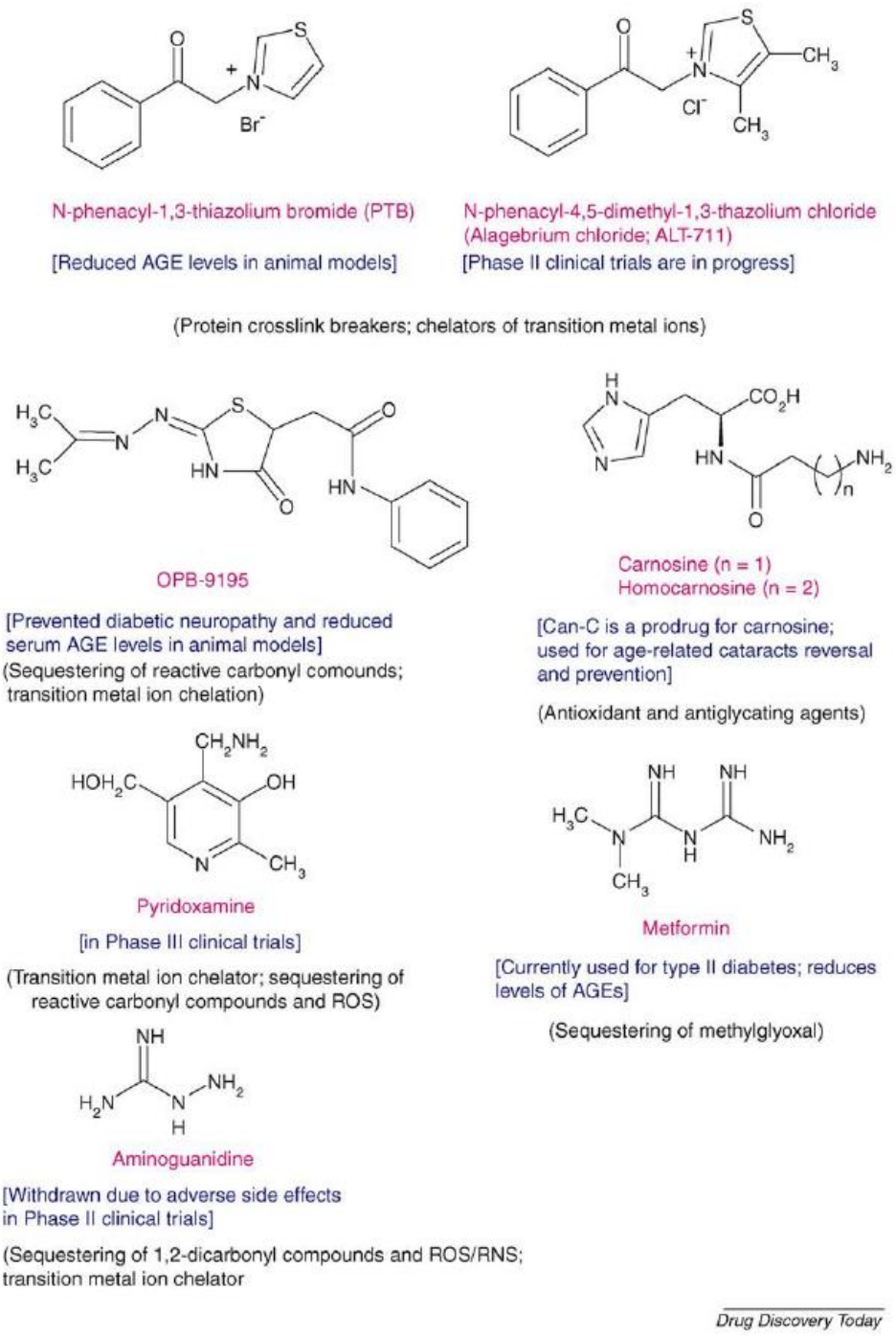

Figure 13 Representative advanced glycation end product (AGE) inhibitors and their modes of action.

(Reddy and Beyaz, 2006)

2.6.2 Natural antioxidant

Many natural compounds have been studied for their ability to prevent glycation. Plant extract such as hydroalcoholic extract of *Cyperus rotundus* and ethyl acetate extract of *Teucrium polium* show the inhibitory effect on AGE formation and protein oxidation, and their effects might be involved by their antioxidant activities the phenolic and flavonoid contents (Ardestani and Yazdanparast, 2007a, b). Isoferulic Acid inhibited the formation of fructosamine, fluorescent AGEs, and non-fluorescent AGEs, and suppressed protein oxidation and crosslinking (Meeprom *et al.*, 2013). Ascorbic acid and some vitamin such as tocopherol, pyridoxal and niacinamide also have been found anti-glycation effect. Supplementation of 1,000 mg of ascorbic acid for 4 weeks resulting in decrease in serum protein glycation (Vinson and Howard Iii, 1996). In addition, thymoquinone, a major quinone from *Nigella sativa* seed, has been reported to be able to inhibit the formation of both early stage glycation products and advanced glycation end products (AGEs) *in vitro* (Losso *et al.*, 2011).

2.7 *Nigella sativa* L.

2.7.1 General information

Nigella sativa L. (Habbatu Sawda, Habatul Baraka, Kalonji, black cumin or black seed) is an aromatic plant in Ranunculaceae family which grows in countries bordering the Mediterranean Sea, Pakistan and India (Gali-Muhtasib *et al.*, 2006). This plant is local to Arab countries and other parts of the Mediterranean region. *Nigella sativa* seeds are angular and dark grey or black color, and also provide slightly bitter and peppery tastes. The seed has been used traditionally in Middle East and India for thousands of years as a spice, seasoning and food preservative as well as a protective and health therapy in traditional folk medicine for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema, as a

diuretic and carminative, lactagogue and vermifuge (Chopra *et al.*, 1956; Gali-Muhtasib *et al.*, 2006). Black cumin seed or black seed is referred by the prophet Mohammed as having healing powers according to his word reported by Bukkori and Musilm, “use the Black seed, because it contains a cure for every type of aliment, except for death” (Al-Jauziyah, 2011). The seed is normally eaten alone or in the combination with honey and also used as the ingredient in many food recipes.

Figure 14 *Nigella sativa* L.

(Source:https://www.flickr.com/photos/14646075@N03/7427890162/in/photostream. Accessed 7 December 2014)

Figure 15 *Nigella Sativa* seed

(Source: http://ambrosianantucket.com/nigella-seeds. Accessed 7 December 2014)

2.7.2 Chemical constituents and active principles in *Nigella sativa* seeds

Nigella sativa seeds contain fixed oil (35.6– 41.6%), volatile oil (0.5–1.6%) and proteins (22.7%) (Gali-Muhtasib *et al.*, 2006). The seeds also contain amino acids, reducing sugars, mucilage, alkaloids, organic acids (al-Gaby, 1998), crude fiber, minerals (such as calcium, iron, sodium and potassium (Gali-Muhtasib *et al.*, 2006), vitamins (Takruri and Dameh, 1998), fatty acids, free sterols, steryl esters, steryl glucosides and acylated steryl glucosides (Menounos *et al.*, 1986; Mohamed Fawzy Ramadan and Mörsel, 2003). In addition, other compounds such as Terpenoids, Alkaloid (nigellicine), isoquinoline alkaloid (nigellimine) and an indazole alkaloid (nigellidine), and saponins are also found in *Nigella sativa* seeds (Gali-Muhtasib *et al.*, 2006).

Black cumin seed volatile oil (Essential oil) contains monoterpenes such as *p*-cymene, γ-terpinene, α-pinene, ß-pinene, α-thujene, carvacrol and thymoquinone. However, the most portion compounds found in volatile oil are *para*-cymene (32.0-37.3%) and thymoquinone (13.7-23.3%) (Botnick *et al.*, 2012; Tauseef Sultan, 2009).

The active components of the seeds comprise the volatile oil consisting of carvone, an unsaturated ketone, carvene, a-pinene and *p*-cymene. The crystalline active constituent which is the only one of the carbonyl fraction of the oil is nigellone. Pharmacologically active principles of volatile oil are thymoquinone, dithymoquinone, thymohydroquinone and thymol; however, the pharmacological properties of *Nigella sativa* are related principally to a major active compound named thymoquinone (Figure 16) which found mainly in seed oil (Ghosheh *et al.*, 1999).

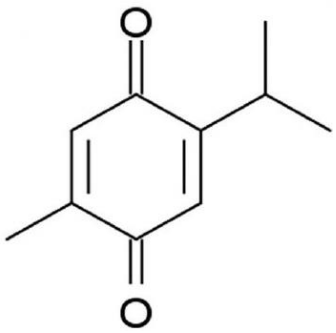

Figure 16 Thymoquinone (2-Isopropyl-5-methyl-1,4-benzoquinone)
(Abukhader, 2013)

Fixed oil contains high proportion of unsaturated fatty acid. The dominate fatty acid are linoleic (57.4-60.8%), oleic (19.7-21.9%) and palmitic acids (11.4-12.1%) (Nergiz and Ötleş, 1993; Tauseef Sultan, 2009). Black cumin fixed oil contains tocopherols and polyphenols. Total tocopherols contain in fixed oil is accounted for 340 µg/g including α-Tocopherol, ß-Tocopherol and γ- Tocopherol (40, 50 and 250

μg/g respectively) (Tauseef Sultan, 2009) . Total polyphenol content in fixed oil was reported at 1744 ug/g oil (Tauseef Sultan, 2009). Thymoquinone is also found in the fixed oil which accounted for 201.31 mg/kg oil (Tauseef Sultan, 2009).

In addition, black cumin seedcake, a residue from oil extraction, also has antioxidant effects. Crude methanolic extract (CME) and its fractions using ethyl acetate (EAF), hexane (HF) and water (WF) of black cumin seedcake also have antioxidant activities. The total phenolic compounds in EAF, CME, WF and HF were found to be 78.8, 27.8, 32.1 and 12.1 mg gallic acid equivalent (GAE)/g in, respectively. The DPPH IC_{50} of EAF, CME, WF and HF were 1.89, 2.26, 2.17 and 2.65 mg/ml respectively (Mariod *et al.*, 2009). The principal phenolic compounds found in crude methanolic extract and water fractions of black cumin seedcake were hydroxybenzoic, syringic and *p*-cumaric acids (Mariod *et al.*, 2009).

2.7.3 Therapeutic effect of *Nigella sativa* and its beneficial effects on diabetes mellitus

Several biological and pharmacological activities of *Nigella sativa* seeds have been reported, including antioxidant effect (Burits and Bucar, 2000; M. F. Ramadan *et al.*, 2003; Suboh *et al.*, 2004), antimicrobial effect (Ferdous *et al.*, 1992; Hanafy and Hatem, 1991), anti-inflammation and immunomodulation (Al-Ghamdi, 2001; Houghton *et al.*, 1995), anticancer (Dilshad *et al.*, 2012; Khan *et al.*, 2011; Woo *et al.*, 2012) as well as the other beneficial effects on metabolic syndromes including hyperglycemia (Losso *et al.*, 2011; Parildar *et al.*, 2011; Woo *et al.*, 2012), dyslipidemia, (Gali-Muhtasib *et al.*, 2006; Sabzghabaee *et al.*, 2012), and cardiovascular disease (Gali-Muhtasib *et al.*, 2006) as demonstrated in figure 17.

Beneficial effects of *Nigella sativa* on diabetes mellitus have been investigated in animal model, clinical trial and *in vitro* studies. In animal study, thymoquinone, a major active compound in black cumin seed volatile oil, can decrease blood glucose and glycated hemoglobin in streptozotocin-induced diabetic hamsters after treatment at a dose of 50mg/kg body weight/day for 4 weeks (Fararh *et al.*, 2005). The anti-diabetic action of thymoquinone is mediated through a decrease in hepatic gluconeogenesis and changes in hepatic enzyme activities (Fararh *et al.*, 2005; Leelavinothan Pari and Chandrasekaran Sankaranarayanan, 2009). Moreover, the amount of 0.2 g/kg of dry aqueous extract of *Nigella sativa* (2 g/kg of original seed power) has been reported to improve glucose tolerant in rat through an inhibition of glucose absorption (Meddah *et al.*, 2009).

Nigella Sativa seed oil, aqueous extract, and thymoquinone have been reported to decrease oxidative stress resulting in preservation of pancreatic ß cell integrity which thereby increased insulin level (Abdelmeguid *et al.*, 2010). In addition, other mechanisms on the decreased blood glucose of *Nigella Sativa* extract are insulin-sensitizing action by activation of AMPK Pathway in the liver and enhancing muscle Glut4 synthesis and translocation (Benhaddou-Andaloussi *et al.*, 2011).

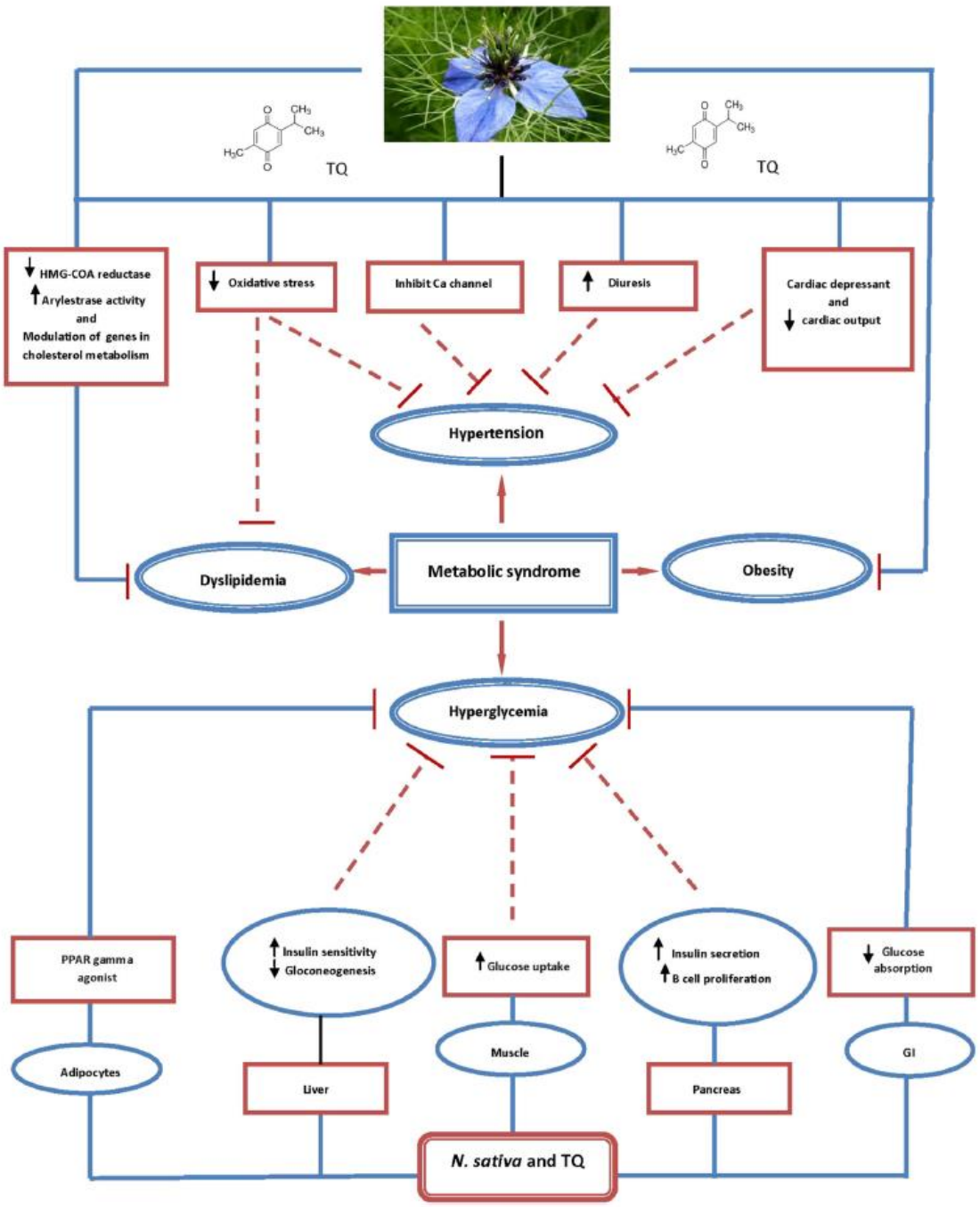

Figure 17 Schematic description for protective effects of *Nigella sativa* and thymoquinone (TQ) in different components of metabolic syndrome.

(Razavi and Hosseinzadeh, 2014)

For the clinical studies in human with metabolic syndrome, black cumin has been reported to be safe and efficacy. Supplementation with 2.5 ml of *Nigella Sativa* oil twice daily addition to normal medication for 6 weeks led to decrease in fasting blood glucose, cholesterol and LDL (Abdelmeguid *et al.*, 2010). The recommended dose of 500 mg in addition to daily drugs for 8 weeks decreased fasting blood glucose, postprandial blood glucose and HbA1c (Ahmad Najmi *et al.*, 2012).

For the inhibitory effect of black cumin extract or thymoquinone on glycation, thymoquinone, a major quinone from black cumin seed, has been reported to have the inhibitory effect on the formation of advance glycation end products (AGEs) in hemoglobin-**δ**-gluconolactone, human serum albumin–glucose and N-acetyl-glycyl-lysine methyl ester–ribose assays. Thymoquinone at 20 μM inhibited 39% of hemoglobin glycation and 82% of post-Amadori glycation products, reduced methyglyoxal- mediated human serum albumin glycation by 68%, and inhibited 78% of late glycation end products. Moreover, thymoquinone has been found to be more effective than 10 mM of aminoguanidine for inhibiting the glycation process (Losso *et al.*, 2011).

2.7.4 Safe and toxicity

Black cumin seeds have been recorded by USDA in Generally Recognized As Safe (GRAS). The acute toxicity of black cumin oil was investigated in mice; the results have showed that the LD_{50} values of black cumin oil were 28.8 ml/kg body weight for oral administration and 2.06 ml/kg body weight for intraperitoneal administration. Administration of black cumin oil in rat at a dose of 2 ml/kg body weight orally for 12 weeks did not change the key hepatic enzymes levels (Zaoui *et al.*, 2002). Giving diets which contained 4.0% of black cumin fixed oil and another formula contained 0.30% black cumin essential oil to normal Sprague dawley rats for

8 weeks indicated that these oil was safe evidenced by normal in liver and kidney functioning tests, serum protein profile, level of cardiac enzymes, and electrolytes balance (Tauseef Sultan *et al.*, 2009).

In addition, a clinical study in safety of thymoquinone in the patients with solid tumors or hematological malignancies demonstrated that the patient tolerated thymoquinone at a dose ranging of 75 to 2,600 mg/day, neither therapeutic responses nor toxicities were reported (Al-Amri A, 2009).

2.8 Analysis of aminophospholipid glycation

2.8.1 Several techniques for analysis of aminophospholipid glycation products.

i Fluorimetric technique

ELISA technique using fluorimeter for analysis was used for detection of fluorescent chromophores AGEs-lipids (Exciting 360 nm, Emitting 440 nm) in system of PE or LDL incubated with glucose, and in LDL specimens isolated from diabetic patient. (Bucala et al., 1993)

ii Chromatographic technique

Gas chromatography-mass spectrometry (GC/MS) was used for analysis of carboxymethylethanolamine (CME), the hydrolysis product of carboxymethylphosphatidylethanolamine (CM-PE), an AEG-PE, in lipid extracts of red blood cell (Requena *et al.*, 1997). CME, a marker of CM-PE, was also be detected in liver mitochondria from mammalian species using a selected-ion-monitoring/gas-chromatography/mass-spectrometry assay (SIM- GC-MS) (Pamplona *et al.*, 1998). This technique also was used for quantification of *N*-(Glucitol) ethanolamine and *N*-

(Carboxymethyl) serine, products of reduction and hydrolysis of glycated phosphatidylethanolamine, and products of oxidation of phosphatidyl serine (PS) (Fountain *et al.*, 1999).

Glycated-PE from the incubation of egg-yolk PE and glucose was analysed by Thin layer chromatography (TLC) and purified by high-performance liquid chromatography with UV diode arrey detector (HPLC-DAD), and then was identitified by Fuorier transform-nuclear magnetic resonance spectrum (FT-NMR). The isolated glycated-PE was indicated as Deoxy-*D*-fructosyl PE (an aminoketose structure), an Amadori product of PE. The authors also isolated and identified glycated-PE from human blood plasma and red blood cells using a two-stage normal- and reversed-phase high-performance liquid chromatography (HPLC) procedure. (Lertsiri *et al.*, 1998).

Identification of pyrrolecarbaldehyde as an AGE from model reactions of PE with D-glucose and 3-deoxyglucosone thanks to using chromatographic and spectroscopic technique by GLC±MS, and using HPLC with diode array detection (DAD) for purification (Lederer and Baumann, 2000). Synthesized Amadori-PE from incubation of Dioleoyl PE with glucose was purified and isolated using solid phase extraction followed by HPLC to be used as standards. For the detection of Amadori-PE presenting in sample, carbonyl group of Amadori-PE was ultraviolet (UV)-labeled with 3-methyl-2-benzothiazolinone hydrazone, and the labeled Amadori-PE was analyzed with normal phase HPLC with UV detector (at 318 nm) (J. H. Oak *et al.*, 2002).

iii Mass spectrometric technique

The identification and quantification methods for phospholipid-linked glycation products mostly contained several processes, including derivatization process, and provide monitoring of one single compound only; therefore, structural characterization by LCMS method have been developed for the determination of such products.

In 2005, an Amadori product of phosphatidylethanolamine (deoxy-D-fructosyl phosphatidylethanolamine, or Amadori-PE) was identified by LC online with hybrid quadrupole/linear ion-trap mass spectrometry (QTRAP LC/MS/MS) (T. Miyazawa *et al.*, 2005; Nakagawa *et al.*, 2005). QTRAP (tandem) mass spectrometer had been developed for detection of detect Amadori-PE of a lipid extract from diabetic plasma and detection of AGE-PE (CM-PE and CE-PE) and Amadori-PE in human red blood cell and blood plasma from healthy subjects and diabetic patients; the neutral loss scanning (NLS) and multiple reaction monitoring (MRM) were used (Nakagawa *et al.*, 2005; Shoji *et al.*, 2010). Liquid chromatography electrospray ionization (ESI) mass spectrometry and ESI-tandem mass spectrometry in both positive and negative modes were used for detecting and identifying products from oxidation of glycated-PE (Simoes *et al.*, 2010). Apart from tandem mass spectrometry, liquid chromatography-evaporative light scattering detection-mass spectrometry (LC-ELSD-MS) was another MS technique used for detecting Schiff-PE and Amadori-PE *in vitro* (Higuchi *et al.*, 2006).

2.8.2 MS/MS mass spectrometric technique for analysis of aminophospholipid glycation products.

i Basic principle of mass spectrometry technique

A mass spectrometry is an analytical technique that determines the molecular weight of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). ("Mass Spectrometry,")The process in the mass spectrometric analysis of compounds is start with the production of gas phase ions of the compound, the ions are generated by inducing ether the loss or gain of a charge. This molecular ion undergoes fragmentation. Then the ions are separated according to their m/z, and are detected in proportion to their abundance. Finally, a mass spectrum of the molecule is produced displaying in the form of a plot between ion abundance and m/z. Ions provide information concerning the nature and the structure of their precursor molecule. The molecular ion of pure compound appears at the highest value of m/z in the spectrum showing the molecular mass of the compound.

Basic components of a mass spectrometer are illustrated in figure 18. When sample is introduced, using sample introduction such as GC and HPLC or direct injection, into the instrument, it will be ionized in the ion source. The charge molecules are then electrostatically moved into the mass analyzer where the ions will be separated according to their mass-to-charge ratio (m/z). After detecting the ions and recording the relative abundance of each of the resolved ionic species by ion detector, the detector signal is transfer to a computer for process and express output information. (Siuzdak, 1996b)

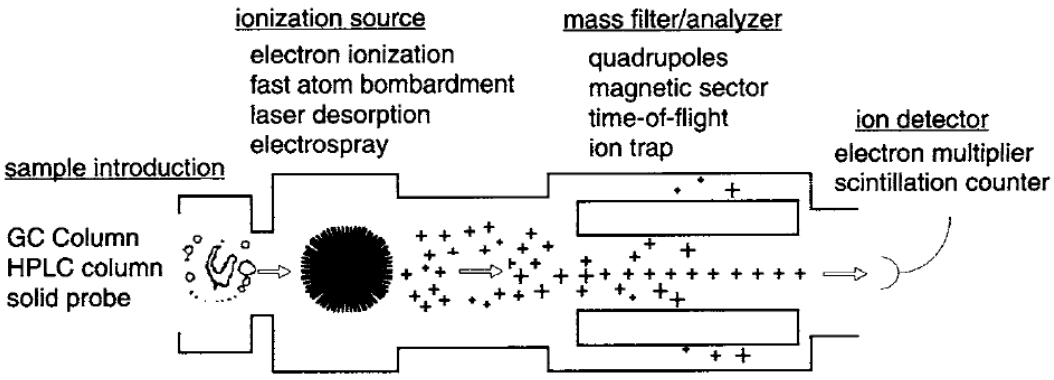

Figure 18 Components of a mass spectrometer

(Siuzdak, 1996b)

ii Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry (MS^n - n refers to the number of generations of fragment ions being analyzed) is a technique allowing for the production and identification of fragment ions from a selected ion. Fragmentation can be achieved by inducing ion-molecule collisions by process called as collision-induced dissociation (CID). CID is accomplished by selecting an interested “parent (precursor) ion” with a mass analyzer/filter and introducing that ion into a collision cell, where the selected ion collides with the argon or nitrogen atoms, resulting in fragmentation. The fragments can be analyzed to obtain a “daughter ion” spectrum. Tandem mass analysis is primary used to obtain structural information.

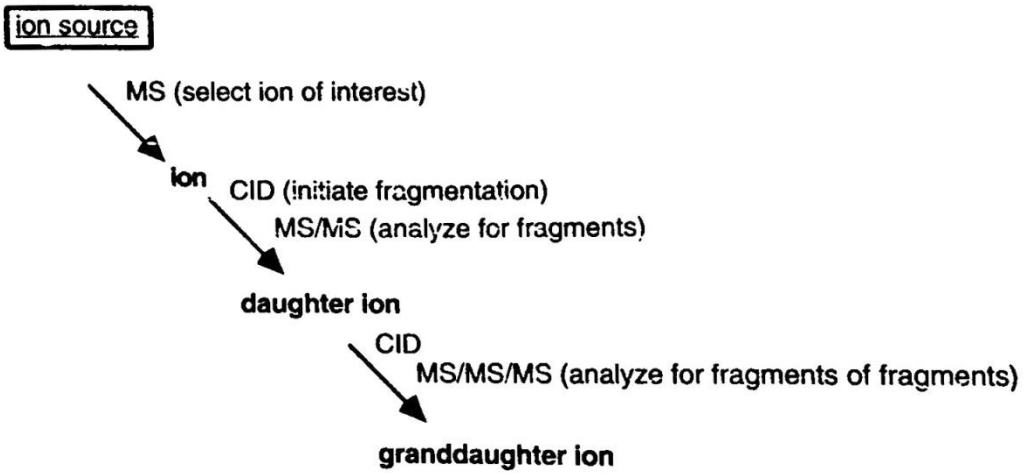

Figure 19 Tandem mass spectrometry, involving generation of fragment ions via CID and the mass analyzer (MS^n) of the progeny fragment ions.

This terms patrent, daughter, and granddaughter ions or precursor, product, and second-generation product ion have been commonly used. (Siuzdak, 1996a)

iii MS/MS analysis for glycated-PE and AGE-PE

Identification of Amadori-PE and AGE-PE using liquid chromatography coupled with mass spectrometry (LC-MS) has been reported. Recently, a MS/MS analysis method of Amadori-PE using a quadrupole/ linear ion-trap mass spectrometer (QTRAP) has been developed. Interfacing liquid chromatography with QTRAP mass spectrometry enabled the separation and determination of glycated-PE species. The MS studies on glycated-PE have focused on fragmentation of glycated-PE in order to identify the characteristic fragment ions in both positive and negative mode. For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the product ion scan, neutral loss scan (NLS), and multiple reactions monitoring (MRM) were used for identification of Amadori-PE (Figure 20). According to spectrum from product ion scan mode, loss of glycated polar head group (-303) was identified as the major

fragment ion in positive mode; a diglyceride ion ($[M+H-303]^+$ generated from collision-induced dissociation of Amadori-PE allowed for neutral loss scanning (NLS) and multiple reaction monitoring (MRM). Therefore, for more specific detection of glycated-PE or Amadori-PE, NLS of 303 in positive mode was used , and the result showed a specific mass spectrum of glycated-PE or Amadori-PE (for example, m/z 906 of 18:1-18:1 Amadori-PE). In the same way, NLS of 141 was a specific detection for nonglycated PE (native-PE) showing a mass spectrum of nonglycated PE (for example, m/z 744 of 18:1-18:1 PE).

Beside the LC-MS/MS analysis, the lipid extract from sample was also directly infused into the QTRAP mass spectrometer without chromatographic separation, called as direct infusion (Nakagawa *et al.*, 2005). For MS/MS analysis, Neutral loss scaning (NLS) was performed to evaluate Amadori-PE and PE molecular species by scanning parent ion that yielded a neutral loss of 303 Da for Amadori-PE or of 141 Da for native PE molecular species (Figure 20). Moreover, the specific detection of AGE-PE had been also developed; the NLS of 199 Da and 213 Da were used for profiling of CM-PE and CE-PE respectively (Figure 21) (Shoji *et al.*, 2010).

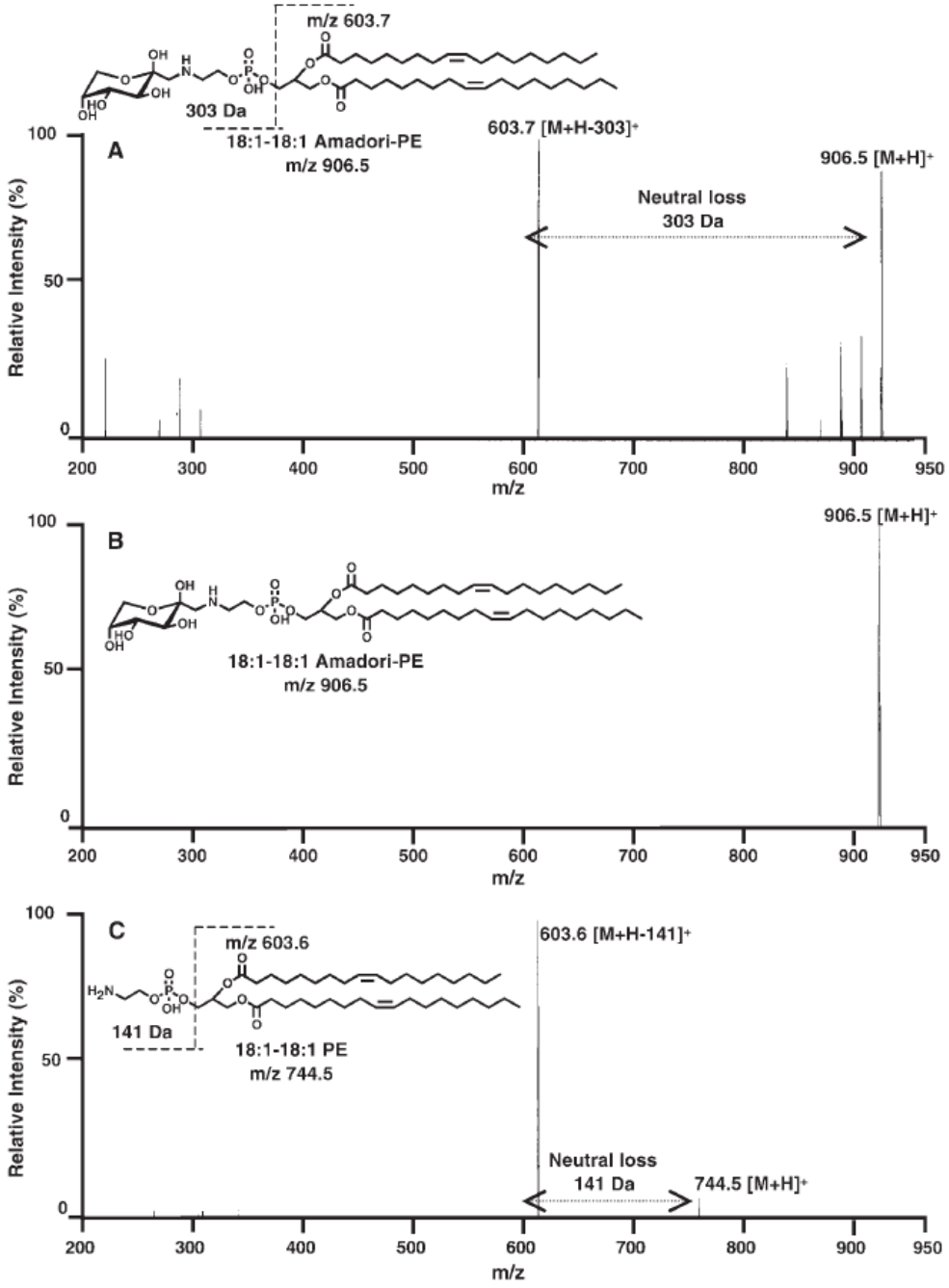

Figure 20 Specific detection of Amadori-PE using the QTRAP mass spectrometer. A: Product ion spectrum of the 18:1-18:1 Amadori-PE (*m/z* 906 $[M+H]^+$). B: Neutral loss scan for 303 Da showing specific detection of 18:1-18:1 Amadori-PE. C: Product ion spectrum of nonglycated PE (18:1-18:1 PE, *m/z* 744 $[M+H]^+$). (Nakagawa *et al.*, 2005)

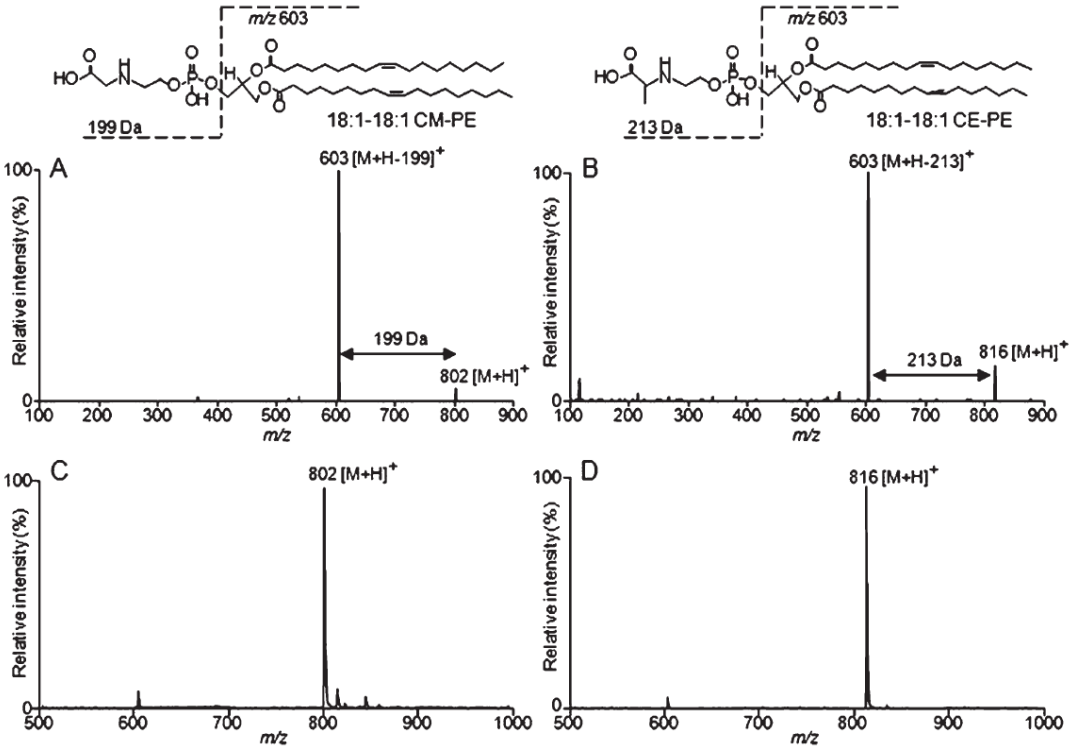

Figure 21 Specific detection of AGE-PE.

Product ion spectrum of the $[M+H]^+$ ion at m/z 802 of 18:1-18:1 CM-PE (A) and m/z 816 of 18:1-18:1 CE-PE (B). Neutral loss scan for 199 Da and 213 showing specific detection of 18:1-18:1 CM-PE (C) and CE-PE (D), respectively. (Shoji *et al.*, 2010)

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.3.1 Chemicals and Materials

i Black cumin seed (*Nigella Sativa* L., India)

ii 1,2-Dioleoyl-sn-glycerol-3-phosphoethanolamine (Sigma-Aldrich, St. Louis, MO, USA)

iii 1,2-Dimyristoyl-sn-glycerol-3-phosphoethanolamine (Sigma-Aldrich, St. Louis, MO, USA)

iv Thymoquinone (Sigma-Aldrich, St. Louis, MO, USA)

v Aminoguanidine (Sigma-Aldrich, St. Louis, MO, USA)

vi D-glucose(Sigma-Aldrich, St. Louis, MO, USA)

vii Sodium Chloride (Sigma-Aldrich, St. Louis, MO, USA)

viii Potassium Chloride (Sigma-Aldrich, St. Louis, MO, USA)

ix Sodium Phosphate, Dibasic (Sigma-Aldrich, St. Louis, MO, USA)

x Potassium Phosphate, Monobasic (Sigma-Aldrich, St. Louis, MO, USA)

xi Methanol (LC grade and analytical grade) (Merck, Darmstadt, Germany)

xii Dichloromethane (LC grade and analytical grade) (Merck, Darmstadt, Germany)

xiii Dimethyl sulfoxide (Merck, Darmstadt, Germany)

xiv Ethanol (analytical grade) (Merck, Darmstadt, Germany)

xv Formic acid (Merck, Darmstadt, Germany)

xvi Ammonia (Merck, Darmstadt, Germany)

xvii Ultrapure water produced from the Millipore Milli-Q purification system (Bedford, MA, USA)

xviii Nitrogen gas

3.1.2 Instruments and Equipment

i **Rotavapor® R-3** Rotary evaporator (BUCHI Labortechnik AG, Switzerland)

ii Stuart® Block heater-sample concentrator (Bibby Scientific Limited, UK)

iii Rocker 300 Oil free vacuum pump (Rocker Scientific, Taiwan)

iv Shimadzu LC-20A HPLC (Shimadzu, Japan)

v VorTemp™ 56 Shaking Incubators (Labnet International Inc, USA)

vi Agilent 1200 High-performance Liquid Chromatrography System (Agilent Technologies, Germany) equipped with the QTRAP® 5500 mass spectrometer (AB SCIEX, CA, USA)

vii Himac CF 7D2 centrifuge (Hitachi, Tokyo, Japan)

viii Eppendorf centrifuge 5430R (Eppendorf AG, Hamburg, Germany)

ix BioTek Powerwave 340 Microplate spectrophotometer (BioTek Instruments, Ltd., Winooski, VT, USA)

x Sartorius TE313S electronic balance (d = 0.001) (Sartorius, Gottingen, Germany)

xi Sartorius CP224S electronic balance (d = 0.0001) (Sartorius, Gottingen, Germany)

xii S220 SevenCompact™ pH/Ion meters (Mettler Toledo, Switzerland)

xiii Ultrasonic bath (Crest Ultrasonics, USA)

xiv Electric food grinder

xv Screw cap tube

xvi Glassware

xvii micro tube

xviii vial

xix Micro plate

xx Automatic pipette

xxi Filter paper (whatman no.1)

xxii Ethylenediaminetetraacetate (EDTA)-containing tube

3.2 Method

3.2.1 Preparation of *Nigella Sativa* seed extract (NSE)

(Farah, 2005 with modification)

i Black cumin seed was grinded and of which 20 grams were mixed with 200 ml of 95% ethanol.

ii The mixture was placed at 4°C in refrigerator overnight.

iii The mixture was filtered through a filtering paper (WhatmanNo. 1) by vacuum system.

iv The solvent was removed by rotary evaporator (at 40°C, pressure not more than 100 mBar)

v Dried residue from the extract was collected under nitrogen stream and then stored at 4°C.

vi Percentage of yield of the extract was calculated and thymoquinone content was measured.

3.2.2 Determination of thymoquinone in NSE by HPLC

(Ghosheh *et al.*, 1999 with modification)

i Ten µl of NSE was dissolved with 90 µl of DMSO and further diluted with methanol to make a desired concentration.

ii The samples was injected to reverse phase HPLC with isocratic elution and the following conditions:

iii Colume: Inertsil C18 (4.6 i.d. x 250 x 5 µm, Japan)

iv Injection volume: 20 µl

v Mobile phase: methanol and water (80:20, v/v)

vi Flowrate: 1.0 ml/min

vii Colum oven: 32°C

viii Detector: UV 254 nm

ix Thymoquinone concentration in NSE was calculated using the standard curves of peak areas (0.1, 1, 10, 50 and 100 µg/ml thymoquinone) and expressed in µg/ml

3.2.3 *In vitro* study of inhibitory effect of *Nigella Sativa* seed extract (NSE) on glycation of commercial PE standard (Dioleoly-PE, DOPE)

i Preparation of glycation of commercial PE standard (Dioleoly-PE, DOPE) in vitro (Shoji et al., 2010)

(1) 0.1M Phosphate buffer solution (PBS) (pH 7.4), Stock solution of 3 mM DOPE in Methanol, 2.5 M D-glucose in PBS, 50 mg/ml Aminoguanidine in PBS and 400 mg/ml NSE in Dimethyl sulfoxide (DMSO) were prepared.

(2) Dioleoly-PE (0.3mM) was mixed with D-glucose (500mM) in 70% methanolic phosphate buffer in micro tube. Test compounds, which were aminoguanidine (AG) (5 mg/ml) or Nigella sativa extracts (NSE) (5, 10, 20, 40 mg/ml) were mixed to the mixture, total volume was 1 ml and each treatment was done in triplicate. Treatments were as follows:

Blank control:	DOPE
Negative control:	DOPE+ glucose
Positive control:	DOPE+ glucose+ AG (5mg/ml)
Samples:	DOPE+ glucose+ NSE (5, 10, 20, 40 mg/ml)

(3) Treatments were incubated in shacking incubator at 37°C for 48 h.

(4) After incubation, treatments were extracted for lipid analysis.

ii Lipid extraction

(Bligh and Dyer, 1959)

(1) 0.8 ml of sample was transferred to the screw cap tube and 200 µl of 1 mM DMPE solution was then added as the internal standard.

(2) Three ml of dichloromethane and methanol (1:2 v/v) was added and the mixture was vigorously mixed.

(3) 1 ml of Dichloromethane was then added and followed by 1 ml of ultrapure water.

(4) The mixture was then mixed and centrifuged at 2,000 rpm, 4°C for 10 minutes.

(5) The lower dichloromethane phase that contained lipid was collected and of which 0.5 ml was transferred to a new tube.

(6) Lipid was dried under nitrogen stream at 40°C by using sample concentrator.

(7) The dried lipid extract was kept at -20°C until analysis.

iii Flow injection-mass spectrometric analysis of glycated-PE and AGE-PE

An Agilent HPLC 1290 system (Agilent, Palo, CA, USA) coupled with quadrupole/ linear ion-trap mass spectrometer (QTRAP) (5500 QTRAP mass spectrometer, AB SCIEX, CA, USA) was used for Glycated-PE and AGE-PE analysis.

(1) Dried lipid extract was reconstituted with 1 ml of dichloromethane and methanol (2:1, v/v).

(2) The mobile phase that composed of methanol/water/formic acid/ammonia (80:19.7:0.1:0.2 v/v/v/v) was delivered to the system at flow rate of 0.4 ml/minute by an Agilent 1290 Infinity Binary Pump (Palo Alto, CA, USA).

(3) Twenty µl of sample was introduced to QTRAP mass spectrometer by flow injection (triplicate per sample). Neutral loss scan (NLS) was used for qualitative analysis of native-PE and glycated-PE. The MS/MS instrument was programmed to scan parent ions that yield these following products (Nakagawa *et al.*, 2005; Shoji *et al.*, 2010):

Table 2 Neutral loss scan (NLS) mode used for detection of products of Dioleoly-PE (18:1-18:1-PE) glycation

Mode of NLS	Products	Specific mass detection
141 Da	native PE	744
303 Da	glycated-PE	906
199 Da	CM-PE (AGE-PE)	802
213 Da	CE-PE (AGE-PE)	816

CM-PE: carboxymethyl-phosphatidylethanolamines; CE-PE: carboxyethyl-phosphatidylethanolamines

Mass spectrometric condition was set as following:

a. Ion source: Turbo Spray

b. Declustering Potential: 80 eV,

c. Collision energy: 30eV

d. Scan rate: 2,000 Da/s

e. Turbo gas temperature: 500°C

f. Spray voltage: 5,500 volts in positive mode

g. Nitrogen pressure values for nebulizer, dried and curtain gas: 40, 50, and 20 psi, respectively.

(4) The data acquisition and analysis were performed by Analyst software version 1.6.

(5) Mass peak areas of each PE species were corrected for overlap of C13 isotope peak.

(6) The mass peak areas were used to calculate % glycated-PE, % CM-PE and % CE-PE which expressed as the percentage relative to native PE species, as the following equation.

$$\% \text{ of each PE molecular species} = \frac{\text{Mass peak area} \times 100}{\text{Mass peak area of Native PE}}$$

3.2.4 *In vitro* study of inhibitory effect of thymoquinone (TQ) on glycation of commercial PE standard (Dioleoly-PE, DOPE)

i Stock solution of 100 mg/ml thymoquinone was prepared in methanol.

ii Dioleoly-PE (0.3mM) was mixed with D-glucose (500mM) in 70% methanolic phosphate buffer (0.1 M, pH 7.4) in the absence or presence of test compound which were aminoguanidine (5 mg/ml) and thymoquinone at 0.5, 1, 2.5, 5 and 10 mg/ml. Total volume was 1 ml and each treatment was done in triplicate. Treatments were as follows:

Blank control:	DOPE
Negative control:	DOPE+ glucose
Positive control:	DOPE+ glucose+ AG (5mg/ml)
Samples:	DOPE+ glucose+ thymoquinone (0.5, 1, 2.5, 5 and 10 mg/ml)

iii Treatments were incubated in shacking incubator at 37°C for 48 h.

iv After incubation, lipid was extracted from each treatment (as described in 3.1.3.ii)

v Dried lipid was then analyzed for native-PE, glycated-PE, CM-PE, CE-PE and thymoquinone-phosphatidylethanolamine adduct (TQ-PE adduct) by flow injection-mass spectrometric method (as described in 3.3). NLS of 287 was used and scan rate was set to 200 Da/s for TQ-PE adduct.

vi The percentages of glycated-PE, CM-PE, CE-PE and TQ-PE adduct were calculated and expressed as a percentage relative to native PE species. The dose-response relationship between the concentration of thymoquinone and products of PE glycation was also investigated.

3.2.5 Study of inhibitory effect of *Nigella Sativa* seed extract (NSE) on the glycation of human erythrocyte PE

i Study design

Human venous blood samples from 10 healthy volunteers were used as biological materials for *in vitro* model of PE glycation. Washed packed erythrocyte from 10 healthy volunteers (10 replicates) was incubated in phosphate buffer saline (Hematocrit during incubation is 40%) with glucose (100 mM) and with 50, 100, 200 ug/ml of NSE. PBS and aminoguanidine (20 mM) was used instead of NSE for blank control and positive control respectively. The mixture was gently shaken in the incubator at 37°C for 48 h. After incubation, lipid was extracted from erythrocyte according Bligh and Dyer method. The products of PE glycation in erythrocyte were investigated by direct injection-mass spectrometric method.

ii Subject

The sample size was set according to the maximal number used in previous red blood cell experiment (Nandhini and Anuradha, 2003; Resmi *et al.*, 2005; Riquelme *et al.*, 2005). Ten healthy volunteers were recruited according to the following criteria;

Inclusion criteria

1. Healthy male or female age 20–50 years old
2. Non-obese (BMI <23 kg/m^2)
3. Non-smokers
4. non-alcoholism
5. Normal blood pressure

6. Blood glucose <110 mg/dL
7. Normal plasma lipid profile including total cholesterol < 200 mg/d, triglyceride < 150 mg/dl, and LDL< 130 mg/dl
8. Free of any medication, drugs or dietary supplementation which affect blood lipids within 3 month

Exclusion criteria

1. Participating in other research project that has food/ drug intervention which can affects lipid profile or blood glucose.
2. Abnormal liver function (AST and ALT test) or renal function (BUN and creatinine test)
3. Abnormality in red blood cell (abnormal result in complete blood count test)

The project and inform consents were reviewed and approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Sciences Group, Chulalongkorn University (ECCU) (Approved No. 064/2014). The informed consents were obtained from all subjects after explanation details of this study.

Subjects were asked to maintain their lifestyle and dietary patterns for one week before blood collection; the habitual nutrient intakes of the subjects were estimated using 3-day food records. After 12 hours fasting, blood was collected from the subjects.

iii Erythrocyte preparation

(1) Fasting blood samples were collected into ethylenediamine tetraacetate (EDTA)-containing tube from 10 healthy participants and kept at 4°C until analysis.

(2) The blood samples were centrifuged at 3,000 rpm for 10 min at 4°C using Himac CF 7D2 centrifuge (Hitachi, Tokyo, Japan).

(3) Plasma and buffy coat were removed and the erythrocyte was washed three times with ice-cold 0.15 M (0.9%) sodium chloride solution. Washed packed erythrocyte (red blood cell, RBC) was then stored at 4°C.

iv *In vitro* glycation of erythrocyte PE

(1) Phosphate buffer solution (PBS) pH 7.4 was prepared (NaCl 8.1 g, Na_2HPO_4 2.302g, and NaH_2PO_4 0.194 g in 1 lit). Stock solution of 1 M D-glucose in PBS and 2 M Aminoguanidine in PBS were prepared.

(2) Stock solution of 200 mg/ml NSE in Dimethyl sulfoxide (DMSO) was prepared and then further diluted using PBS to make working solution 20, 10, and 5 mg/ml NSE. The final concentration of DMSO in each tube of treatments was less than 0.1%.

(3) The RBC (400 µl) was mixed with 100 µl of D-glucose (final concentration=100mM) in PBS in micro tube. Ten µl of test compounds, aminoguanidine (AG) or *Nigella sativa* extracts (NSE), were mixed to the mixture, total volume was 1 ml. Treatments were as follows:

Blank control:	RBC + PBS
Negative control:	RBC + PBS + glucose (100mM)
Positive control:	RBC + PBS + glucose (100mM) + AG (20 mM)
Sample:	RBC + PBS + glucose (100mM) + NSE (50, 100, and 200 µg/ml)

(4) Because NSE also contain phospholipid, treatments containing only NSE (50, 100, and 200 µg/ml) and PBS were also prepared to be the blank for subtraction of amount of PE after MS/MS analysis.

(5) Treatments were incubated in shacking incubator (Labnet International Inc, USA) (150 rpm) at 37°C for 48 h.

(6) After incubation, treatments were centrifuged (Eppendorf 5430R, Hamburg, Germany) at 1,500 g and 4° C for 10 min. The 30 µl of supernatant was transfered to a new tube for hemolysis measurement, and the rest was used for lipid extraction.

v Hemolysis measurement

After centrifugation, the supernatant was diluted 10 fold with PBS and absorbance of 100 µl of that was determined at 540 nm. Reference values (100% hemolysis) were determined using the same amount of erythrocyte in ultrapure water (400 µl in 1ml). The percentage of hemolysis was calculated using the following equation (Hseu *et al.*, 2002);

$$\% \text{ hemolysis} = \frac{\text{Absorbance of sample supernatant}}{\text{Absorbance of reference value}} \times 100$$

vi Lipid extraction (Bligh and Dyer with modification)

(1) RBC solution (970 µl) was added with 230 µl of ultrapure water and then vigorously mixed for 1 min. The 1.2 ml of sample was transferred to the screw cap tube and 20 µl of 1 mM DMPE solution was then added as the internal standard.
(2) A 4.5 ml of the solution of dichloromethane and methanol (1:2 v/v) was added and the mixture was vigorously mixed for 30 s.
(3) Dichloromethane (1.5 ml) was then added before mixing for 30 s.
(4) The treatment was then added with 1.5 ml of ultrapure water and mixed for 30 s.
(5) The mixture was then centrifuged at 2,000 rpm, 4°C for 10 minutes.
(6) The lower phase that contained lipid was collected and of which 2 ml was transferred to a new tube.
(7) Lipid was dried under nitrogen stream to less volume than 1 ml by using sample concentrator (Stuart®, Bibby Scientific Limited, UK) and then was collected by rinsing with dichloromethane to a vial.
(8) The sample was dried again with nitrogen steam and the dried lipid extract was kept at -20°C until analysis.

vii Mass spectrometric analysis of erythrocyte glycated-PE and AGE-PE.

(1) The dried lipid extract was reconstituted with 500 µl of dichloromethane and methanol (2:1, v/v).
(2) The solution (10 µl) was further mixed with 990 µl of the solvent composed of methanol, dichloromethane, water, formic acid, and ammonia (7.0: 2.0: 0.8: 0.1: 0.1 v/v/v/v/v).

(3) Sample was directly injected to QTRAP mass spectrometer with flow rate of 10 µl/ min.

(4) Neutral loss scan (NLS) was used for analysis of native-PE and glycated-PE. The MS/MS instrument was programmed to scan parent ions that yield these following products (Nakagawa *et al.*, 2005; Shoji *et al.*, 2010):

NLS of 141 for native PE

NLS of 303 for glycated-PE (including Amadori-PE)

NLS of 199 for CM-PE (AGE-PE)

NL of 213 CE-PE (AGE-PE)

MS/MS condition was set as following:

Ion source: Turbo Spray

Declustering Potential: 80 eV,

Collision energy: 30eV

Scan rate: 2,000 Da/s

Turbo gas temperature: 500°C

Spray voltage: 5,500 volts in positive mode

Nitrogen pressure values for nebulizer, dried and curtain gas: 40, 50, and 20 psi, respectively.

(5) Mass peak areas of each following PE species were corrected for overlap of C13 isotope peak.

Table 3 Mass per charge (Da) of Native-PE, glycate-PE, CM-PE, and CE-PE for selected Phosphatidylethanolamine (PE) species in red blood cell membrane lipid

Species	Native-PE	Glycate-PE	CM-PE	CE-PE
PE16:0/18:1	718	880	776	790
PE16:0a/22:6	750	912	808	822
PE18:1a/20:4	752	914	810	824
PE18:0a/20:4	754	916	812	826
PE16:0/22:6	764	926	822	836
PE18:1/20:4	766	928	824	838
PE18:0a/22:6	778	940	836	850
PE18:0/22:6	792	954	850	864

a = alkenyl-acyl species (plasmalogen)

(6) The mass peak areas were used to calculate % glycated-PE, % CM-PE and % CE-PE which expressed as the percentage relative to native PE species, as the following equation.

$$\% \text{ of each PE molecular species} = \frac{\text{Mass peak area} \times 100}{\text{Mass peak area of Native PE}}$$

3.2.6 Statistical analysis

Data are expressed as means ± SEM. The statistical differences among treatments were analyzed by using one-way ANOVA for experiment of DOPE model and repeated measure ANOVA for experiment of erythrocyte. Tukey and Bonferroni post hoc test were used for experiment of DOPE model and experiment of erythrocyte, respectively. The dose-response relationship between the concentration of NSE or TQ and products of PE glycation was analyzed by pearson's correlation analysis. $p<0.05$ was considered statistical significance. SPSS version 17.0 software (Chicago, IL, USA) was used. All experiments except the erythrocyte experiment were performed in triplicate.

CHAPTER IV
RESULTS

4.1 Thymoquinone content in *Nigella Sativa* seed extract (NSE)

The NSE appeared dark yellowish oil. Because the extract was not in the form of dried powder, the stability may change when keeping for long time, the extract was not be used after stored (at 4°C in the dark) for 2 month. Two batchs of extraction were prepared (triplicate) for using in the commercial PE standard and using in the erythrocyte model which had percentage of yield of 24.43 ± 0.50 % and 30.93 ± 1.03 % (w/w), respectively. After getting the extract of each batch, equal volume of each replicates was pooled together in order to be NSE used in the experiment. Thymoquinone contents of two batch of NSE were quantified by HPLC technique. Chromatogram from HPLC analysis of NSE presented in figure 23. Thymoquinone concentration in NSE was showed in table 4.

Figure 22 Ethanolic extract of *Nigella Sativa* seed (NSE)

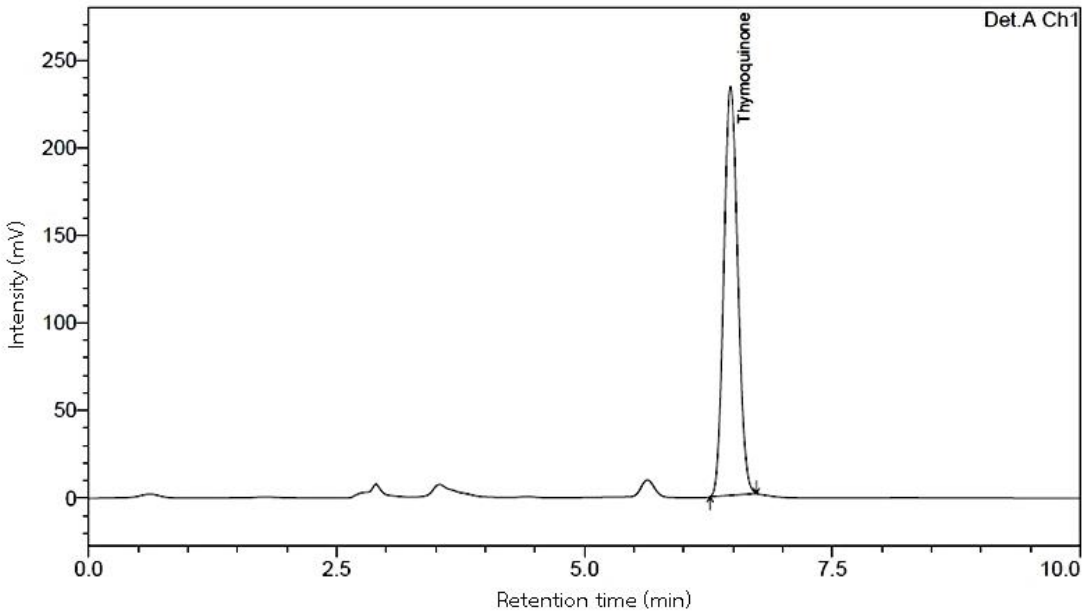

Figure 23 Chromatogram from HPLC analysis of NSE presenting peak of thymoquinone

Table 4 Thymoquinone concentration in *Nigella Sativa* seed extract

	Thymoquinone (µg/ml)*
Commercial PE experiment	7,831.71 ± 346.06
Erythrocyte experiment	6,106.19 ± 313.11

* T-test showed a significant different between two batches of NSE ($p < 0.05$); Data are expressed as mean ± SEM, n=3

4.2 Inhibitory effect of *Nigella Sativa* seed extract (NSE) on glycation of Dioleoly-PE (DOPE)

After incubation of DOPE (0.3 mM) with glucose (500 mM) for 48 hr., the PE glycation products including glycated-PE (m/z= 906.8) and advance glycation end product (AGE-PE) which were carboxymethyl-phosphatidylethanolamines; CM-PE (m/z= 802.8) and carboxyethyl-phosphatidylethanolamines; CE-PE (m/z= 816.8) were found and detected by mass spectrometric technique (figure 24-26).

Efficacy of NSE at 5-40 mg/ml against lipid glycation was tested. The effect of NSE on the formation of glycated-PE which was known as the early PE glycation product was shown in figure 27. The result demonstrated that NSE at the concentration of 5, 10, and 20 mg/ml and also AG (5mg/ml) significantly increased the glycated-PE formation when compared to negative control ($p< 0.05$). Conversely, the higher concentration of NSE (40 mg/ml) significantly reduced the glycated-PE formation when compared to negative control ($p< 0.05$) with 40.04% inhibition.

For CM-PE, the extract at 5, 10, 20 and 40 mg/ml significantly reduced the glycated-PE formation when compared to negative control ($p< 0.05$) (Figure 28). NSE at 5, 10, 20 and 40 mg/ml inhibited CM-PE formation accounted for 47.61, 36.71, 78.28 and 92.86% respectively whereas AG (5 mg/ml) inhibited the product around 19.83%. The results showed that the inhibitory effect of NSE was higher than that of AG when compared at the same concentration (5 mg/ml) ($p< 0.05$).

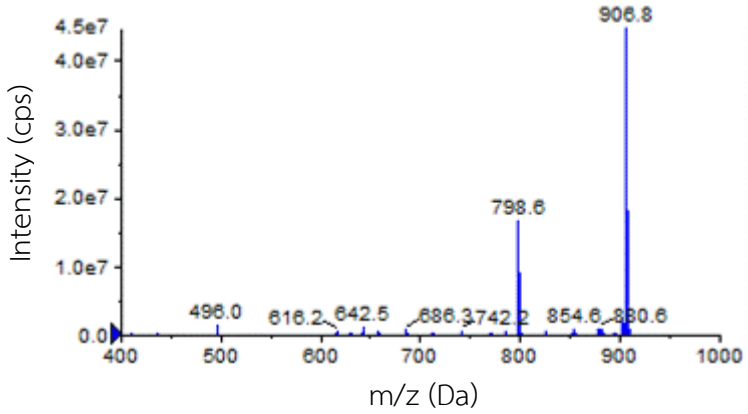

Figure 24 Mass peak of 18:1-18:1 glycated-PE (m/z of 906.8 Da) detected by the specific neutral loss scan of 303 Da

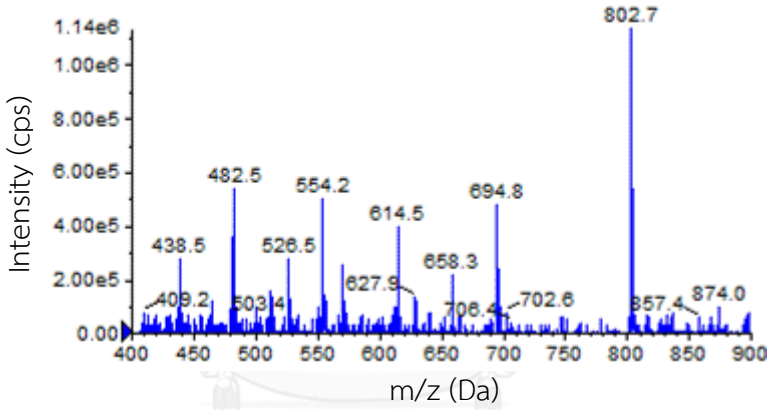

Figure 25 Mass peak of 18:1-18:1 CM-PE (m/z of 802.7 Da) detected by the specific neutral loss scan of 199 Da

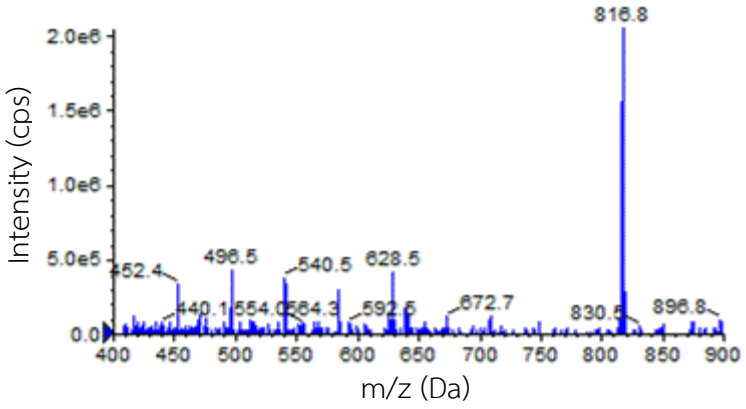

Figure 26 Mass peak of 18:1-18:1 CE-PE (m/z of 816.8 Da) detected by the specific neutral loss scan of 213 Da

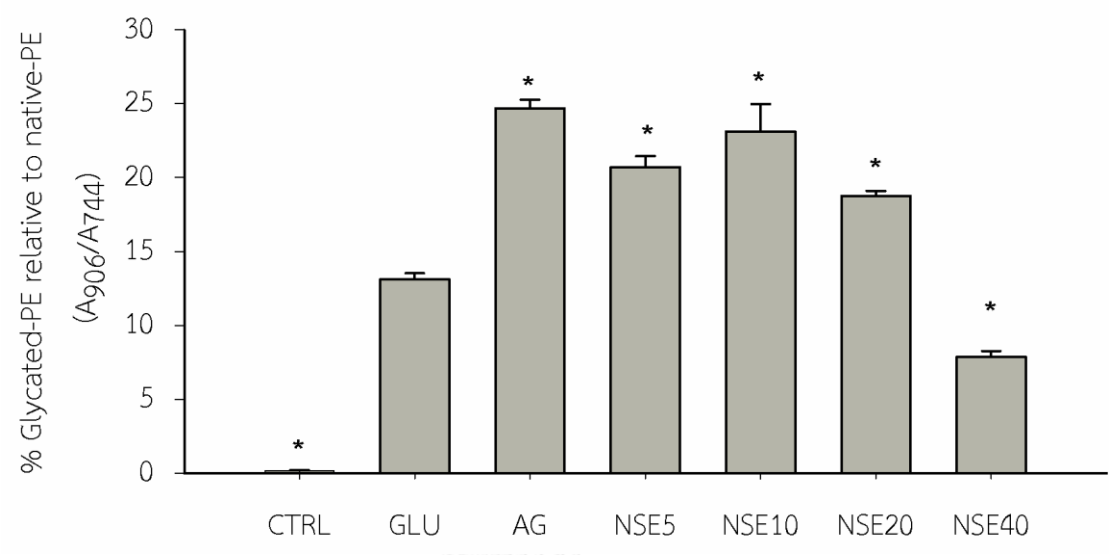

Figure 27 Effect of NSE on formation of glycated-PE.

Data are expressed as mean ± SEM (n=3). CTRL= PE (blank control), GLU=PE+glucose (negative control), AG= PE+glucose+ 5.0 mg/ml of AG (positive control), NSE5, NSE10, NSE20 and NSE40 = PE+ glucose + NSE at 5, 10, 20 and 40 mg/ml, respectively. * Significant difference from negative control ($p< 0.05$).

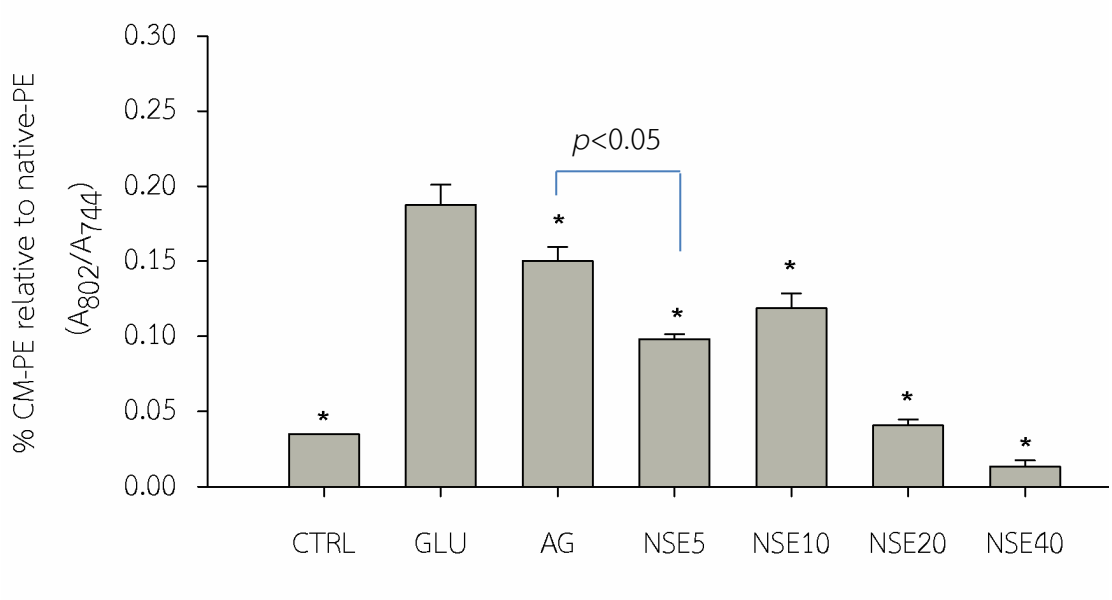

Figure 28 Effect of NSE on formation of carboxymethyl- phosphatidylethanolamines (CM-PE).

Data are expressed as mean ± SEM (n=3). CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE + glucose + 5.0 mg/ml of AG (positive control), NSE5, NSE10, NSE20 and NSE40 =PE+ glucose+ NSE at 5, 10, 20 and 40 mg/ml, respectively). * Significant difference from negative control ($p< 0.05$).

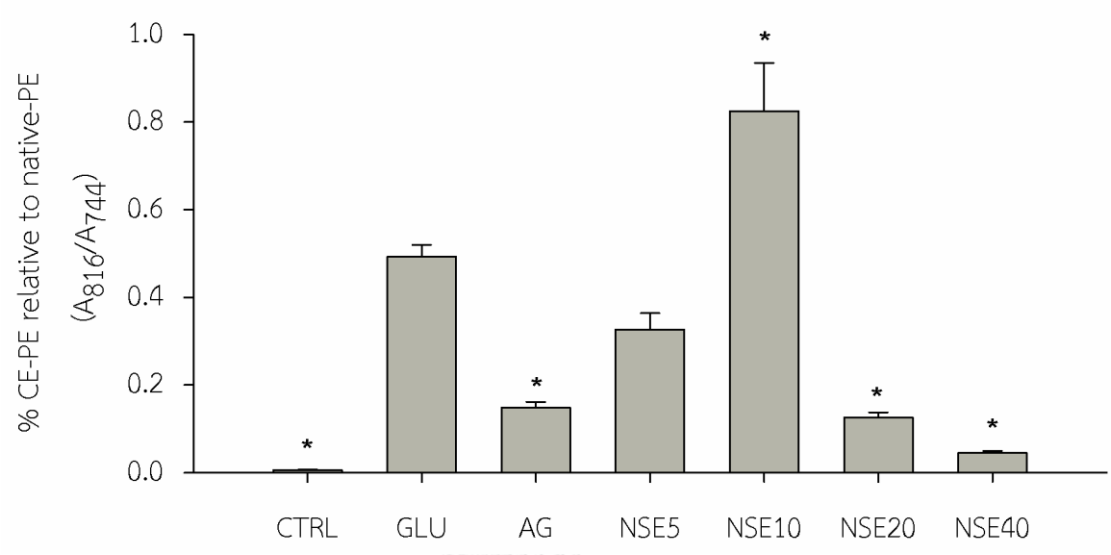

Figure 29 Effect of NSE on formation of carboxyethyl- phosphatidylethanolamines (CE-PE).

Data are expressed as mean ± SEM (n=3). CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE + glucose + 5.0 mg/ml of AG (positive control), NSE5, NSE10, NSE20 and NSE40 =PE+ glucose+ NSE at 5, 10, 20 and 40 mg/ml, respectively). * Significant difference from negative control ($p< 0.05$).

Figure 29 demonstrated an inhibitory effect of NSE on formation of CE-PE. NSE at 20 and 40mg/ml significantly decreased CE-PE formation ($p< 0.05$) and the effect were same as that in AG treatment ($p> 0.05$). The percentages of inhibition were accounted for 69.82 %, 74.43% and 90.85% for AG 5mg/ml, NSE 20mg/ml and NSE 40 mg/ml respectively.

There was a correlation between concentration of NSE and products of glycation of DOPE which showed in figure 30-32. Amount of glycated PE, CM-PE, and CE-PE significantly inverse correlated with concentration of NSE ($p< 0.05$ or $p< 0.01$). It can be implied that the extract showed an inhibitory effect in dose-dependent manner.

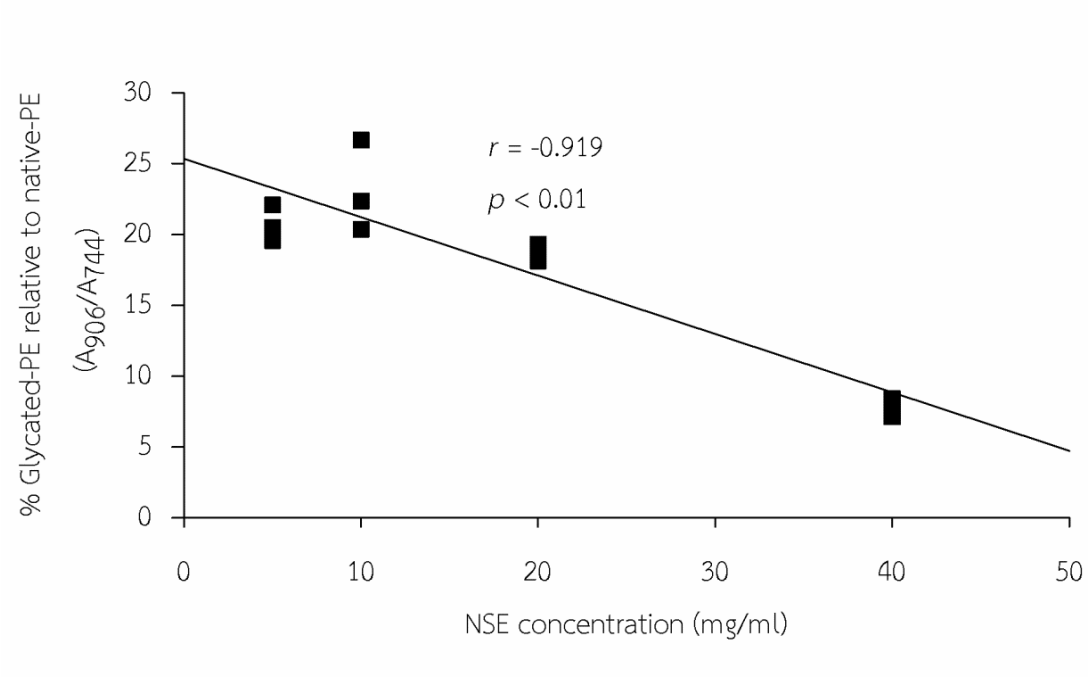

Figure 30 Correlation between concentration of NSE and glycated-PE formation.
r = Pearson correlation coefficient.

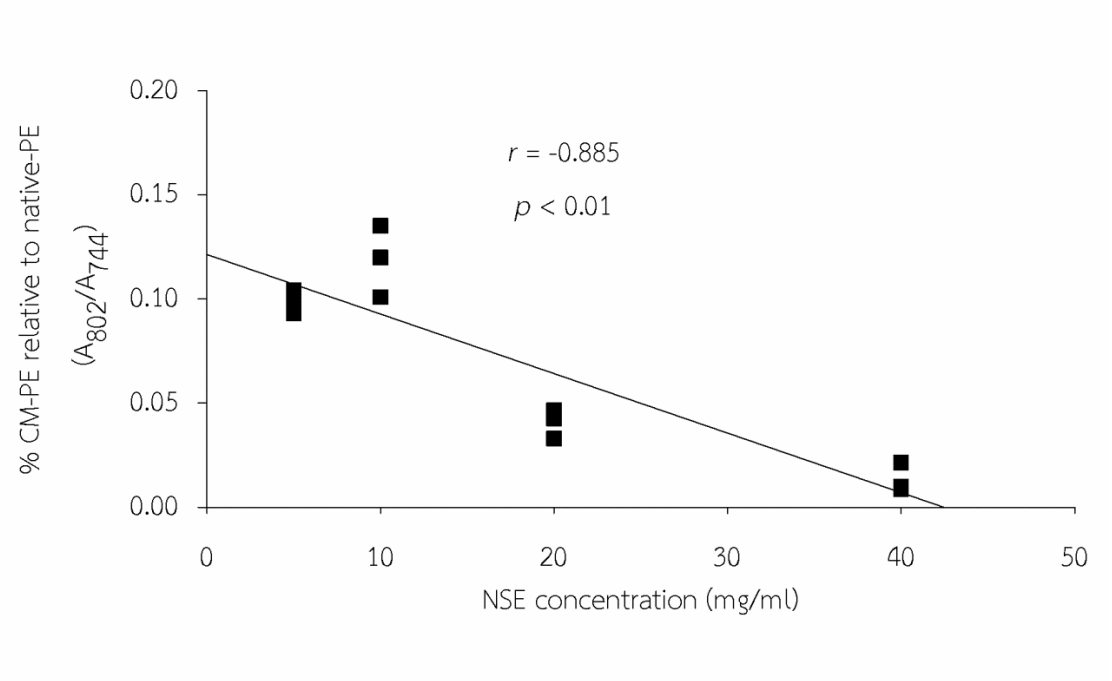

Figure 31 Correlation between concentration of NSE and CM-PE formation.

r = Pearson correlation coefficient.

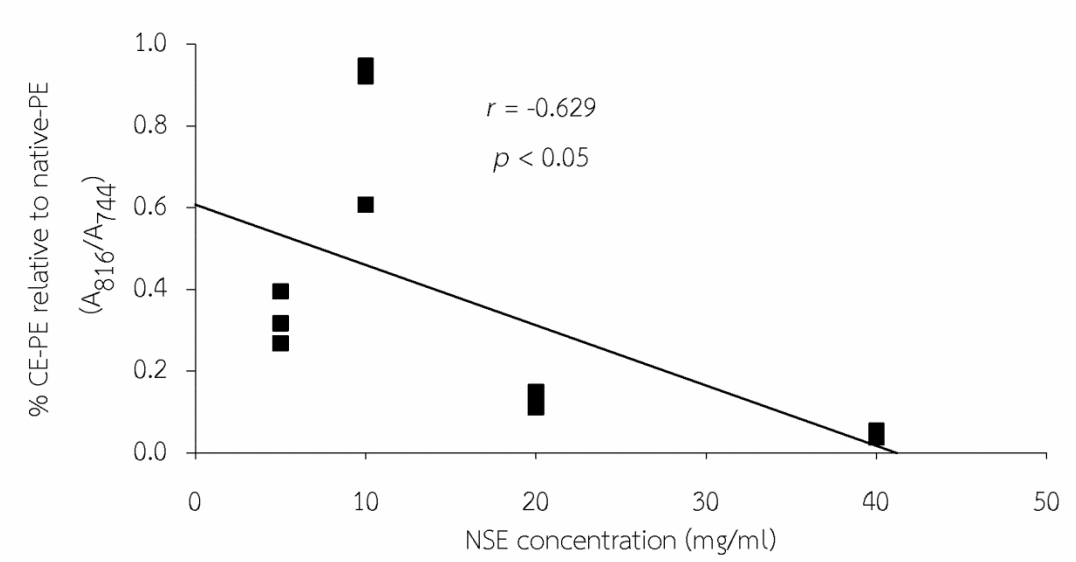

Figure 32 Correlation between concentration of NSE and CE-PE formation.

r = Pearson correlation coefficient.

4.3 Inhibitory effect of thymoquinone (TQ) on glycation of Dioleoly-PE (DOPE)

4.3.1 Inhibitory effect of TQ on formation of glycated-PE, CM-PE and CEPE

Thymoquinone (TQ) at the concentration of 0.5-10.0 mg/ml was tested its efficacy against lipid glycation by adding it into the *in vitro* lipid glycation system containing DOPE (3 mM) and glucose (2.5 M) with incubation time of 48 hr. Products from DOPE glycation (glycated-PE, CM-PE and CEPE) were measured by mass spectrometric technique. Figure 33 illustrated inhibitory effect of TQ on formation of glycated-PE. It was found that all concentration of thymoquinone (0.5, 1.0, 2.5, 5.0 and 10.0 mg/ml) exhibited a significantly lowered the percentage of glycated-PE peak area relatively to native-PE peak area compared to negative control ($p < 0.05$). In contrast, aminoguanidine (5.0 mg/ml) showed a significantly increase in this product ($p < 0.05$). In addition, a lower concentration of TQ had higher inhibitory effect on the glycated-PE formation than a higher concentration. The lowest concentration of TQ (0.5 mg/ml) showed the highest percentage of inhibition (83.67%) whereas the highest concentration of TQ (10.0 mg/ml) inhibited glycated-PE formation by 19.57%. However, percentage of glycated-PE was positively correlated with TQ concentration ($p < 0.01$) (figure 36).

Apart from glycated-PE, TQ also exhibited an inhibitory effect on CM-PE. CM-PE formation was significantly decreased accounted for 22.23%, 46.46% and 61.56% by 2.5, 5.0 and 10.0 mg/ml TQ, respectively as compared to negative control ($p<0.05$) (Figure 34). Moreover, TQ at 5.0 and 10.0 mg/ml exert a similar inhibition to that of AG ($p>0.05$) which can reduce CM-PE by 57.14 %. Figure 37 demonstrated that the reduction of CM-PE was significantly invert correlated with TQ concentration ($p < 0.01$), meaning that TQ inhibited CM-PE formation in a dose-dependent manner.

For CE-PE, AG significantly inhibited CE-PE formation (p<0.05) whereas TQ (0.5-5.0 mg/ml) significantly increased that compared to negative control (p<0.05) (Figure 35). However, TQ tend to reduce the amount of CE-PE when its concentration increased (Figure 38).

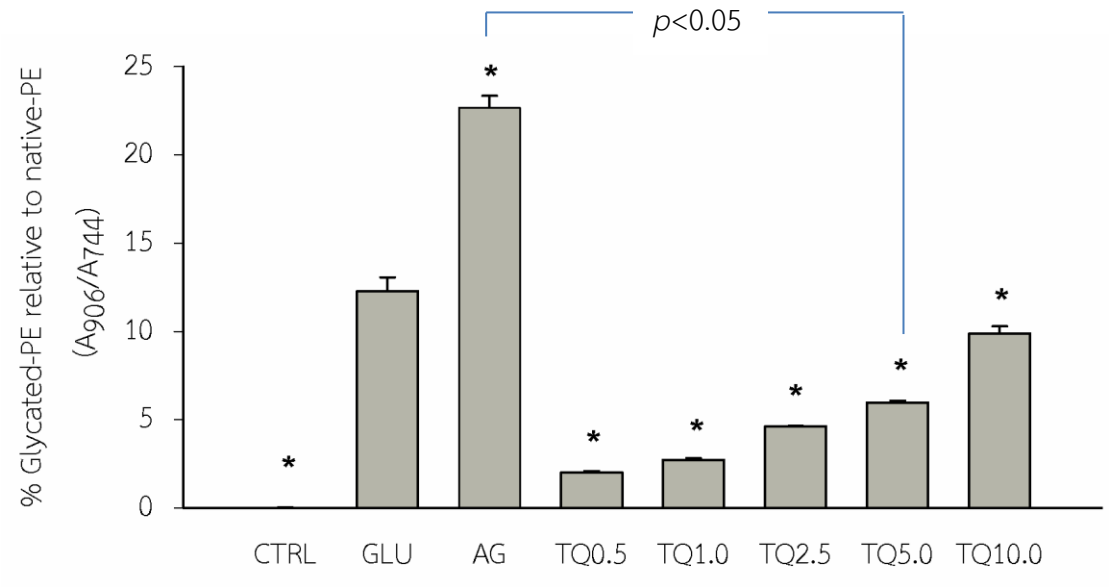

Figure 33 Effect of TQ on formation of glycated-PE.

Data are expressed as mean ± SEM (n=3). CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE + glucose + 5.0 mg/ml of AG (positive control), TQ0.5, TQ1.0, TQ2.5, TQ5.0 and TQ10.0 = PE+ glucose + 0.5, 1.0, 2.5, 5.0 and 10.0 mg/ml of TQ, respectively. * Significant difference from negative control (p < 0.05).

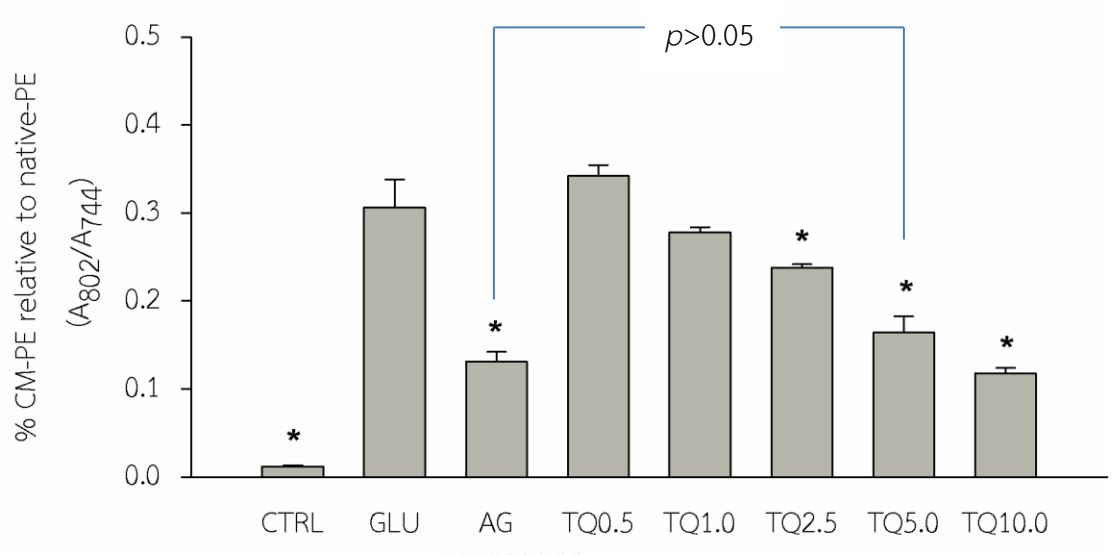

Figure 34 Effect of TQ on formation of carboxymethyl- phosphatidylethanolamines (CM-PE).

Data are expressed as mean ± SEM (n=3). CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE + glucose + 5.0 mg/ml of AG (positive control), TQ0.5, TQ1.0, TQ2.5, TQ5.0 and TQ10.0 = PE+ glucose + 0.5, 1.0, 2.5, 5.0 and 10.0 mg/ml of TQ, respectively. * Significant difference from negative control ($p < 0.05$).

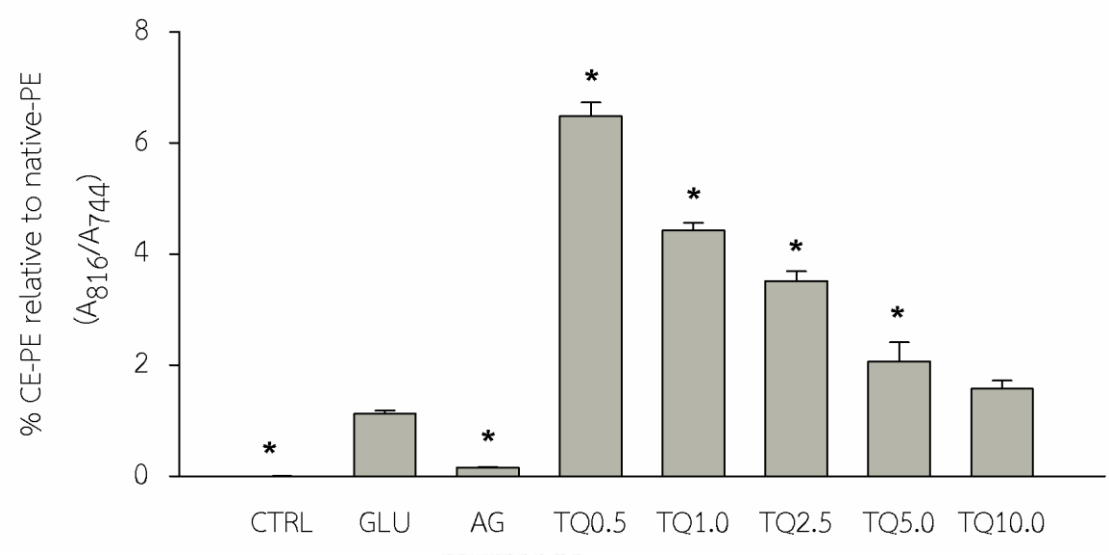

Figure 35 Effect of TQ on formation of carboxyethyl- phosphatidylethanolamines (CE-PE).

Data are expressed as mean ± SEM (n=3). CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE + glucose + 5.0 mg/ml of AG (positive control), TQ0.5, TQ1.0, TQ2.5, TQ5.0 and TQ10.0 = PE+ glucose + 0.5, 1.0, 2.5, 5.0 and 10.0 mg/ml of TQ, respectively. * Significant difference from negative control ($p < 0.05$).

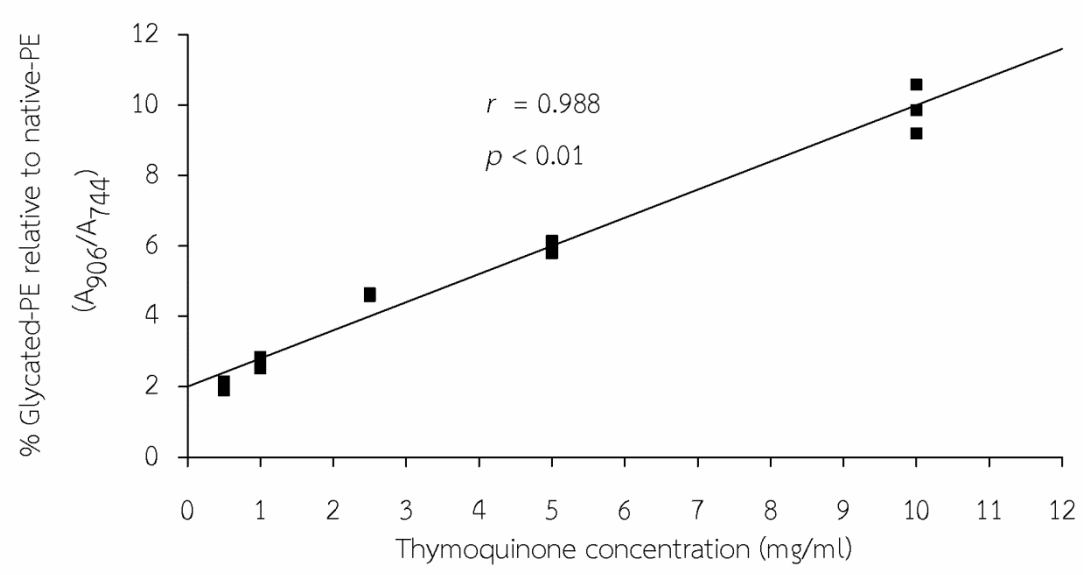

Figure 36 Correlation between concentration of thymoquinone and glycate-PE formation.

r = Pearson correlation coefficient.

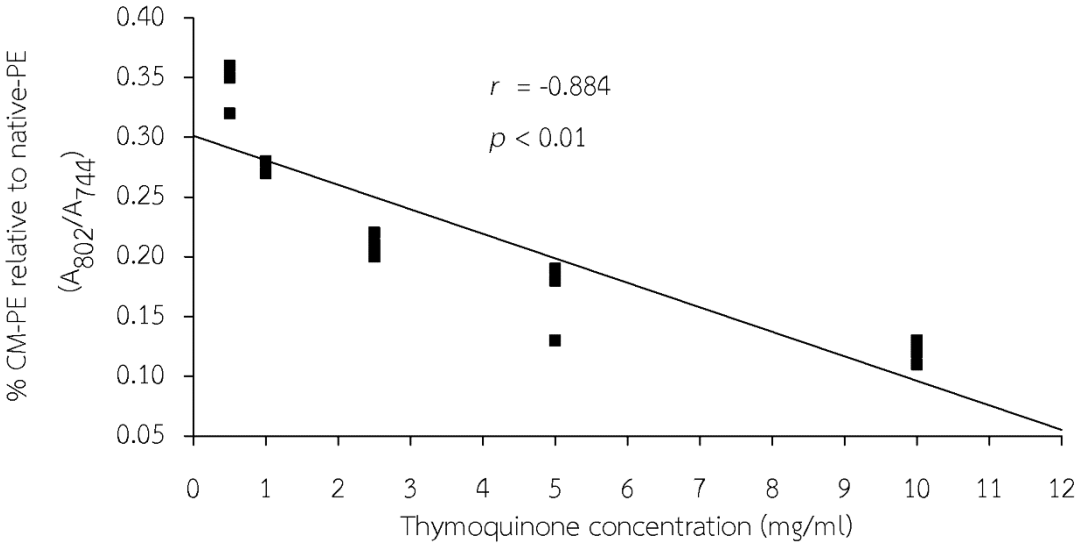

Figure 37 Correlation between concentration of thymoquinone and CM-PE formation.

r = Pearson correlation coefficient.

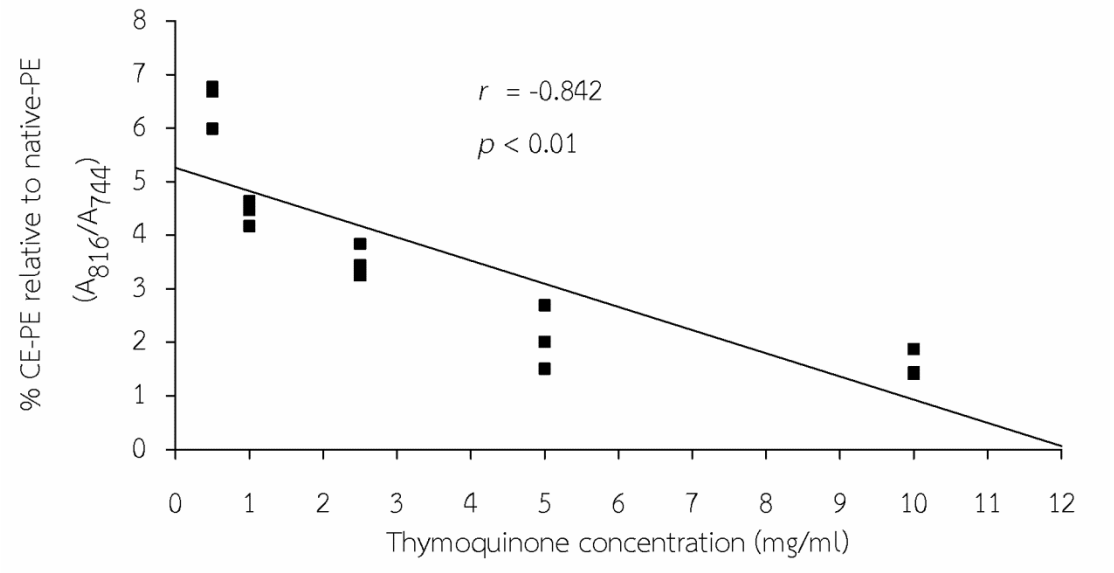

Figure 38 Correlation between concentration of thymoquinone and CE-PE formation. *r* = Pearson correlation coefficient.

4.3.2 Formation of thymoquinone - phosphatidylethanolamine adduct (TQ-PE adduct)

TQ-PE adduct was assumed to be able to condense with PE, thereby inhibiting lipid glycation. Therefore, in the TQ/DOPE/glucose system, it was scanned and detected by mass spectrometric technique. In positive ion mode, collision-induced dissociation (CID) of a reduced form of thymoquinone-phosphatidylethanolamine adduct produced a diglyceride ion ($[M+H-287]^+$) permitting neutral loss scanning. Figure 39 illustrated a scheme for the condensation of TQ with DOPE. The carbonyl group of TQ might be condensed with the amino group of PE to form TQ-PE adduct. Our result found the mass peak indicating TQ-PE adduct (m/z 890.6 $[M+H]^+$) which detected by specific neutral loss scan of 287 Da (Figure 40).

In addition to the occurrence of TQ-PE adduct, its formation was also found to be significantly correlated with TQ concentration ($p < 0.01$) as shown in Figure 41. Moreover, the pearson's correlation analysis showed the significantly inverse

relationship between TQ-PE adduct and the CM-PE formation ($p < 0.01$) (Figure 42). Nevertheless, this relationship was not found in case of glycated-PE.

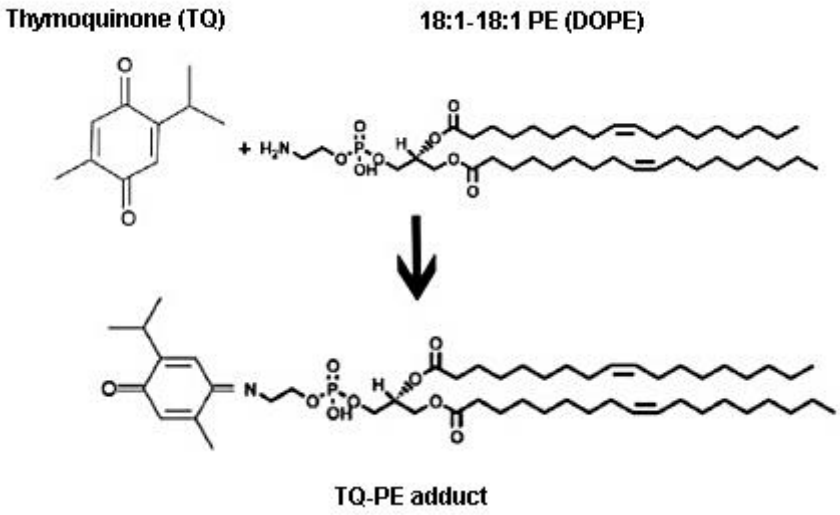

Figure 39 A proposed scheme for the condensation of TQ with PE.

The carbonyl group of TQ was condensed with the amino group of PE to form TQ-PE adduct.

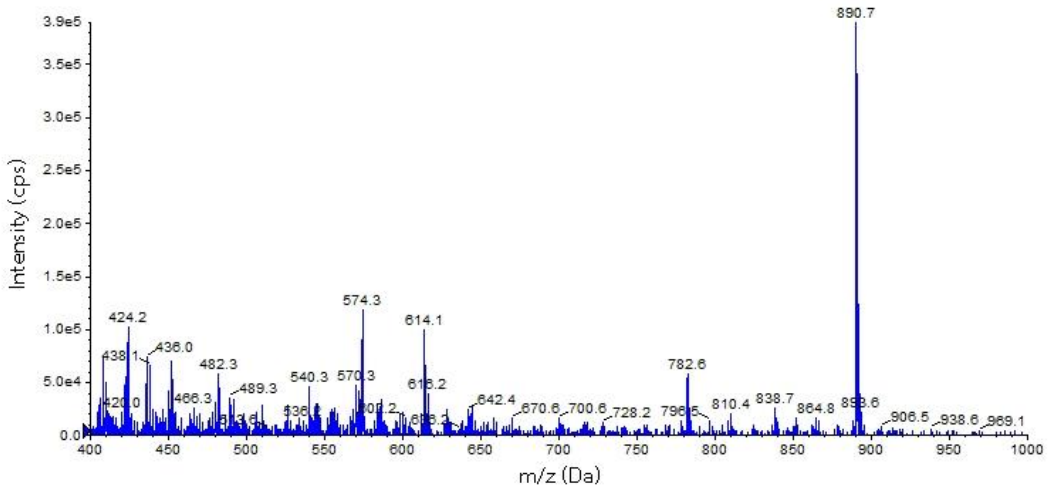

Figure 40 Mass peak of TQ-PE adduct found in TQ/18:1-18:1PE/glucose system.

The specific neutral loss scan of 287 Da showed m/z of 890.7 Da $[M+H]^+$ of TQ-18:1-18:1PE adduct.

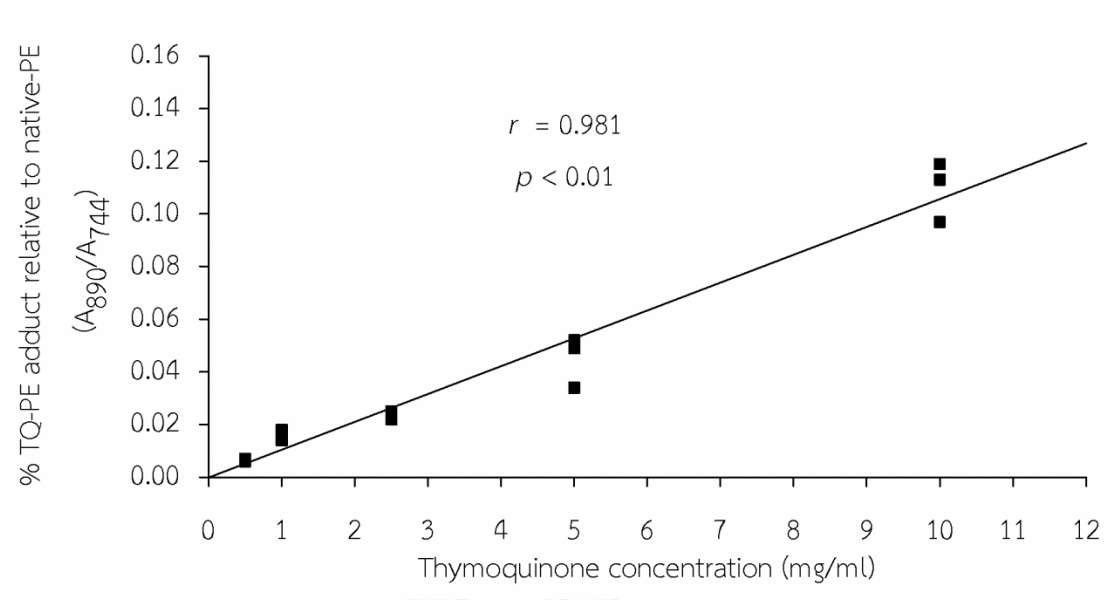

Figure 41 Correlation between concentration of thymoquinone and formation of TQ-PE adduct.

r = Pearson correlation coefficient.

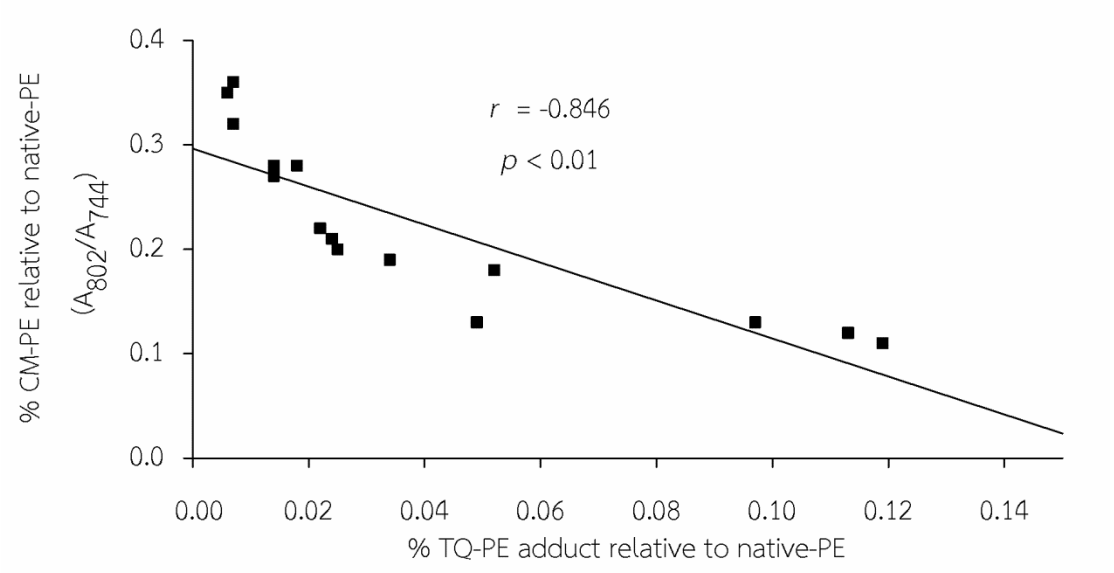

Figure 42 Correlation between TQ-PE adduct and CM-PE formation.

r = Pearson correlation coefficient.

4.4 Effect of *Nigella Sativa* seed extract (NSE) on glycation of human erythrocyte PE

4.4.1 Baseline characteristics of the subjects

Baseline characteristics on the day of blood collection and habitual nutrient intakes estimated by using 3-day food records and based on food exchange list of 10 healthy subjects were shown in table 5. All subjects had normal biochemical parameters. From 3-day food records, it was found that subject had high fat intake observed by 39.6% of total energy intake (normal distribution is %carbohydrate: %protein: %fat = 55-60: 10-15: 25-30).

Table 5 Baseline characteristics and caloric intake of the subjects

Parameters	Values
BMI (kg/m^2)	20.8 ± 0.6
Glucose (mg/dL)	80.9 ± 2.5
Cholesterol (mg/dL)	171.4 ± 7.1
Triglyceride (mg/dL)	75.5 ± 12.1
HDL (mg/dL)	55.7 ± 4.5
LDL (mg/dL)	100.5 ± 4.1
Caloric distribution (%carbohydrate: %protein: %fat)	41.8±3.2: 18.6±1.2: 39.6±2.4

Data are expressed as mean ± SEM, n=10

4.4.2 Hemolysis of erythrocyte

The percentage of hemolysis of erythrocyte after incubation with glucose in different treatments for 48 h was presented in table 6. It was found that incubating of erythrocyte (RBC) with glucose significantly increased hemolysis as compared to that without glucose ($p < 0.05$). Adding of aminoguanidine (AG) or *Nigella Sativa* seed extract (NSE) prior to glucose seemed to increase the percentage of hemolysis; however, there was no significant difference among that ($p > 0.05$).

Table 6 The percentage of hemolysis of erythrocyte after 48 h incubation

Treatments	% Hemolysis
RBC	3.4 ± 0.8^{b}
RBC + 100mM glucose	26.6 ± 3.8^{a}
RBC + 100mM glucose +20 mM AG	32.1 ± 3.5^{a}
RBC + 100mM glucose + 50 µg/ml NSE	38.3 ± 3.9^{a}
RBC + 100mM glucose + 100 µg/ml NSE	31.5 ± 4.9^{a}
RBC + 100mM glucose + 200 µg/ml NSE	39.7 ± 4.4^{a}

Results are mean ± SEM (n=10). Difference among groups was tested by using repeated measure ANOVA with Bonferroni post hoc test. Groups with different letter superscripts are significantly different ($p < 0.05$).

4.4.3 Profiles of PE molecular species in erythrocyte

After incubation of erythrocyte (RBC) with 100 mM glucose at 37°C for 48 h, lipid fraction of erythrocyte was extracted and then analyzed by neutral loss scan mode for scanning of its PE molecular species. Native-PE, glycated-PE, CM-PE and CE-PE species in erythrocyte were scanned by NLS of 141, 303, 199 and 213 Da, respectively. A NLS spectra of the native-PE (nonglycated-PE) erythrocyte extracted

from a healthy human subject was shown in figure 43. Figure 44-46 presented NLS spectrum of erythrocyte glycated-PE species and erythrocyte AGE-PE species (CM-PE and CE-PE) from glucose-treated erythrocyte.

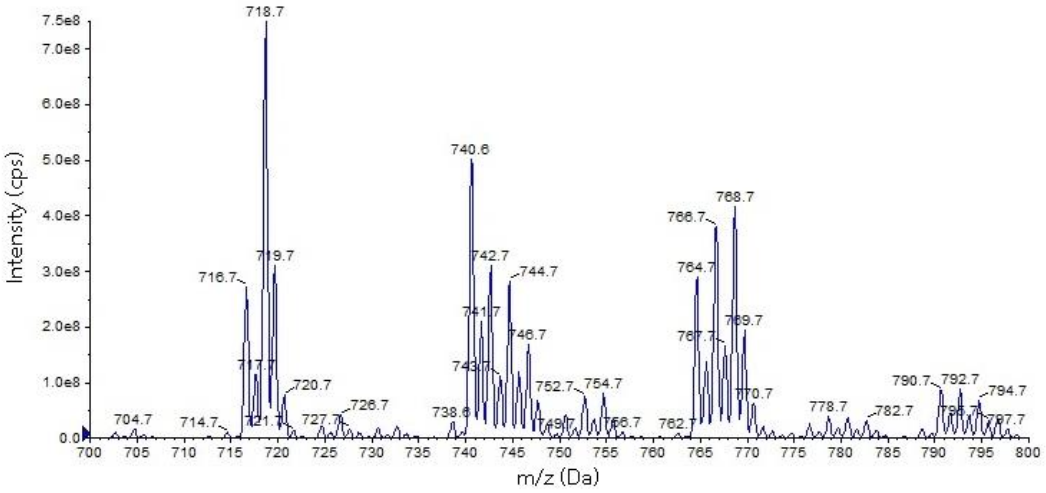

Figure 43 NLS spectra of erythrocyte native-PE (nonglycated-PE) of a healthy human subject.

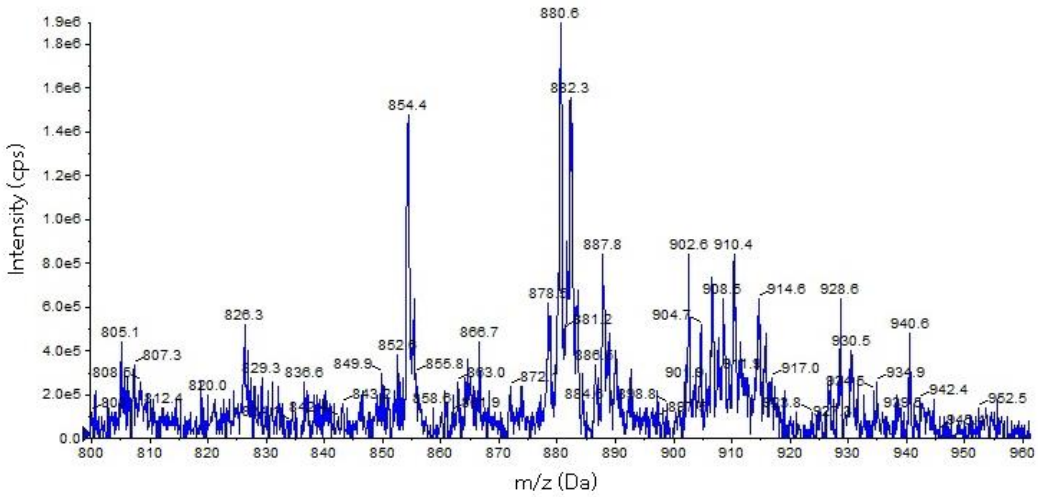

Figure 44 NLS spectrum of erythrocyte glycated-PE species of glucose-treated erythrocyte.

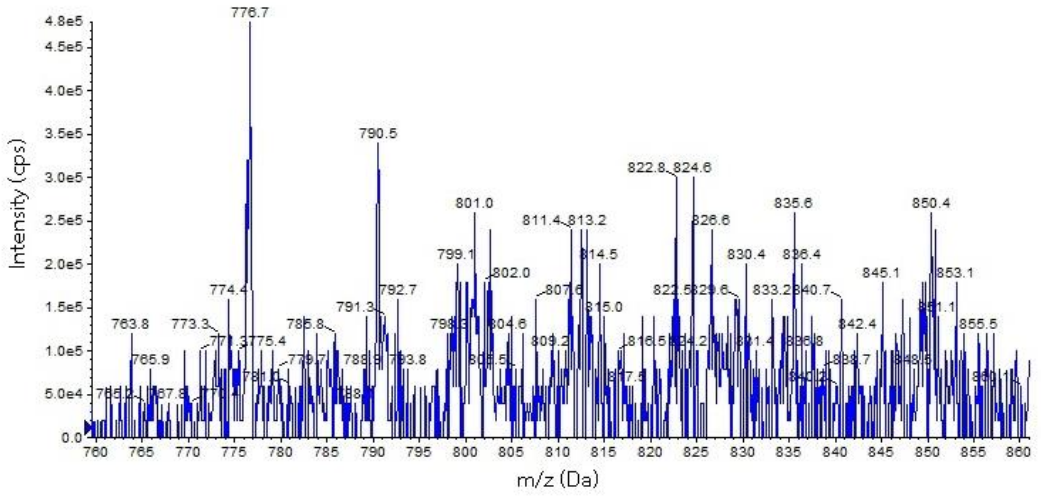

Figure 45 NLS spectrum of erythrocyte CM-PE species of glucose-treated erythrocyte.

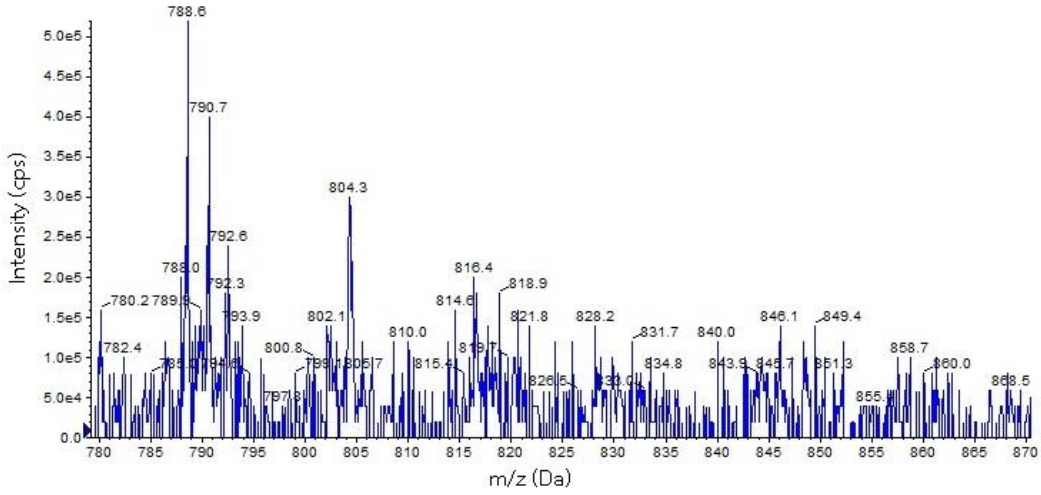

Figure 46 NLS spectrum of erythrocyte CM-PE species of glucose-treated erythrocyte.

4.4.4 Effect of NSE on formation of erythrocyte glycated-PE, CM-PE and CE-PE.

The amount of erythrocyte glycated-PE and AGE-PE species after treated with glucose, in the absence (negative control) or presence of aminoguanidine; AG (positive control) and *Nigella Sativa* ethanolic extract (NSE) were presented in the percentage of each species related to its native species. For treatments of NSE, both peak areas of glycated-PE or AGE-PE and native-PE were subtracted with that of NSE (50, 100 and 200 µg/ ml) in PBS. It was found that, after 48 h incubation with glucose, mostly of erythrocyte glycated-PE species was not significantly different in the amount with blank control (p>0.05), except for palmitoyl-docosahexaenoyl-plasmalogen-PE (16:0a-22:6), octadecenoyl-eicosatetraenoyl-plasmalogen-PE (18:1a-20:4) and stearoyl-docosahexaenoyl-plasmalogen-PE (18:0a-22:6), and for the percentage of total glycated-PE (figure 45). Furthermore, there was no significant difference in the percentage of any glycated-PE species among the treatment of NSE, positive control (AG) and negative control.

In the same way, all treatments (negative control, positive control, NSE50, 100 and 200 µg/ ml) expressed no significant difference in CM-PE formation of all species when compared to blank control (p>0.05) (figure 46). For erythrocyte CE-PE species, incubation of erythrocyte with glucose significantly increased only 16:0a-22:6 CE-PE formation compared with blank control (p< 0.05), but not for the rest species (figure 47). However, 16:0a-22:6 CE-PE from erythrocyte treated with AG or NSE did not show significant difference compared with the negative control (p>0.05).

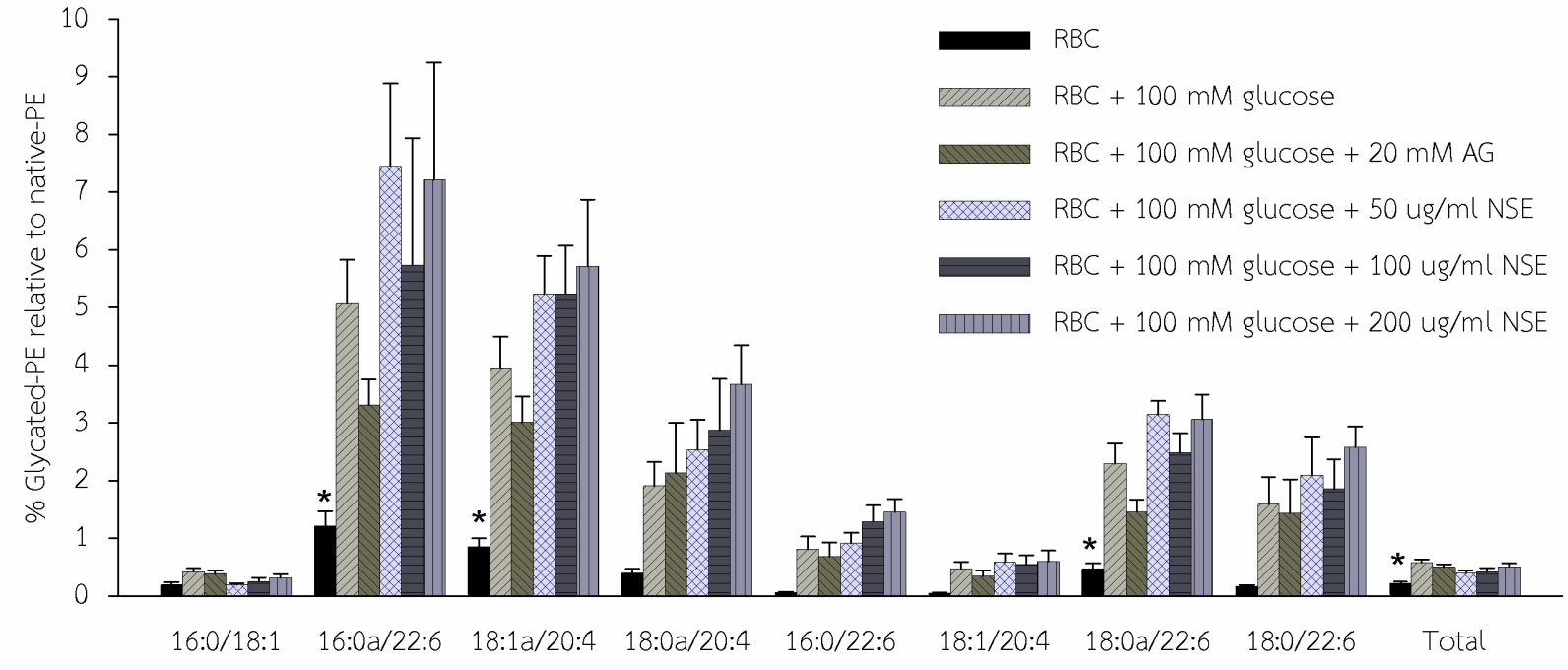

Figure 47 Effect of NSE against formation of etythrocyte glycated-PE.

Data are expressed as mean ± SEM (n=10). Total = % total glycated-PE relative to total native-PE. a = alkenyl-acyl species (plasmalogen). RBC is refered to erythrocyte. * Significant difference from negative control (RBC+100mM glucose) ($p< 0.05$).

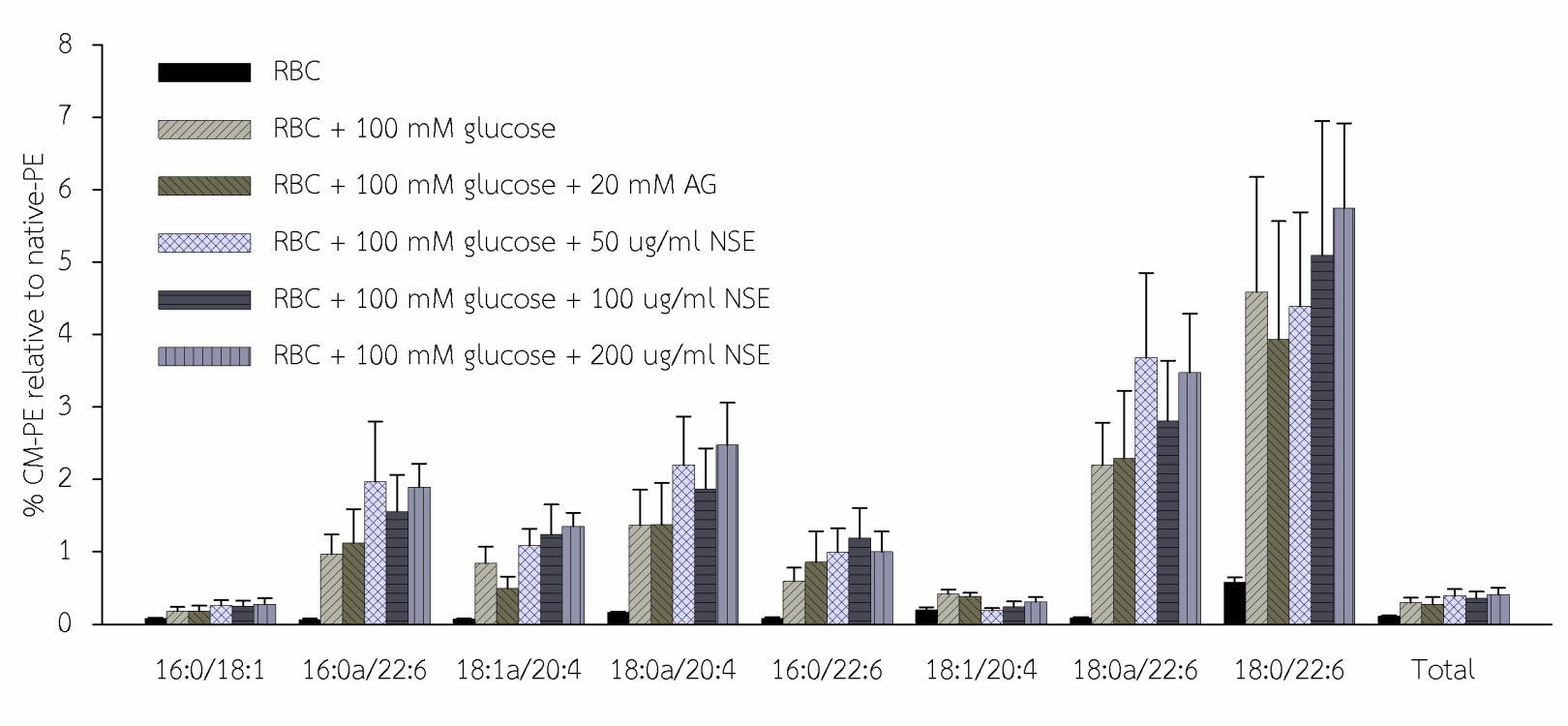

Figure 48 Effect of NSE against formation of etythrocyte CM-PE.

Data are expressed as mean ± SEM (n=10). Total = % total CM-PE relative to total native-PE. a = alkenyl-acyl species (plasmalogen). RBC is refered to erythrocyte. * Significant difference from negative control (RBC+100mM glucose) ($p< 0.05$).

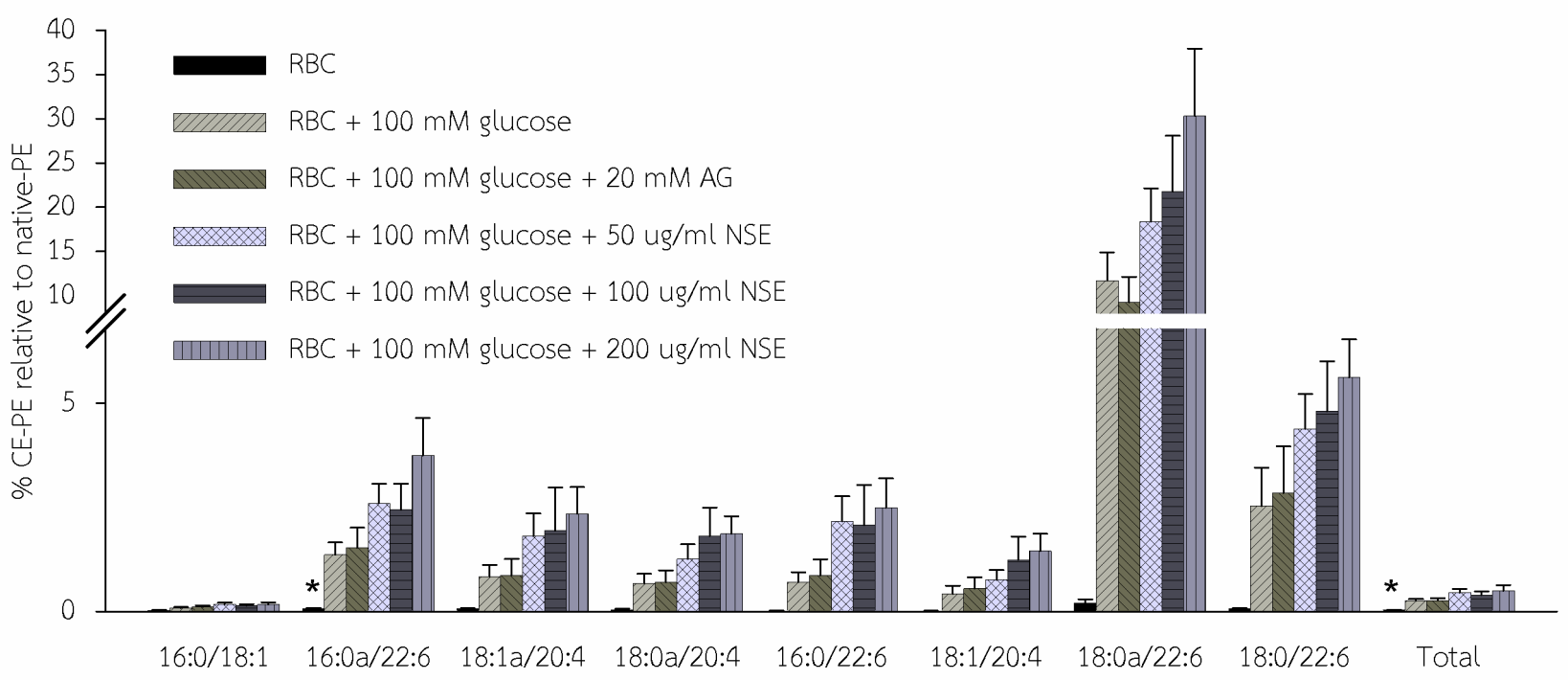

Figure 49 Effect of NSE against formation of etythrocyte CE-PE.

Data are expressed as mean ± SEM (n=10). Total = % total CE-PE relative to total native-PE. a = alkenyl-acyl species (plasmalogen). RBC is refered to erythrocyte. * Significant difference from negative control (RBC+100mM glucose) ($p< 0.05$).

CHAPTER V
DISCUSSION

5.1 Thymoquinone content in *Nigella Sativa* seed extract (NSE)

The result demonstrated that thymoquinone (TQ) was the highest quinone compound found in ethanolic extract of *Nigella Sativa* seed. Thymoquinone content in ethanolic extract has not been reported; however, the result was similar with the previous study in *Nigella Sativa* oil showing the highest quantity of thymoquinone among other quinone in *Nigella Sativa* oil**.** The commercial oil of *Nigella Sativa* seed contain TQ accounting for $5.26x10^{-2}$ % w/w followed by thymol ($9.12x10^{-3}$ % w/w) and thymohydroquinone ($7.67x10^{-4}$% w/w) (Ghosheh *et al.*, 1999)**.** Regarding to our study, TQ content in NSE was about 6,800-8,600 µg/g or $6.8\text{-}8.6x10^{-1}$ % w/w which higher than the oil from previous study. In addition, previous study demonstrated TQ content in fixed oil which accounted for 0.2 mg/g oil (Tauseef Sultan, 2009). Another study reported TQ content in *Nigella Sativa* seed oil extracted using soxhlet and supercritical carbon dioxide, which were 1.06 mg/g oil and 4.07 mg/g oil, respectively (Solati *et al.*, 2014).

5.2 Inhibitory effect of thymoquinone (TQ) on glycation of Dioleoly-PE (DOPE)

Previous study reported that Amadori-PE, an early stage product from phosphatidylethanolamine glycation, was found abnormal quantity in diabetic plasma and was found at high level in the blood and in organs that are affected by diabetes in streptozotocin–induced diabetic rat (Teruo Miyazawa et al., 2005; Nakagawa et al., 2005; Sookwong et al., 2011). Moreover, the accumulation of Amadori-PE was faster than that of Carboxymethyl Lysine (CML), a protein glycation product; therefore, Amadori-PE was suggested to be a helpful predictive marker for

early state of diabetes (Sookwong *et al.*, 2011). Carboxymethyl-phosphatidylethanolamine (CM-PE), a major AGE-PE, has also been reported to present *in vivo*, in human erythrocyte and mitochondrial membranes of mammalian (Pamplona *et al.*, 1998; Requena *et al.*, 1997). Glycation of phospholipid can stimulate pathogenesis which may play a role in the development of diabetic complications. Moreover, it also alter the physical and biological properties of cell membrane such as changes in the biosynthesis and turnover of membrane phospholipids, the activities of membrane-bound enzymes, and the susceptibility of oxidative stress (Naudi *et al.*, 2013; J.-H. Oak *et al.*, 2003; J. Oak *et al.*, 2000; T. Obšil *et al.*, 1999; T. A. Obšil, Evžen; Pavlíček, Zdeněk, 1998; Simões *et al.*, 2013). Therefore, prevention or inhibition of lipid glycation may be benefitial to diabetic patient.

From the result of *in vitro* glycation of Dioleyl-phosphatidylethanolamine, Thymoquinone (2.5-10 mg/ml) was successful to inhibit both early stage product and Advanced glycation end product of glucose-mediated phosphatidylethanolamine glycation, except CE-PE. Comparing with aminoguanidine at the same concentration, efficacy of TQ on the inhibition of glycated-PE and CM-PE were higher and the same, respectively. Moreover, TQ seemed to pay role in the inhibition of early stage than late stage observed by its efficacy even at 0.5 mg/ml which can show the strong inhibition. In addition, inhibitory effects of thymoquinone on products of PE glycation were in dose-dependent manner.

Our results were similar with previous study showing anti-glycation effects of thymoquinone which demonstrated that TQ can inhibit glycated hemoglobin (HbA1c), an early stage of protein glycation, and also reduce post-Amadori products from glucose-mediated human serum albumin glycation (Losso *et al.*, 2011). However, in *in vitro* protein glycation, TQ inhibited methylglyoxal-mediated human

serum albumin glycation whereas in *in vitro* lipid glycation, a carboxyethyl-phosphatidylethanolamine (CE-PE), which is possible to be formed by reaction between methylglyoxal and PE (Naudi *et al.*, 2013; Shoji *et al.*, 2010) was not inhibited by TQ. The explanation of this result is still unclear. Therefore, further study conducting on the effect of TQ against methylglyoxal-mediated lipid glycation should be investigated by incubation of PE with methylglyoxal and then mornitoring the formation of carboxyethyl-phosphatidylethanolamines (CE-PE).

On the basis of our results, the possible mechanism for efficacy of TQ might be involved with the TQ-PE adduct. It was hypothesized that carnonyl group of TQ may condense with amine group of phosphatidylethanolamine and then form thymoquinone-phosphatidylethanolamine adduct (TQ-PE adduct). The occurrence of TQ-PE adduct was found in this study and its concentration was significantly correlated with concentration of TQ and inversely correlated with the products of PE glycation. This adduct was suggested to be an explanation for role of TQ in inhibition of PE glycation by competing with glucose to bind with PE. This mechanism has been previously described in the study using pyridoxal 5‘-phosphate and pyridoxal (vitamin B6 derivatives). It demonstrated that pyridoxal 5‘-phosphate and also pyridoxal were rapidly condensed with PE than that of glucose to form PE-pyridoxal 5‘-phosphate adduct (Higuchi *et al.*, 2006). This thereby reduce the Schiff-PE formation and prevent the production of Amadori-PE.

Some interesting results from previous study can support hypothesis. Pre-incubation (24 h) of TQ with blood or human serum albumin (HSA), before adding of delta-gluconolactone or glucose, increased its inhibitory effect on formation of glycated hemoglobin HbA1c and fluorescence AGE compared to the concurrent incubation of blood with delta-gluconolactone and TQ or HAS with glucose and TQ (Losso *et al.*, 2011).

Apart from competitive binding activity of TQ, antioxidant activies of thymoquinone may be pay role in its inhibitory effect on CM-PE. Thymoquinone is a strong quencher of superoxide anion radical ($O_2^{\cdot -}$), one of reactive oxigen species (ROS) (Badary *et al.*, 2003; Kruk *et al.*, 2000) and it also was found the inhibitory effect on non-enzymatic lipid peroxidation in ox brain phospholipid (Houghton *et al.*, 1995). Regarding antioxidant activies, TQ may be preventing oxidation of Amadori-PE, which produces CM-PE, resulting in the reduction in CM-PE formation (Naudi *et al.*, 2013; Requena *et al.*, 1997). From the result, it was surprising that percentage of glycated-PE was increased when TQ concentration increased, this might be a result from the prevention of oxidative cleavage of Amadori-PE to be CM-PE, thereby finally lead to an accumulation of glycated-PE.

A clinical study conducting in the patients with solid tumors or hematological malignancies demonstrated that thymoquinone at a dose ranging of 75 to 2,600 mg/day was safe (Al-Amri A, 2009). For the bioavailability of TQ, there are no report on percentage of absorption to the body or circulation in blood of TQ; however, maximum absorption of TQ in rabbit was found 58 % (Alkharfy *et al.*, 2014). From *in vitro* study, the lowest concentration of TQ (0.5 mg/ml) succeeded to reduce glycated PE. Nevertheless, this dose still too high to apply in clinical trial especially when consideration on its amount after absorption (the intake would be around 3,950 mg TQ which more than safety dose). For that reason, the *in vitro* anti-lipid glycation effect of TQ at concentration less than 0.5 mg/ml should be further investigated.

5.3 Inhibitory effect of *Nigella Sativa* seed extract (NSE) on glycation of Dioleoly-PE (DOPE)

From the result of *in vitro* glycation of Dioleyl-phosphatidylethanolamine, *Nigella Sativa* ethanoic extract (NSE) showed an inhibitory effect on formation of glycated-PE, including Schiff-PE and Amadori-PE, at the high concentration (40 mg/ml), and also that effect on CE-PE and CM-PE at the concentration of 20 mg/ml and 5 mg/ml, respectively. All products were inhibited by NSE in dose-dependent manner. NSE seemed to have more efficacies against AGE-PE, the late stage products of PE glycation, especially CM-PE formation than products from early stage (Amadori-PE and Schiff-PE). Additionally, at the same concentration, NSE can decrease CM-PE formation to a greater extent than aminoguanidine, a common anti-glycation agent for the efficacy in anti-glycation.

It was found in these two experiments that aminoguanidine was unable to inhibit glycated-PE, except AGE-PE. Our result was in consistent with previous study that showed the failure of aminoguanidine to inhibit the Schiff-PE formation (Higuchi *et al.*, 2006). Therefore, it can be suggested that aminoguanidine did not play role in the inhibition of early stage of lipid glycation process. Generally, aminoguanidine pay major role on inhibition of Advance glycation end products (AGEs) formation stage by act as a dicarbonyl scavenger, scavenging dicarbonyl glycating agents such as methylglyoxal and glyoxal, thereby reduction in AGEs (Ahmed, 2005; Thornalley *et al.*, 2000). However, previous study on protein glycation also found that aminoguanidine failed to inhibit the increase in pentosidine and carboxymethyl-lysine (CML) in diabetic rat skin collagen (Degenhardt *et al.*, 1999).

Anti-glycation activity of *Nigella Sativa* seed has not been reported yet. Our result showed the high amount of TQ contained in NSE and also revealed its

inhibitory activity on PE glycation, therefore, these can support that TQ might involve in the inhibitory effect of NSE on lipid glycation.

One of the possible explanations for efficacy of NSE and TQ might be involved with the antioxidant properties of NSE. Regarding CM-PE formation formed by two major pathways including (1) oxidative cleavage of Amadori-PE, and (2) the reaction between PE and glyoxal derived from oxidation of glucose or polyunsaturated fatty acids (PUFAs) (Naudi *et al.*, 2013; Requena *et al.*, 1997). Therefore, the antioxidant might be useful for inhibition of CM-PE. *Nigella Sativa* extract, *Nigella Sativ*a oil and thymoquinone have been reported to have the antioxidant properties comprising free radicals and superoxide radical scavenging activities (Badary *et al.*, 2003; Burits and Bucar, 2000; M. F. Ramadan *et al.*, 2003; Solati *et al.*, 2014; Suboh *et al.*, 2004). Therefore, it can be implied that NSE and also thymoquinone might prevent the oxidation of glucose and unsaturated fatty acid to generate the glyoxal and/or inhibit the oxidative cleavage of glycated-PE, resulting in the decrease of the late stage products of PE glycation, especially CM-PE formation.

Previous studies have demonstrated that fixed oil, methanolic extract, and ethanolic extract of *Nigella Sativa* seed contained polyphenolic compounds, an antioxidant compound that have an anti-glycation activities by trapping the dicarbonyl compounds such as glyoxal and methylglyoxal (Houghton *et al.*, 1995; Mahmood *et al.*, 2013; Meziti A, 2012; Xie and Chen, 2013). This may be related to the decrease of glycated-PE and AGE-PE by NSE observed in our study. Moreover, this can also explain the mechanism that NSE, not TQ, can inhibit CE-PE, an AGE-PE formed by the reaction between methylglyoxal and PE. However, the potential of NSE on inhibition of methylglyoxal-mediated lipid glycation should be further study.

5.4 Inhibitory effect of *Nigella Sativa* seed extract (NSE) on glycation of human erythrocyte PE

Incubation of erythrocyte with 100mM at 37ºC for 48h resulted increased hemolysis. Previous study demonstrated that the elevated levels of glucose lead to an increased osmotic fragility of erythrocyte, as well as membrane lipid peroxidation and changed morphological properties (Jain, 1989; Soares *et al.*, 2006). Therefore, high concentration of glucose used in this study to accelerate the glycation of PE may be a cause of hemolysis.

The formation of erythrocyte PE glycation products of glucose-treated erythrocyte was not significant in this study. Although the amount of glycated-PE, CM-PE, and CE-PE were detected after incubation of erythrocyte with glucose for 48 h, most of those were not significantly changed as compared to blank control, except some species of glycated-PE.

Generally, Amadori-PE and AGE-PE have been reported to be found in erythrocyte from healthy individuals (Breitling-Utzmann *et al.*, 2001; Requena *et al.*, 1997; Shoji *et al.*, 2010). Moreover, for the *in vivo study*, Some studies have reported that the level of Amadori-PE in red blood cell of diabetic patient were significant higher than that of healthy subject, whereas there was no significant difference in AGE-PE in red blood cell of those subjects (Shoji *et al.*, 2010). This demonstrated that the formation of erythrocyte Amadori-PE and especially AGE-PE in hyperglycemic condition may require prolong period. Therefore, the unexpected results in our study may be from the factor of incubation time which was not enough to see the changes of products of lipid glycation in erythrocyte.

Another factor may be involved with other target protein presenting in the system of treatments. Actually, hemoglobin is a major protein in erythrocyte which rapidly reacts with glucose to form glycated hemoglobin (HbA1c) (Zhang *et al.*, 2008). Hemolysis, founded in this study, may cause hemoglobin release from inside the cell and then exposed to glucose before the glycation reaction of PE was completed. Therefore, some amount of glucose was utilized by other protein resulting in the inadequate glucose for reacting with PE.

From our study, *Nigella Sativa* ethanolic extract (NSE) did not attenuate the occurrence of lipid glycation products. This may be due to its concentrations used in this study which were not enough to see the effect. The possible explanation for this might be involved with the fact that the final concentration of dimethylsulfoxide (DMSO), used for dissolving of NSE, was limited to 0.1% in order to avoid DMSO-induced cell hemolysis.

5.5 Limitation

There were some limitations in this study. First, The Shiff-PE and Amadori-PE were simultaneously detected as the glycated-PE since the flow injection MS analysis without chromatographic separation was unable to separate between those species (isobaric species). Moreover, the concentration of the products from PE glycation in this study was shown as relative percentage, not absolute concentration. This was due to the lack of commercial glycated PE standard available for quantitation.

5.6 Future study

1. The *in vitro* anti-lipid glycation effect of thymoquinone at concentrations lower than 0.5 mg/ml should be further investigated. The finding of effective dose that is not higher than the safe dose for human will be useful for other study involved with food product developement or clinical trial.
2. The occurence of TQ-PE adduct measured by mass spectrometric technique was unable to show the molecular structrue of such adduct. Therefore, purification and identification of structure of TQ-PE adduct should be future elucidated by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) technique.
3. *In vitro* study of effect of *Nigella Sativa* seed extract and thymoquinone against methylglyoxal-mediated lipid glycation, especially Carboxyethyl-phosphatidylethanolamines (CE-PE) formation should be investigated.

CHAPTER VI

CONCLUSION

This study demonstrated that *Nigella Sativa* seed extract had a potential to inhibit glucose-mediated lipid glycation evidenced by the reduction in the early stage products (glycated-PE) and Advance glycation end products of phosphatidlyethanolamine glycation (carboxymethyl-phosphatidylethanolamine ,CM-PE and carboxyethyl-phosphatidylethanolamine; CE-PE) *in vitro*. Thymoquinone, a major active compound found in ethanolic extract of *Nigella Sativa* seed might play an important role for the inhibitory effect of *Nigella Sativa* on the lipid glycation. The possible mechanism of such effect may be involved with its competitive binding activity against glucose to form thymoquinone- phosphatidylethanolamine adduct (TQ-PE adduct). Moreover, the degree of inhibition in the formation of early and late stage products of phosphatidylethanolamine glycation were depend on the concentration of the extract or thymoquinone. Therefore, from the study, it can be concluded that *Nigella Sativa* (Black cumin) might be the other therapeutic option, which may be potentially developed in form of nutraceutical or functional food, for preventing or reducing the risk of lipid glycation-induced pathogenesis of diabetic complications. Further study should be conducted in human, particularly in the diabetic patients, to determine the efficacy of *Nigella Sativa* seed on lipid glycation.

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APPENDIX

Appendix A

Standard Curves of Thymoquinone

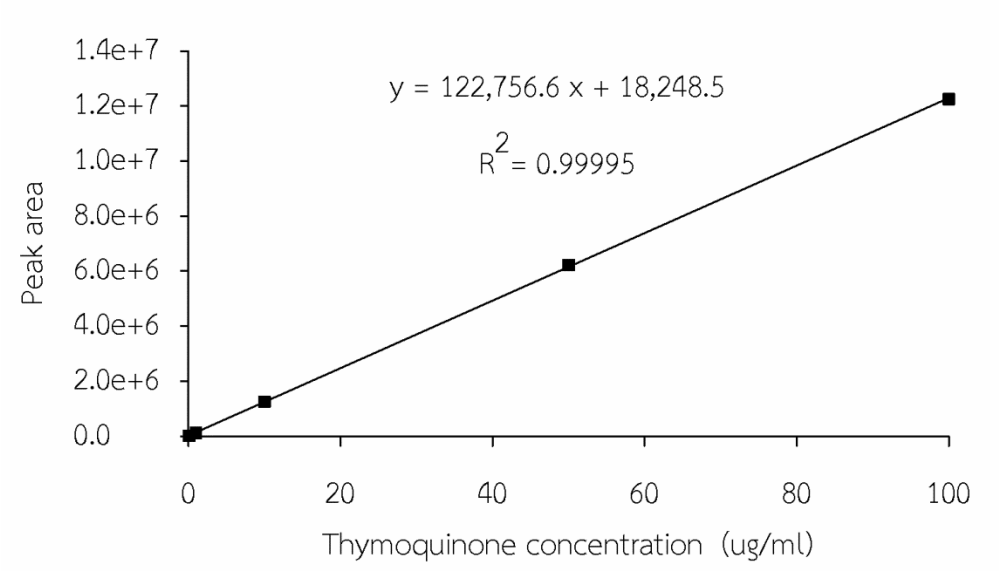

Figure 50 Standard curve of thymoquinone used in commercial PE experiment.

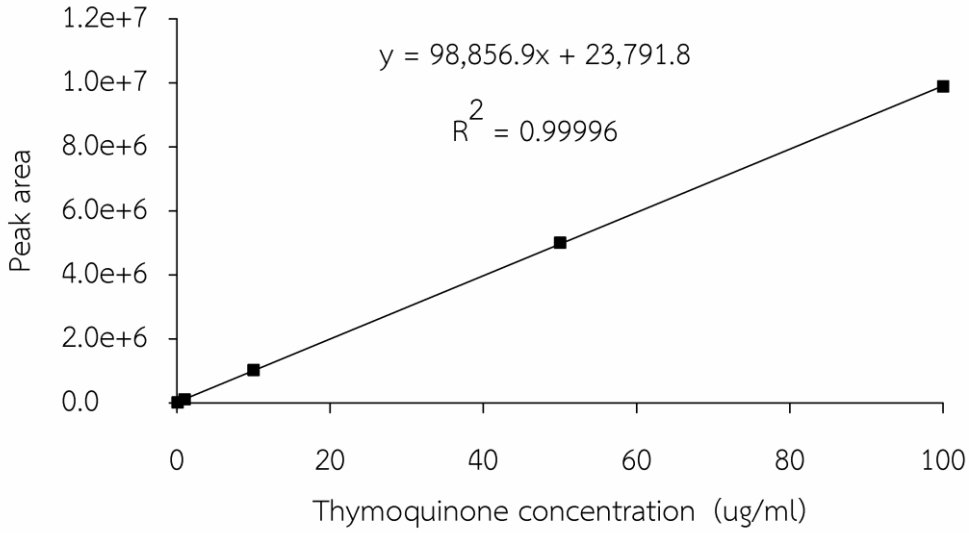

Figure 51 Standard curve of thymoquinone used in erythrocyte experiment.

Appendix B

NLS spectra of *Nigella Sativa* seed extract

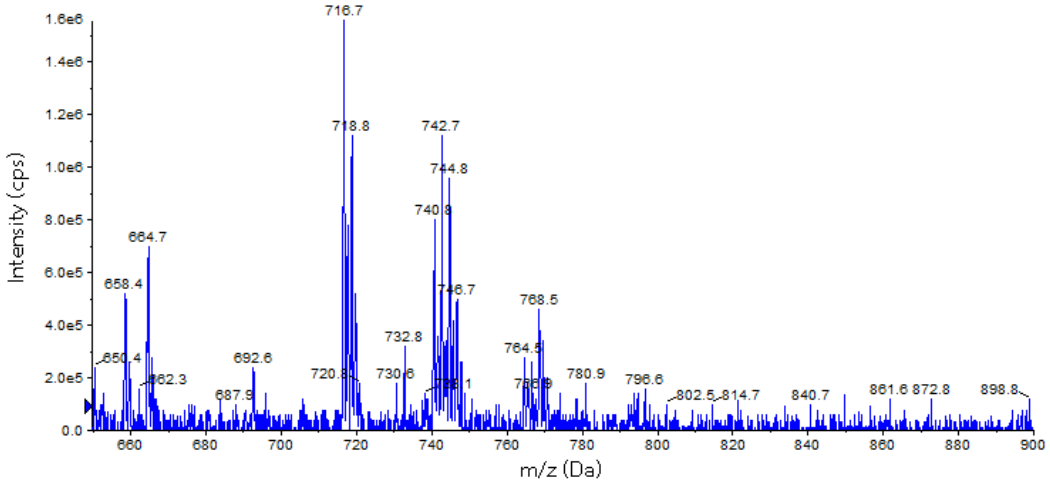

Figure 52 141 Da NLS spectra of *Nigella Sativa* seed extract showing the detection of native-PE species.

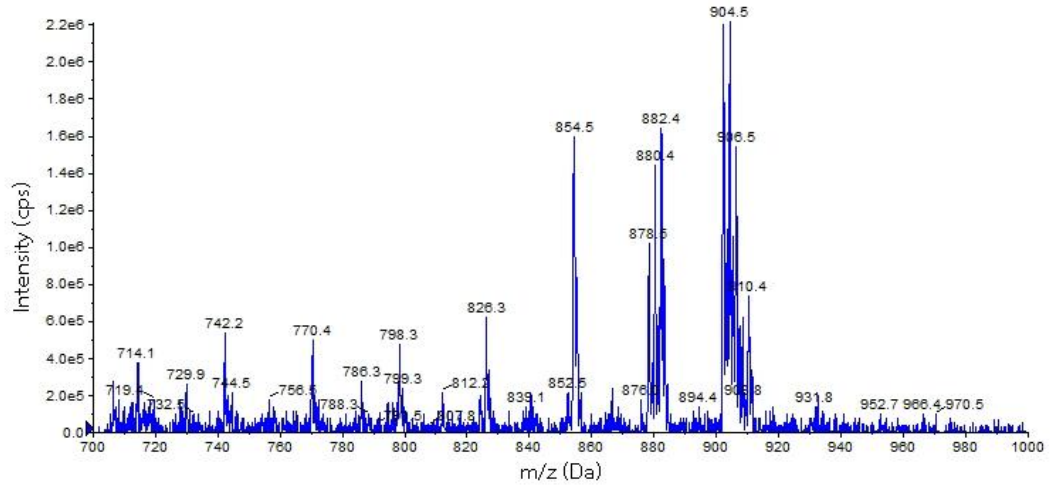

Figure 53 303 Da NLS spectra of *Nigella Sativa* seed extract showing the detection of glycated-PE species.

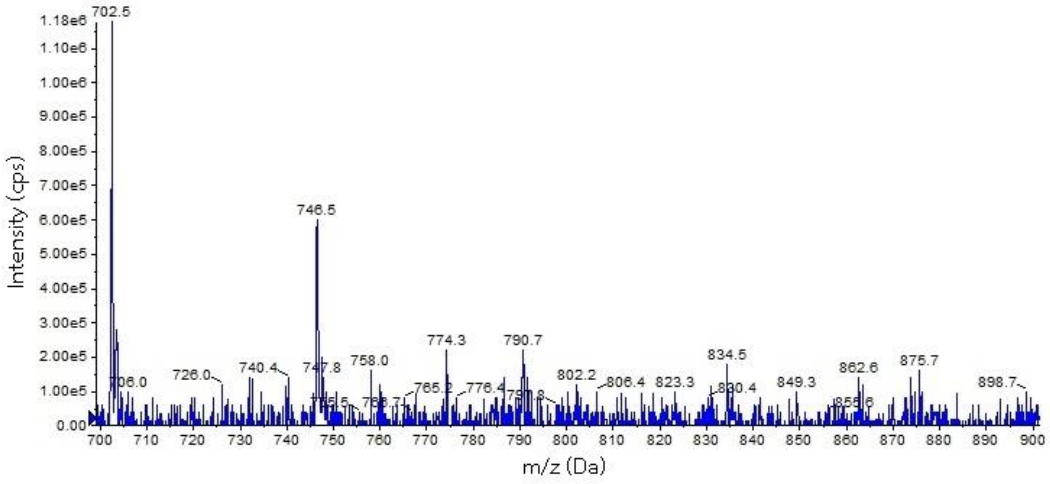

Figure 54 199 Da NLS spectra of *Nigella Sativa* seed extract showing the detection of CM-PE species.

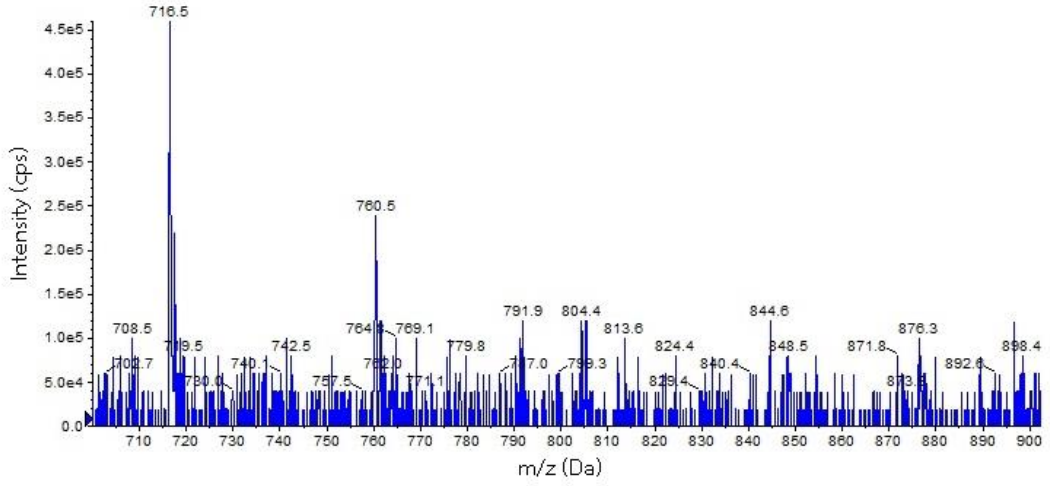

Figure 55 213 Da NLS spectra of *Nigella Sativa* seed extract showing the detection of CE-PE species.

Appendix C

An ethic approval of the experiment

The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University
Institute Building 2, 4 Floor, Soi Chulalongkorn 62, Phyat hai Rd., Bangkok 10330, Thailand,
Tel: 0-2218-8147 Fax: 0-2218-8147 E-mail: eccu@chula.ac.th

COA No. 064/2014

Certificate of Approval

Study Title No.004.1/57 : **INHIBITORY EFFECT OF *NIGELLA SATIVA* (BLACK CUMIN) SEED EXTRACT ON THE GLYCATION OF PHOSPHATIDYLETHANOLAMINE**

Principal Investigator : MS.MARISA MARNPAE

Place of Proposed Study/Institution : Faculty of Allied Health Sciences, Chulalongkorn University

The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University, Thailand, has approved constituted in accordance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) and/or Code of Conduct in Animal Use of NRCT version 2000.

Signature: ..... Signature: .....

(Associate Professor Prida Tasanapradit, M.D.) (Assistant Professor Dr. Nuntaree Chaichanawongsaroj)

Chairman Secretary

Date of Approval : 8 April 2014 Approval Expire date : 7 April 2015

The approval documents including

1) Research proposal
2) Patient/Participant Information Sheet and Informed Consent Form
3) Researcher
4) Questionnaire

Protocol No. 004.1/57
Date of Approval - 8 APR 2014
Approval Expire Date - 7 APR 2015

The approved investigator must comply with the following conditions:

1. *The research/project activities must end on the approval expired date of the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University (ECCU). In case the research/project is unable to complete within that date, the project extension can be applied one month prior to the ECCU approval expired date.*
2. *Strictly conduct the research/project activities as written in the proposal.*
3. *Using only the documents that bearing the ECCU's seal of approval with the subjects/volunteers (including subject information sheet, consent form, invitation letter for project/research participation (if available).*
4. *Report to the ECCU for any serious adverse events within 5 working days*
5. *Report to the ECCU for any change of the research/project activities prior to conduct the activities.*
6. *Final report (AF 03-12) and abstract is required for a one year (or less) research/project and report within 30 days after the completion of the research/project. For thesis, abstract is required and report within 30 days after the completion of the research/project.*
7. *Annual progress report is needed for a two- year (or more) research/project and submit the progress report before the expire date of certificate. After the completion of the research/project processes as No. 6.*

Appendix D

Screening Form

แบบสอบถามคุณสมบัติของผู้เข้าร่วมวิจัย

การศึกษาผลของสารสกัดเมล็ดเทียนตำต่อการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีน

Subject No.....

เพศ.....อายุ.....ปี น้ำหนัก.....กิโลกรัม ส่วนสูง.....เมตร ดัชนีมวลกาย.....

ความดันโลหิต ตัวบน.....mmHg ตัวล่าง.....mmHg

วัน/เดือน/ปีเกิด..... เบอร์โทรศัพท์.....

ให้ทำเครื่องหมาย ✓ ในช่องว่าง ☐ ที่กำหนดให้

1. ท่านมีอาการไม่สบาย เป็นไข้หวัด เจ็บคอ ปวดศีรษะ

☐ใช่ ☐ไม่ใช่

2. ท่านรับประทานอาหารเสริมหรือวิตามิน ภายใน 3 เดือนที่ผ่านมา

☐ใช่ ☐ไม่ใช่

3. ท่านรับประทานยาแก้อักเสบ หรือยาปฏิชีวนะ ภายใน 7 วันที่ผ่านมา

☐ใช่ ☐ไม่ใช่

3. ท่านมีประวัติการเป็นโรคดังต่อไปนี้หรือไม่

3.1 โรคอักเสบ เช่น ตับอักเสบ, ผิวหนังอักเสบ

☐มี ☐ไม่มี

3.2 โรคเรื้อรัง เช่น เบาหวาน, มะเร็ง

☐มี ☐ไม่มี

3.3 โรคติดเชื้อ เช่น เอดส์, ไวรัสตับอักเสบ

☐มี ☐ไม่มี

3.4 โรคทางภูมิคุ้มกัน เช่น ภูมิแพ้ , Systemic Lupus Erythrematosus (SLE)

☐มี ☐ไม่มี

3.5 โรคพิษสุราเรื้อรัง

☐มี ☐ไม่มี

4. ท่านอยู่ในภาวะตั้งครรภ์

☐ใช่ ☐ไม่ใช่

6. ท่านมีประวัติการสูบบุหรี่

☐ใช่ ☐ไม่ใช่

แบบสอบถามนี้อ้างอิงจากแบบสอบถามคุณสมบัติของผู้บริจาคเลือดสภากาชาดไทย โดยคัดเลือกมาเป็นบางข้อ เพื่อให้ตรงกับวัตถุประสงค์ของงานวิจัย

Appendix E

Participant Information Sheet

ข้อมูลสำหรับกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย

ชื่อโครงการวิจัย การศึกษาผลของสารสกัดเมล็ดเทียนดำต่อการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีน

ชื่อผู้วิจัย นางสาวมาริสา มารแพ้

ตำแหน่ง นิสิตปริญญาโท สาขาอาหารและโภชนาการ
คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สถานที่ติดต่อผู้วิจัย

(ที่ทำงาน1) ภาควิชาโภชนาการและการกำหนดอาหาร คณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
154 ซ.จุฬา 12 ถ.พระราม1แขวงวังใหม่ เขตปทุมวัน กรุงเทพมหานคร 10330
โทรศัพท์02-218-1116โทรสาร 02-218-1116

(ที่ทำงาน2) ศูนย์วิทยาศาสตร์การแพทย์ จุฬาลงกรณ์มหาวิทยาลัย
254อาคารวิจัยจุฬาลงกรณ์มหาวิทยาลัยชั้น 11
แขวงวังใหม่ เขตปทุมวัน กรุงเทพมหานคร 10330
โทรศัพท์02-218-1054 โทรสาร 02-218-1105
โทรศัพท์มือถือ 084-707-513 Email: marisa_6401@hotmail.com

เอกสารชุดนี้ได้อธิบายถึงวัตถุประสงค์และขั้นตอนการดำเนินการวิจัย ซึ่งผู้เข้าร่วมวิจัยได้รับเชิญให้เข้าร่วมโครงการวิจัยนี้ ก่อนที่ท่านจะตัดสินใจเข้าร่วมในการวิจัย มีความจำเป็นที่ท่านควรทำความเข้าใจว่างานวิจัยนี้ทำเพราะเหตุใด และเกี่ยวข้องกับอะไร กรุณาใช้เวลาในการอ่านข้อมูลต่อไปนี้อย่างละเอียดรอบคอบ และสอบถามข้อมูลเพิ่มเติมหรือข้อมูลที่ไม่ชัดเจนได้ตลอดเวลา โปรดเก็บเอกสารนี้ไว้เพื่อเป็นเอกสารอ้างอิงต่อไป

1. โครงการวิจัยนี้เกี่ยวข้องกับ

โครงการวิจัยนี้เกี่ยวข้องกับการศึกษาฤทธิ์ของสารสกัดจากเมล็ดเทียนดำในการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีนบนเซลล์เม็ดเลือดแดง โดยจะมีการใช้เซลล์เม็ดเลือดแดงที่แยกได้จากเลือดของอาสาสมัครที่มีสุขภาพดี มาเป็นโมเดลสำหรับการศึกษาไกลเคชั่นในหลอดทดลอง

2. วัตถุประสงค์ของการวิจัย

เพื่อศึกษาฤทธิ์ของสารสกัดจากเมล็ดเทียนดำในการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีนบนเซลล์เม็ดเลือดแดง

3. รายละเอียดของกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย

ในการทดลองจะใช้เลือดจากกลุ่มอาสาสมัครสุขภาพดีที่สมัครใจเข้าร่วมโครงการวิจัย จำนวน 10 คนซึ่งมีคุณสมบัติต่างๆ ดังนี้

เกณฑ์คัดเข้า

- เป็นผู้มีสุขภาพดี อายุระหว่าง 20-50 ปี เพศชายหรือหญิง
- มีดัชนีมวลกาย (BMI) ต่ำกว่า 23.0 กิโลกรัม ต่อ ตารางเมตร
- ไม่มีประวัติการสูบบุหรี่
- *ไม่เป็นโรคพิษสุราเรื้อรัง*
- มีความดันโลหิตปกติ
- มีระดับกลูโคสในเลือดน้อยกว่า *110* มก./ดล.
- มีระดับของไขมันในเลือดปกติ คือ คอเลสเตอรอลรวมน้อยกว่า 200 มก./ดล. ไตรกลีเซอไรด์น้อยกว่า150 มก./ดล. และแอลดีแอล น้อยกว่า130 มก./ดล.
- ไม่ได้อยู่ในระหว่างการรักษาโรคใช้ยาหรือผลิตภัณฑ์เสริมอาหารที่มีผลต่อระดับไขมันในเลือดในช่วง 3 เดือนก่อนเข้ารับการทดสอบ

เกณฑ์คัดออก

- *อยู่ระหว่างเข้าร่วมงานวิจัยอื่นที่ต้องมีการได้รับหรือบริโภคอาหารหรือสารทดสอบต่างๆ ที่มีผลให้เกิดการเปลี่ยนแปลงต่อระดับน้ำตาลและไขมันในเลือด*
- มีผลการตรวจการทำงานของตับ *(AST และ ALT)* หรือไต *(BUN และ creatinine)* ผิดปกติ
- *มีผลการตรวจความสมบูรณ์ของเม็ดเลือดผิดปกติ*

เหตุผลที่อาสาสมัครได้รับเชิญเข้าร่วมโครงการวิจัยเนื่องจากอาสาสมัครมีคุณสมบัติตามเกณฑ์ที่กำหนดและมีความสะดวกที่จะสามารถเข้าร่วมงานวิจัยได้

4. วิธีการคัดกรองผู้เข้าร่วมวิจัย

ขั้นแรก ผู้เข้าร่วมวิจัยจะได้รับการซักประวัติการเจ็บป่วยในอดีตที่ผ่านมาและในปัจจุบัน ประวัติการใช้ยา ผลิตภัณฑ์เสริมอาหาร วิตามินบำรุง หรือสารต้านอนุมูลอิสระในอดีตและในเวลา 3 เดือนที่ผ่านมา รวมถึงข้อมูลผลการตรวจความสมบูรณ์ของเม็ดเลือด ระดับไขมัน น้ำตาลและการทำงานของตับและไตในเลือดใน 1 เดือนที่ผ่านมา (หากมี) เพื่อคัดกรองผู้สมัครเข้าร่วมโครงการที่มีคุณสมบัติเข้าเกณฑ์ที่กำหนด

ขั้นต่อมาผู้วิจัยจะชี้แจ้งรายละเอียดของโครงการวิจัยและให้เอกสารข้อมูลสำหรับผู้มีส่วนร่วมในการวิจัยก่อนเพื่อพิจารณาก่อนสมัครใจลงนาม เมื่อผู้สมัครยินดีตกลงเข้าร่วมการวิจัยและลงนามในเอกสารยืนยันการยินยอมเข้าร่วม โครงการ ผู้วิจัยจะให้เอกสารชุดนี้แก่ผู้เข้าร่วมวิจัยพร้อมทั้งสำเนาการลงนามเอกสารยืนยันการยินยอมเข้าร่วมโครงการ

จากนั้นจะมีการชั่งน้ำหนัก วัดส่วนสูง และความดันโลหิตของผู้เข้าร่วมวิจัย ถ้าผู้เข้าร่วมวิจัยไม่มีข้อมูลผลการตรวจทางชีวเคมีในเลือดไม่เกิน1 เดือนที่ผ่านมา ผู้เข้าร่วมวิจัยจะได้รับการเจาะเลือดไม่เกิน 15 มิลลิลิตร (ประมาณ 1 ช้อนโต๊ะ) เพื่อตรวจระดับไขมันและน้ำตาลในเลือด ความสมบูรณ์ของเม็ดเลือดตลอดจนระดับค่าการทำงานของตับและไต โดยการตรวจทางชีวเคมีในเลือดก่อนดำเนินการวิจัย ทั้งนี้ผู้วิจัยจะรับผิดชอบค่าใช้จ่ายในการตรวจเลือดในส่วนของการตรวจคัดกรองนี้

ในกรณีที่ผู้เข้าร่วมวิจัยไม่มีคุณสมบัติตามที่กำหนดไว้ เป็นเหตุให้ผู้วิจัยจำเป็นต้องคัดผู้เข้าร่วมวิจัยออกจากโครงการวิจัย ผู้วิจัยจะติดต่อและแจ้งให้ผู้เข้าร่วมวิจัยทราบโดยเร็วในกรณีหากพบผลเลือดผิดปกติ ผู้เข้าร่วมวิจัยจะได้รับทราบผลเลือด คำแนะนำในการปฏิบัติตัว และเพื่อทำการรักษาต่อไป

5. รูปแบบและกระบวนการการวิจัยที่กระทำต่อผู้มีส่วนร่วมในการวิจัย

รูปแบบ

การทดลองจะเป็นการเก็บตัวอย่างเลือดจากผู้เข้าร่วมวิจัย เพื่อนำมาแยกเซลล์เม็ดเลือดแดง โดยผู้ร่วมวิจัยไม่ต้องเข้ารับการทดสอบ การบริโภคอาหาร หรือยาใดๆ

วิธีดำเนินการทดลอง

ผู้เข้าร่วมวิจัยที่ผ่านเกณฑ์การคัดเข้าจะได้รับการนัดหมายให้มาพบผู้วิจัยครั้งที่สองภายในเวลาไม่เกินหนึ่งสัปดาห์ ซึ่งเป็นการนัดเพื่อทำการเก็บตัวอย่างเลือด

ก่อนถึงกำหนดนัดหมาย ผู้เข้าร่วมวิจัยจะได้รับการแนะนำทางด้านโภชนาการเพื่อให้รักษารูปแบบการบริโภคอาหารและการใช้ชีวิตประจำวันให้คงที่ก่อนเข้ารับการเก็บตัวอย่างเลือด1 สัปดาห์โดยระหว่างนี้ผู้เข้าร่วมวิจัยสามารถใช้ชีวิตได้ตามปกติยกเว้น ต้องไม่รับประทานยา วิตามินบำรุง หรือผลิตภัณฑ์เสริมอาหารใดๆ รวมถึงงดดื่มเครื่องดื่มแอลกอฮอล์ จนถึงวันเก็บตัวอย่างเลือด นอกจากนี้ ผู้เข้าร่วมวิจัยจะได้รับการแนะนำเพื่อบันทึกประวัติการบริโภคอาหาร 3 วัน

การทดลองจะดำเนินการในวันที่ผู้เข้าร่วมวิจัยสามารถเข้าร่วมได้ โดยไม่กระทบเวลาในการทำงานหรือการเรียน หรือ อาจเป็นวันหยุดราชการหรือวันหยุดนักขัตฤกษ์ที่ได้มีการนัดหมายไว้ล่วงหน้า เป็นต้น โดยในการทดลอง *ผู้วิจัยจะขอให้ผู้เข้าร่วม*งดอาหารและเครื่องดื่มต่างๆ (ยกเว้น น้ำเปล่าธรรมดาซึ่งดื่มได้เพียงเล็กน้อย) เป็นเวลา 12 ชั่วโมง โดยเริ่มงดอาหารตั้งแต่ 2 ทุ่มของวันก่อนเข้าทำการทดลอง เป็นต้นไป จากนั้น ผู้เข้าร่วมวิจัยจะเริ่มทำการทดลองโดยได้รับการเจาะเลือดโดยนักเทคนิคการแพทย์ โดยเจาะไม่เกิน 15 มิลลิลิตร (ประมาณ 1 ช้อนโต๊ะ) บริเวณข้อพับแขน เป็นจำนวน 1 ครั้ง ณ เวลา 8.00 น.

หลังจากการเจาะเลือดแล้วเสร็จ ผู้เข้าร่วมวิจัยพร้อมรับประทานอาหารว่างที่ผู้วิจัยได้จัดเตรียมไว้ หลังจากนั้นผู้เข้าร่วมวิจัยสามารถเดินทางกลับได้

6. สถานที่ทำการวิจัย

ภาควิชาโภชนาการและการกำหนดอาหาร คณะสหเวชศาสตร์ และ ศูนย์วิทยาศาสตร์การแพทย์ จุฬาลงกรณ์มหาวิทยาลัย

7. ความเสี่ยงหรือความไม่สบายที่อาจเกิดขึ้นกับผู้เข้าร่วมวิจัย

1. ผู้เข้าร่วมวิจัยอาจเกิดความเจ็บปวด วิตกกังวล หรือเส้นเลือดเขียวช้ำจากการเจาะเลือด แต่ความเสี่ยงนี้เป็นสิ่งปกติที่อาจเกิดขึ้นได้เมื่อมีการเจาะเลือด

ผู้วิจัยได้เตรียมการดังนี้ :

- การเจาะเลือดทำโดยนักเทคนิคการแพทย์ผู้ชำนาญการเจาะเลือดจากคณะสหเวชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย โดยใช้เข็มเจาะเลือดที่ใช้ครั้งเดียวเฉพาะแต่ละคน จึงไม่มีความเสี่ยงต่อการติดเชื้อ และผู้เจาะเลือดกระทำด้วยความระมัดระวังในการแทงเข็ม

- มีการอธิบายให้ผู้เข้าร่วมวิจัยเข้าใจเพื่อคลายความวิตกกังวล และแนะนำให้ใช้น้ำแข็งประคบ เพื่อลดการขยายตัวของรอยช้ำนั้น รอยช้ำนี้จะค่อยๆ จางลงและไม่มีอันตรายใด ๆ

2. ผู้เข้าร่วมวิจัยต้องงดอาหารเพื่อมารับการเจาะเลือด อาจมีภาวะหน้ามืดหรือหมดสติเนื่องจากระดับน้ำตาลในเลือดต่ำ

ผู้วิจัยได้เตรียมการดังนี้ :

- สังเกตและเฝ้าดูอาการของผู้เข้าร่วมวิจัยอย่างใกล้ชิด

- แนะนำผู้เข้าร่วมวิจัยว่าถ้ามีอาการเหงื่อออก มือเท้าสั่น รู้สึกหิวจัด อ่อนเพลีย หน้ามืดคล้ายจะเป็นลม หรือมีอาการผิดปกติใดๆให้รีบบอกให้ผู้วิจัยทราบ และหยุดทำการวิจัยทันทีพร้อมทั้งส่งต่อให้แพทย์ดูแลต่อไป

- ผู้วิจัยเตรียมน้ำหวานสำหรับผู้เข้าร่วมวิจัยที่มีอาการดังกล่าว

8. การดูแลรักษาเมื่อเจ็บป่วยหรือจากผลไม่พึงประสงค์จากการวิจัย

ระหว่างดำเนินการวิจัย ผู้วิจัยจะระมัดระวังในการทำวิจัยโดยปฏิบัติตามระเบียบและวิธีวิจัยอย่างเคร่งครัด เฝ้าติดตามและประเมินอาการของผู้เข้าร่วมวิจัยเป็นระยะ ถ้ามีเหตุการณ์อันไม่พึงประสงค์เกิดขึ้นกับผู้เข้าร่วมวิจัย ผู้วิจัยมีความพร้อมที่จะส่งต่อให้แพทย์ดูแลอย่างใกล้ชิดและทันท่วงที หากเกิดการเจ็บป่วยอันเนื่องมาจากการวิจัย ผู้เข้าร่วมวิจัยจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่าตามมาตรฐานวิชาชีพ

9. วิธีการพิทักษ์สิทธิ์กลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย

ข้อมูลที่เกี่ยวข้องกับท่านจะเก็บเป็นความลับ โดยข้อมูลต่างๆ ระหว่างวิจัยจะอ้างอิงเฉพาะหมายเลขของผู้เข้าร่วมวิจัยและอักษรย่อของชื่อและนามสกุลเท่านั้น หากมีการเสนอผลการวิจัยจะเสนอเป็นภาพรวม ข้อมูลใดที่สามารถระบุถึงตัวท่านได้จะไม่ปรากฏในรายงาน

10. วิธีการเก็บและทำลายเลือดหลังสิ้นสุดการวิจัย

ผู้วิจัยจะไม่เก็บเลือดของผู้เข้าร่วมวิจัยเพื่อใช้ในการทดลองอื่นใดนอกเหนือจากที่ระบุในโครงการวิจัยนี้ เมื่อสิ้นสุดการวิจัย ผู้วิจัยจะทำลายเลือดและส่วนประกอบใดๆที่ได้จากเลือดของผู้เข้าร่วมวิจัยที่เหลือจากการทดลอง โดยแช่สารฆ่าเชื้อก่อนที่จะทำลายทิ้งทั้งหมด

11. ประโยชน์ที่คาดว่าจะได้รับจากโครงการวิจัย

1.ผู้มีส่วนร่วมวิจัยได้ทราบถึงระดับของน้ำตาลและไขมันในเลือดในช่วงอดอาหาร รวมถึงความสมบูรณ์ของเม็ดเลือดและค่าเกี่ยวกับตับและไต ซึ่งสามารถบ่งชี้ถึงภาวะสุขภาพและการเป็นการตรวจสุขภาพทางหนึ่ง

2. ทำให้ทราบว่าเมล็ดเทียนตำมีผลดีต่อการลดการเกิดปฏิกิริยาไกลเคชั่นที่ทำให้เกิดโรคแทรกซ้อนในผู้ป่วยเบาหวานหรือไม่ซึ่งจะสามารถนำความรู้ที่ได้จากการวิจัยไปต่อยอดงานวิจัยในการทดสอบการบริโภคเมล็ดเทียนตำในคน และใช้ในการแนะนำให้ความรู้แก่ประชาชนทั่วไปในเรื่องของผลของสารสกัดจากเมล็ดเทียนตำต่อสุขภาพในเชิงโภชนาการเกี่ยวกับโรคเบาหวานต่อไป

12. ค่าตินทางหรือค่าเสียเวลา

การวิจัยนี้ไม่มีการจ่ายค่าพาหนะ ค่าชดเชยการเสียเวลาให้กับผู้เข้าร่วมวิจัย

13. การเข้าร่วมในการวิจัยของท่านเป็นโดยสมัครใจ และสามารถปฏิเสธที่จะเข้าร่วมหรือถอนตัวจากการวิจัยได้ทุกขณะ โดยไม่ต้องให้เหตุผลและไม่สูญเสียประโยชน์ที่พึงได้รับ ทั้งนี้รวมถึงสิทธิ์ในการได้รับการดูแลลดความเสี่ยงและการไม่สบายอันเกิดจากการเจาะเลือดในระหว่างคัดกรองด้วย

14. หากท่านไม่ได้รับการปฏิบัติตามข้อมูลดังกล่าวสามารถร้องเรียนได้ที่ คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย ชั้น 4 อาคารสถาบัน 2 ซอยจุฬาลงกรณ์ 62 ถนนพญาไท เขตปทุมวัน กรุงเทพฯ 10330 โทรศัพท์ 0-2218-8147 หรือ 0-2218-8141 โทรสาร 0-2218-8147 E-mail:eccu@chula.ac.th

Appendix F

Informed Consent Form

AF 05-07

หนังสือแสดงความยินยอมเข้าร่วมการวิจัย

ทำที่.....จุฬาลงกรณ์มหาวิทยาลัย.....

วันที่.....เดือน.....พ.ศ. .....

เลขที่ ประชากรตัวอย่างหรือผู้มีส่วนร่วมในการวิจัย.....

ข้าพเจ้า ซึ่งได้ลงนามท้ายหนังสือนี้ ขอแสดงความยินยอมเข้าร่วมโครงการวิจัย

ชื่อโครงการวิจัย การศึกษาผลของสารสกัดเมล็ดเทียนดำต่อการยับยั้งการเกิดไกลเคชั่นของฟอสฟาทิดิลเอทาโนลามีน

ผู้วิจัย นางสาวมาริสา มารแพ้

ที่อยู่ที่ติดต่อ ภาควิชาโภชนาการและการกำหนดอาหาร คณะสหเวชศาสตร์ จุฬาฯ เลขที่โครงการวิจัย 004-1/57

เลขที่ 154 ถ.พระราม 1 วังใหม่ ปทุมวัน กรุงเทพฯ 10330 วันที่รับรอง - 8 เม.ย. 2557

โทรศัพท์ 084-707-5135 E-mail: marisa_6401@hotmail.com วันหมดอายุ - 7 เม.ย. 2558

ข้าพเจ้า **ได้รับทราบรายละเอียดเกี่ยวกับที่มาและ วัตถุประสงค์ในการทำวิจัย** รายละเอียดขั้นตอนต่างๆ ที่จะต้องปฏิบัติหรือได้รับการปฏิบัติ ความเสี่ยง/อันตราย และประโยชน์ซึ่งจะเกิดขึ้นจากการวิจัยเรื่องนี้ โดยได้อ่านรายละเอียดในเอกสารชี้แจงผู้เข้าร่วมการวิจัยโดยตลอด และ**ได้รับคำอธิบายจากผู้วิจัย จนเข้าใจเป็นอย่างดีแล้ว**

ข้าพเจ้าจึงสมัครใจเข้าร่วมในโครงการวิจัยนี้ ตามที่ระบุไว้ในเอกสารชี้แจงผู้เข้าร่วมการวิจัย โดยข้าพเจ้ายินยอม *เจาะเลือด 2 ครั้ง ครั้งที่ 1 จำนวน 15 มิลลิลิตร หรือประมาณ 1 ช้อนโต๊ะ เพื่อคัดกรอง หากผ่านเกณฑ์จะยินยอมเจาะเลือดครั้งที่ 2 จำนวน 15 มิลลิลิตร*

ข้าพเจ้ามีสิทธิถอนตัวออกจากการวิจัยเมื่อใดก็ได้ตามความประสงค์ โดย**ไม่ต้องแจ้งเหตุผล** ซึ่งการถอนตัวออกจากการวิจัยนั้น จะไม่มีผลกระทบในทางใดๆ ต่อข้าพเจ้าทั้งสิ้น

ข้าพเจ้าได้รับคำรับรองว่า ผู้วิจัยจะปฏิบัติต่อข้าพเจ้าตามข้อมูลที่ระบุไว้ในเอกสารชี้แจงผู้เข้าร่วมการวิจัย และข้อมูลใดๆ ที่เกี่ยวข้องกับข้าพเจ้า ผู้วิจัยจะเก็บรักษาเป็นความลับ โดยจะนำเสนอข้อมูลการวิจัยเป็นภาพรวมเท่านั้น ไม่มีข้อมูลใดในการรายงานที่จะนำไปสู่การระบุตัวข้าพเจ้า

หากข้าพเจ้าไม่ได้รับการปฏิบัติตรงตามที่ได้ระบุไว้ในเอกสารชี้แจงผู้เข้าร่วมการวิจัย ข้าพเจ้าสามารถร้องเรียนได้ที่คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย ชั้น 4 อาคารสถาบัน 2 ซอยจุฬาลงกรณ์ 62 ถนนพญาไท เขตปทุมวัน กรุงเทพฯ 10330 โทรศัพท์ 0-2218-8147, 0-2218-8141 โทรสาร 0-2218-8147 **E-mail: eccu@chula.ac.th**

ข้าพเจ้าได้ลงลายมือชื่อไว้เป็นสำคัญต่อหน้าพยาน ทั้งนี้ข้าพเจ้าได้รับสำเนาเอกสารชี้แจงผู้เข้าร่วมการวิจัย และสำเนาหนังสือแสดงความยินยอมไว้แล้ว

ลงชื่อ.....
(นางสาวมาริสา มารแพ้)
ผู้วิจัยหลัก

ลงชื่อ.....
(.....)
ผู้มีส่วนร่วมในการวิจัย

ลงชื่อ.....
(.....)
พยาน

ปรับปรุงเมื่อ 23 พฤษภาคม 2554

Appendix G

3- Day Food Record Form

เพิ่มเติมรายละเอียดด้านหลังได้

แบบบันทึกการรับประทานอาหารในรอบ 24 ชั่วโมงของวันที่บันทึก

รหัสผู้เข้าร่วม.....วันเดือนปีที่บันทึกข้อมูล .....บันทึกวันที่ ☐ วันที่1 ☐ วันที่2 ☐ วันที่3

มื้อ	เมนูที่รับประทาน	ส่วนประกอบ	ข้าว/ก๋วยเตี๋ยว (ทัพพี)	ไข่ (ฟอง)	เนื้อสัตว์ (ช้อนโต๊ะ)	ไขมัน (ช้อนชา)	ผักดิบ (ทัพพี)	ผักสุก (ทัพพี)	ผลไม้ (ผล, คำ)	นม (แก้ว, มล.)	เครื่องดื่ม (มล.)	ผลิตภัณฑ์เสริมอาหาร (มิลลิกรัม)
มื้อเช้า ตั้งแต่ตื่นนอน	....	....										
กลางวัน	....	....										
เย็น จนถึงเข้านอน	....	....										

VITA

Miss Marisa Marnpae was born on 9th February 1987 in Phranakorn Sri Ayutthaya, Thailand. She graduated with Bachelor degree of Science in Food Science and Technology with first class honors and Bhumipol Award for Academic Excellence from Thammasat University in 2009. Then, in 2012 she entered the Master of Science Program in Food and Nutrition, Department of Nutrition and Dietetics, Faculty of Allied Health Science, Chulalongkorn University and majored in Applied Food and Nutrition.