In Vitro Inhibitory Potential of Ethanolic Extract from *Nigella Sativa* Seed against Glucose-Induced Lipid Glycation

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Abstract

This research investigated the inhibitory effect of *Nigella sativa* (NS) seed extract on glucose-induced lipid glycation by using phosphatidylethanolamine (PE) as a model. The anti-lipid glycation assay was conducted by incubating the Dioleoyl-PE (DOPE) with glucose at 37°C for 48 hr in the presence of various concentration of ethanolic NS seed extract (NSE) ranging from 5 to 40 mg/ml. Glycated-PE and carboxymethyl-PE (CM-PE) were analyzed by QTRAP mass spectrometer. The results showed that the highest concentration of NSE (40 mg/ml) significantly reduced the glycated-PE formation whereas the aminoguanidine (AG) failed to inhibit. All concentrations of NSE can inhibit the CM-PE formation, of which the reduction was greater in NSE compared to AG with the same concentration of 5 mg/ml. The significantly inverse correlations between glycated-PE and CM-PE formation with NSE concentration were also found (r = -0.919 and -0.885, respectively). Thus, our findings concluded that the ethanolic NSE had a potential to attenuate glucose-induced lipid glycation.

Keywords: Lipid glycation, *Nigella sativa*, Phosphatidylethanolamine, Thymoquinone (TQ)

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Introduction

Long-term chronic hyperglycemia is a major cause of the progression of diabetic complications which include the macrovascular and microvascular complications (Forbes and Cooper, 2013). One of the factors that can accelerate such complications is involved with the glycation reaction. Glycation is a non-enzymatic reaction between the carbonyl group of a reducing sugar and the free amino group of proteins, lipids or nucleic acids (Ahmed, 2005). The reaction between glucose and the amino group of protein forms a reversible Schiff base which further rearranges to a fructosamine group or Amadori product and then, finally yields the advanced glycation end products (AGEs) (Ansari and Dash, 2013). AGEs accumulates in vascular wall tissues and plasma lipoproteins, and they can also bind to AGE receptors (RAGEs) to activate many biological processes involved in the development of diabetic complications (Ahmed, 2005; Ramasamy et al., 2011; Ansari and Dash, 2013; Yamagishi et al., 2015). Apart from protein glycation, this reaction also occurs between glucose and lipid, especially amino-containing lipid called lipid glycation (Bucala et al., 1993; Miyazawa et al., 2012; Suzuki et al., 2014). Under hyperglycaemic conditions, phosphatidylethanolamine (PE), the most abundant aminophospholipid in the cell membrane, has been found to rapidly condense with glucose, resulting in the production of glycated lipid species (Bucala et al., 1993). These species include Schiff base- and Amadori-PE product, moreover, of which the latter can further undergo the complex reactions to form PE-linked advanced glycation end products (AGE-PE) which consists of Carboxymethyl-PE (CM-PE) and Carboxyethyl-PE (CE-PE) (Bucala et al., 1993; Requena et al., 1997). The products of lipid glycation have been found to have the ability to stimulate the inflammatory cytokines production, lipid peroxidation and angiogenesis (Oak et al., 2000; Oak et al., 2003; Simões et al., 2013). In addition, the physical and biological properties of the membrane phospholipid bilayer have been reported to be affected by the lipid glycation (Naudi et al., 2013).

To inhibit the glycation reaction, the synthetic compound such as aminoguanidine (AG), the common anti-glycation agent, has been widely used for inhibiting the AGEs formation both in vitro and in vivo studies (Luo et al., 2012; Nagai et al., 2012). Unfortunately, AG has been reported to have some toxicity and was withdrawn from the crucial phase III of clinical trials due to the safety concerns and lack of efficacy (Freedman et al., 1999). Therefore, the natural products including medicinal plants and herbs have currently become an alternative agent for testing their anti-glycation properties (Elosta et al., 2012; Kazeem et al., 2012; Ramkissoon et al., 2013; Aljohi et al., 2016).

Nigella sativa L., also known as Habbatu Sawda, Kalonji, black cumin, or black seed, is an aromatic plant in the Ranunculaceae family that is commonly found in the Mediterranean region, Pakistan and India and has been traditionally used for thousands of years as a spice and as a therapeutic agent in traditional folk and Islamic medicine (Gali-Muhtasib et al., 2006). The pharmacological properties of *Nigella sativa* includes antioxidant, anti-microbial, anti-inflammation, immunomodulation and anticancer properties which have been investigated (Dilshad et al., 2012; Duncker et al., 2012; Aftab et al., 2013; Singh et al., 2014; Agbaria et al., 2015; Amin and Hosseinzadeh, 2016; Gholamnezhad et al., 2016;). Previous study has demonstrated the various phytochemical compounds in different extracts from *Nigella sativa* seed including alkaloids, steroids, terpenoids, flavonoids, phenols and tannins (Shafodino et al. 2022) In addition, thymoquinone (TQ), one of the major active compound found in *Nigella sativa* seed, has been found to play an important role with many beneficial effects (Rajput et al., 2013; Darakhshan et al., 2015). For antidiabetic properties, the glucose-lowering effects of *Nigella sativa* have been clearly observed (Najmi et al., 2012; Heshmati and Namazi, 2015; Gray et al., 2016). However, the inhibitory effect of *Nigella sativa* against glycation especially lipid glycation, has not been investigated. Therefore, the aim of this study was to determine the inhibitory effects of *Nigella sativa* seed extract (NSE) on the formation of PE glycation. The products from early and late stage of PE glycation induced by glucose were detected and identified by mass spectrometric technique.

Materials and methods

1. Chemicals and materials

Nigella sativa seed was imported from India by a local distributor. D-glucose, 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (Dioleoly-PE; DOPE), 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine (DMPE), aminoguanidine (AG), dimethylsulfoxide (DMSO) and thymoquinone (TQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All HPLC grade solvents, analytical grade chemicals and solvents were from Merck (Darmstadt, Germany). Ultrapure water was produced from the Millipore Milli-Q purification system (Bedford, MA, USA).

2. Preparation of *Nigella sativa* seed extract (NSE)

The extraction protocol was conducted according to the method from a previous study with some modifications (Farah, 2005). Briefly, *Nigella sativa* seed was ground with a clean electric grinder. Twenty grams of seed powder was mixed with 200 ml of 95% ethanol solution. After vigorous shaking, the mixture was left overnight at 4°C in a refrigerator. The mixture was then filtered through Whatman No. 1 filter paper and the filtrate was evaporated by a rotary evaporator (Buchi Labortechnik, AG, Switzerland). The final NSE was collected and stored under nitrogen gas at 4°C in the dark until usage.

3. Determination of thymoquinone (TQ) in NSE by reverse phase HPLC

The quantitative analysis of TQ content in NSE was conducted according to the previous method with modifications (Ghosheh et al., 1999). HPLC analysis was performed on LC-20A HPLC system (Shimadzu, Tokyo, Japan) with binary pump system and UV detector. The chromatographic separation was done on reversed phase mode using an Inertsil ODS-3, C18 column (4.6 mm i.d. x 250 mm x 5µm). The mobile phase was composed of methanol and water (80:20 v/v). The isocratic elution with the flow rate of 1.0 ml/min was used. The UV wavelength was set at 254 nm for TQ detection. A 10 µl of NSE was dissolved in DMSO and further diluted with methanol. A 20 µl of diluted TQ sample was injected to the HPLC system. TQ concentration in NSE was quantified by using the TQ calibration curve.

4. Anti-lipid glycation assay

The stock solution of 3 mM DOPE and 400 mg/ml NSE were prepared in methanol and DMSO, respectively. Phosphate buffer saline (0.1 M, pH 7.4) was used to prepare the stock solution of 2.5 M D-glucose and 50 mg/ml AG. The *in vitro* glycation of DOPE was conducted according to the method described previously with slight modification (Shoji et al., 2010). Briefly, DOPE (0.3 mM) was incubated with D-glucose (500mM) in 70% methanolic phosphate buffer (0.1 M, pH 7.4) in the absence (negative control) or presence of NSE at the concentration of 5, 10, 20 and 40 mg/ml in a total volume of 1 ml. A 5 mg/ml of AG was used as a positive control. The mixture was then incubated at 37°C, 500 rpm in a shaking incubator (Labnet International, NJ, USA) for 48 hr. The experiment was done in triplicates.

5. Lipid extraction

After incubation, 0.8 ml of the mixture was transferred to a screw cap tube and 200 µl of 1 mM DMPE in methanol was then added as the internal standard. Lipid was extracted by using Bligh and Dyer method with some modifications (Bligh and Dyer, 1959). Briefly, a 3 ml of dichloromethane: methanol

(1:2 v/v) was added and the mixture was vigorously mixed. In addition, 1 ml of dichloromethane was added and then followed by 1 ml of ultrapure water. The mixture was then mixed and centrifuged at 2,000 rpm, 4°C for 10 min. The lower phase containing lipid was collected. Lipid extract was dried under nitrogen stream at 40°C by using sample concentrator (Stuart Scientific, UK). The dried lipid extract was kept at -20°C until analysis.

6. Flow injection-mass spectrometric analysis of glycated-PE and CM-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. The dried lipid extract was reconstituted with 1 ml of dichloromethane and methanol (2:1, v/v). Mobile phase containing methanol /water/formic acid/ ammonia (80:19.7:0.1:0.2 v/v/v/v) was delivered to the system with the flow rate of 0.4 ml/min by an Agilent 1290 Infinity Binary Pump (Palo Alto, CA, USA). Injection volume was 20 µL and the analysis was done without chromatographic separation. Mass spectrometric condition was set as follows: nitrogen gas for nebulizer, dried and curtain gas were 40, 50 and 20 psi, respectively. Spray voltage was 5,500 volts in positive mode. The neutral loss scans (NLS) of 141, 303 and 199 Da were used to detect native PE, glycated-PE and CM-PE, respectively (Nakagawa et al., 2005; Shoji et al., 2010). Data acquisition and analysis were performed by Analyst software version 1.6 (AB SCIEX, CA, USA). The mass peak areas of glycated-PE and CM-PE were calculated and expressed as percentage relative to that of native PE.

7. Statistical analysis

Data were expressed as mean ± SEM. The differences of PE glycation products among treatments were tested by one-way ANOVA followed by Tukey post hoc multiple comparison. The dose-response relationships between NSE concentration and PE glycation products were analyzed by Pearson's correlation analysis with $p<0.05$ being considered as statistical significance. SPSS version 17.0 software (Chicago, IL, USA) was used.

Results

1. TQ content in NSE

The active compound, TQ in ethanolic extract from *Nigella sativa* seed was analyzed by reverse phase HPLC and the chromatogram is shown in Figure 1. As compared with the TQ standard, the peak of TQ (Retention time ~ 6.5 min) was found to be highest among the others found in the NSE. Moreover, the quantitative analysis revealed that the concentration of TQ in NSE used in this study was approximately 7,831.71±346.06 µg/ml.

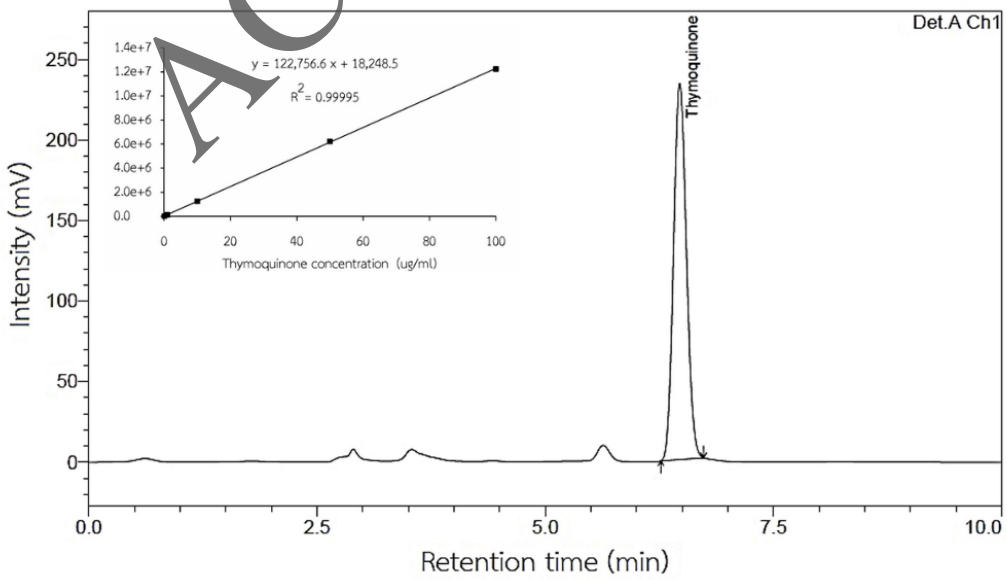

Fig.1 The reverse phase HPLC chromatogram of NSE presenting the predominant peak of TQ and related calibration curve (inset). Preparation of NSE and HPLC condition were described in materials and methods section.

2. The formation of glycated-PE and CM-PE

After 48 hr of incubation, the glycated-PE and AGE-PE were extracted from the reaction mixtures and then identified by the QTRAP mass spectrometer with specific NLS. In the presence of glucose, as shown in Figure. 2A, the mass spectrum obtained from NLS of 303 Da revealed the most abundant peak of glycated-PE (m/z 906.8, $[M+H]^+$) which was the product from the condensation between glucose and amino group of DOPE. For CM-PE, the late stage product of lipid glycation, the mass peak of m/z 802.8, $[M+H]^+$ obtained from specific NLS of 199 Da was also detected, however, the intensity of such a peak was found to be lower than that of glycated-PE as seen in Figure 2B.

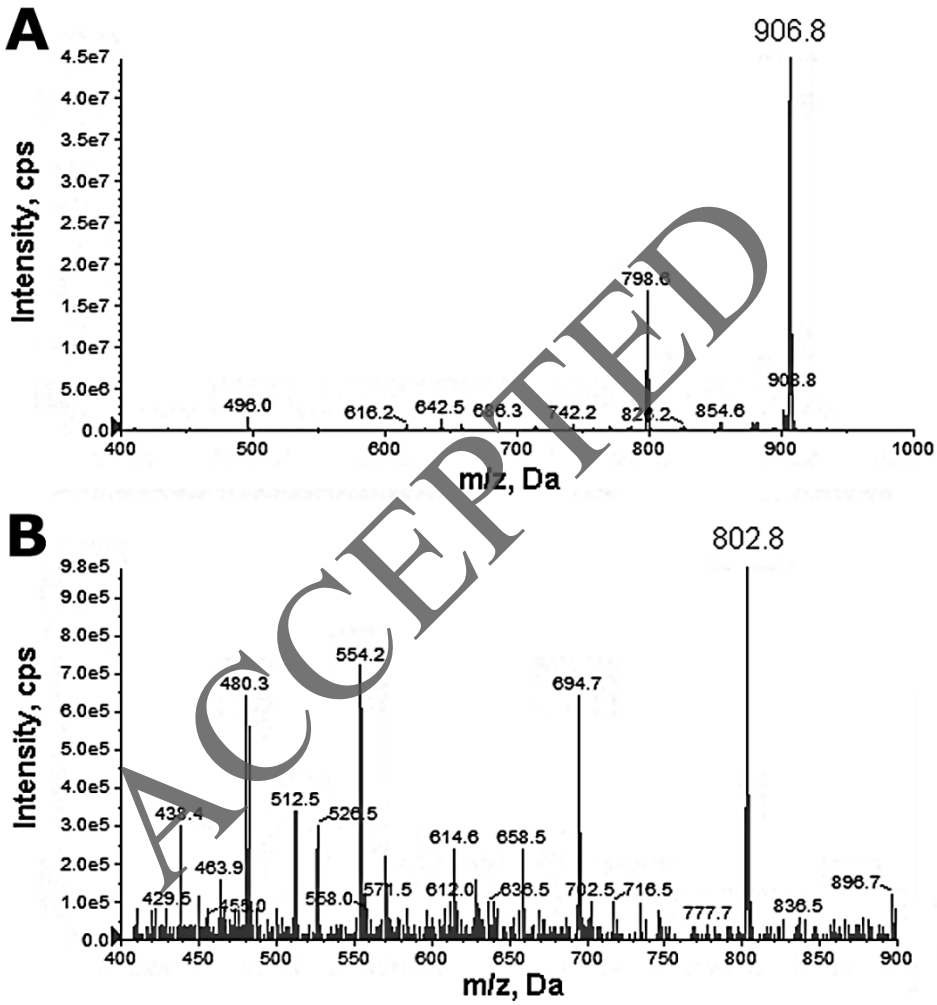

Fig.2 Mass spectra of PE glycation products obtained from PE–glucose experiment. Specific neutral loss MS scan of 303 Da and 199 Da showed m/z of 906.8 Da of, 18:1-18:1 glycated-PE (A) and m/z of 802.8 Da of 18:1-18:1, CM-PE (B), respectively

3. Inhibitory effect of NSE on the formation of glycated-PE and CM-PE

Various concentrations of NSE were added to the reaction mixture of DOPE and glucose and the results are shown in Figures 3 and 4. Although most NSE concentrations as well as AG were unable to show the inhibition of glycated-PE formation, however, the 40 mg/ml of NSE was only found to be able to decrease the glycated-PE formation ($p < 0.05$) by approximately 40% as compared to that of negative control (Figure 3). In contrast with glycated-PE, all concentrations of NSE and AG significantly inhibited the CM-PE formation by approximately 20% – 50% as compared to that of negative control (Figure 4).

Interestingly, our result revealed that NSE had a potential to inhibit the CM-PE formation to a greater extent than that of AG when compared with the same concentration of 5 mg/ml ($p< 0.05$)

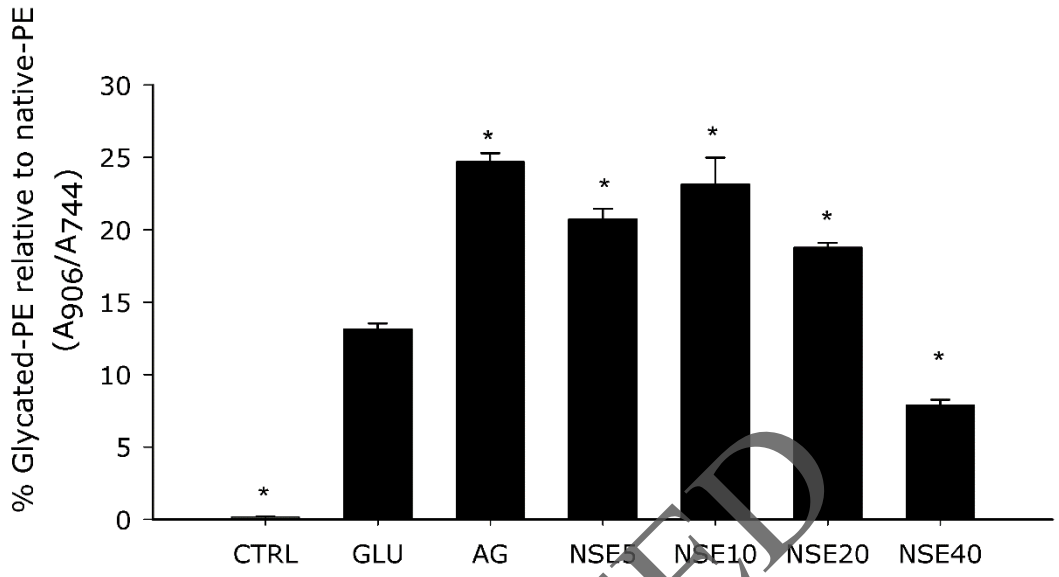

Fig.3 Effect of NSE on the formation of glycated-PE. CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE+glucose + 5.0 mg/ml of AG (positive control), NSE5, NSE10, NSE20 and NSE40 = PE + glucose + NSE at 5, 10, 20 and 40 mg/ml, respectively), * Significant difference from negative control (p< 0.05).

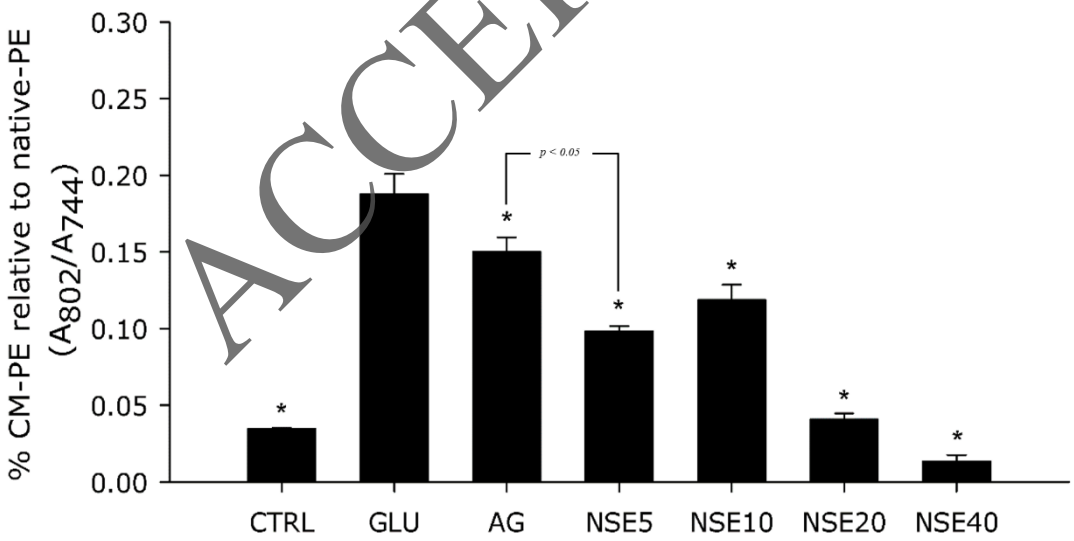

Fig.4 Effect of NSE on the formation of CM-PE. CTRL = PE (blank control), GLU = PE + glucose (negative control), AG = PE + glucose + 5.0 mg/ml of AG (positive control), NSE5, NSE10, NSE20 and NSE40 = PE + glucose + NSE at 5, 10, 20 and 40 mg/ml, respectively), * Significant difference from negative control (p< 0.05).

4. The association between NSE and the products of PE glycation

The association between the concentration of NSE and the early and late-stage products of lipid glycation was also investigated and presented in Figure 5. The formation of glycated-PE was inversely correlated with the NSE concentration ($p<0.05$, Fig. 5A). Moreover, the same trend was also observed for

the CM-PE formation (p<0.05, Fig. 5B). Therefore, it can be implied that NSE exhibited the inhibitory effect on glucose-mediated PE glycation in the dose-dependent manner.

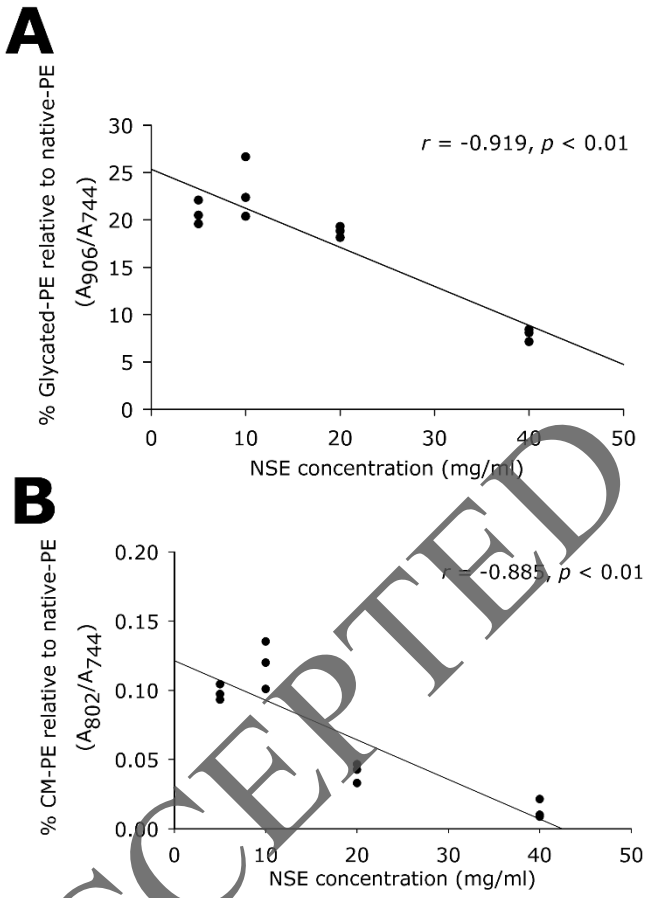

Fig.5 Pearson correlation between concentration of NSE and the formation of PE glycation products; (A) glycated-PE and (B) CM-PE.

Discussion

Apart from protein glycation, the lipid glycation, especially the formation of Amadori-PE, the early-stage product of PE glycation, was found to be increased in plasma, blood and organs in both human and animal with diabetes (Nakagawa et al., 2005; Sookwong et al., 2011). Moreover, previous finding has demonstrated that the accumulation of Amadori-PE was faster than that of Carboxymethyl lysine (CML) and HbA_1c, the products from protein glycation. Thus, Amadori-PE was suggested to be a sensitive predictive marker for early state of diabetes (Sookwong et al., 2011). In addition, CM-PE, the late stage of PE glycation, has also been reported to be found in the surfaces of biological membranes (Solís-Calero et al., 2015). Since the increasing of lipid glycation products has been shown to be associated with the development of diabetic complications and can cause the alteration of physical and biological properties of the cell membrane such as the increased susceptibility of oxidative stress, therefore the prevention or inhibition of lipid glycation reaction might be beneficial to diabetic patients.

In this study, NSE was used for testing its anti-lipid glycation properties and our results demonstrated that only high concentration of NSE (40 mg/mL) exhibited a strong effect on the inhibition of glycated-PE formation, whereas the other concentrations were unable to show such effects. Since the anti-lipid glycation properties of NSE has not been investigated before, hence, this finding could be explained based on the activity from the major active compound known as “Thymoquinone (TQ)” in *Nigella sativa* seed. Our results from HPLC analysis revealed that TQ was the major compound in NSE, a consistent with the findings from the analysis of black seed oil, where TQ was identified as the major

quinone compound (Ghosheh et al., 1999). To support the anti-lipid glycation properties of TQ, our previous study has demonstrated that TQ was able to inhibit the glycated-PE formation and the lowest concentration of TQ has shown a greater potential than the others to reduce the glycated-PE formation. Furthermore, the new compound TQ-PE adduct has also been proposed. The carbonyl group of TQ might be condensed with the amino group of PE to form TQ-PE adduct which was detected and identified by neutral loss MS scan of 287 Da, $[M+H-287]^+$. This has indicated that TQ might compete with glucose for condensing with PE, resulting in the decrease of lipid glycation products (Marnpae et al., 2014). The mechanism for the condensation between PE and some active compounds has been previously reported. The initial study done by Higuchi and colleagues (Higuchi et al., 2006) has firstly revealed the structural evidence for the condensation of pyridoxal 5-phosphate or pyridoxal with PE to form PE-pyridoxal 5-phosphate adduct. The study concluded that pyridoxal 5′-phosphate and pyridoxal exhibited the strong anti-glycation properties and could be used for diabetic prevention. In considering the evidence, it can be implied that TQ in NSE could play the important role in preventing the lipid glycation reaction. For the late-stage products of lipid glycation, our results demonstrated that NSE had a potential to inhibit the formation of CM-PE, the prominent product from late PE glycation. In fact, CM-PE can be formed by not only the oxidative cleavage of Schiff-PE and Amadori-PE, but also by the reaction between PE and glyoxal, the reactive carbonyl species derived from glucose or polyunsaturated fatty acids (PUFAs) oxidation (Requena et al., 1997; Naudi et al., 2013). Since the antioxidant properties including the free radical and superoxide radical scavenging activities of *Nigella sativa* extract and oil have been reported (Badary et al., 2003; Mohammed et al., 2016). Therefore, the anti-lipid glycation properties of NSE, particularly against the CM-PE formation, might be explained by the antioxidant capacity of NSE. The antioxidant compounds including TQ in NSE could prevent the oxidation of glucose and PUFAs and also inhibit the oxidative cleavage of glycated-PE to generate CM-PE. Consequently, this thereby resulted in the reduction of the late-stage products of PE glycation.

In comparison with AG, the most common anti-glycation agent, NSE seemed to have the inhibitory effect on the formation of lipid glycation product to a greater extent than that of AG. Our findings showed that AG failed to inhibit the glycated-PE formation, and this was in agreement with the previous study that showed the failure of AG to inhibit the Schiff-PE formation (Higuchi et al., 2006). Conversely, the formation of CM-PE was found to be inhibited by AG. This may be due to the fact that AG can react with glyoxal, methylglyoxal and dicarbonyl compounds derived from glucose or lipid oxidation, thus, it may thereby prevent the formation of AGEs (Nagai et al., 2012; Yoon et al., 2012) including CM-PE by scavenging such compound and/or prevent the oxidative cleavage of glycated-PE.

Conclusion

In conclusion, the thymoquinone (TQ)-rich ethanolic NSE has the potential to attenuate glucose-induced PE glycation by reducing both early (glycated-PE) and late-stage product (CM-PE). Therefore, *Nigella sativa* might be the alternative therapeutic option for preventing or reducing the risk of lipid glycation-induced pathogenesis of diabetic complications.

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