Nat. Volatiles & Essent. Oils, 2019; 6(1): 25-33

NVEO
Natural Volatiles & Essential Oils

RESEARCH ARTICLE

Antiglycation and antiaggregation potential of thymoquinone

Dinesh Kumar and Ahmad Ali*

Department of Life Sciences, University of Mumbai, Vidyanagari, Santacruz (East), Mumbai, Maharashtra, INDIA

*Corresponding author. Email: ahmadali@mu.ac.in

Abstract

The consequences of Diabetes are manifested due to the accumulation of glucose. The carbonyl group of sugars reacts with the amino group of proteins leading to generation of harmful products collectively known as advanced glycation end products (AGEs). These products have been shown to be involved in the various secondary complications of Diabetes and neurodegenerative disorders. The present study involves the assessment of role of Thymoquinone in the process of glycation.

The *in vitro* glycation system consisted of BSA and glucose and incubated in the presence and absence of thymoquinone for four weeks at 37°C. The amount of glycation products was measured by standard methods like browning, total AGEs by spectrofluorimetry. The aggregation of protein was checked by aggregation index and Congo red assays. The effect of thymoquinone was also checked on the glycation of DNA and the sample was analysed by agarose gel electrophoresis. The presence of thymoquinone resulted in the decrease in browning and amount of total AGEs significantly. There was also a drastic decrease in the glycation-induced aggregation of BSA and reversal of glycoxidative damage of DNA in the presence of thymoquinone. It can be concluded from these results that thymoquinone is potential antiglycating agent and it can be used to prevent the glycation-induced damage of biomolecules.

Keywords: Advanced glycation end-products (AGEs), antiglycation, aggregation, DNA damage, thymoquinone

Introduction

Diabetes has affected millions of people worldwide and has become a major socioeconomic issue. The condition begins with a slow accumulation of glucose in the body and reaches a toxic level after sometime. The reactive group of sugars, carbonyl, interacts covalently with the amino group of other biomolecules like proteins by a process known as glycation or Maillard reaction after its discover Louise Camille Maillard (Kikuchi et al., 2003). This reaction leads to generation of Schiff's bases which get converted to Amadori products. The rearrangement of these metabolites lead to generation of a group of harmful compounds commonly known as advanced glycation end products (AGEs) (Ahmad, 2005). All these intermediates of glycation process are also known to generate free radicals and cause weakening of the antioxidant defense mechanisms which damages cellular organelles and enzymes (Ahmad et al., 2014). The rate of formation of AGEs increases during the oxidative and carbonyl stress caused due to accumulation of reactive oxygen species (ROS) and dicarbonyl compounds (Sadowska-Bartosz & Bartosz, 2015). The biological reactions leading to AGE formation and free radical generation are closely related and are often called as glycoxidation process. They have been shown to be implicated in various pathophysiological conditions like diabetes, neurodegenerative disorders and cancer (Ahmad et al., 2014).

Glycation has received a lot of attention in the last two decades because of its involvement in the secondary complications of diabetes and many neurodegenerative disorders. There has been effort to develop a drug which can inhibit the generation of AGEs in the body or treat the conditions due to accumulation of AGEs. Although some synthetic drugs have been approved for the treatment but they have been found to exert harmful effects on the body for example, aminoguanidine (Thornalley, 2003). Recent developments in the

field of photochemistry and analytical methods have led to discovery of natural products from plants or derivatives of the natural products synthesized and tested in the laboratories. Some of these natural compounds such as curcumin, eugenol, rutin, garcinol etc. have shown to possess significant antioxidant and antiglycating potentials (Khan & Gothalwal, 2018).

Nigella sativa (black cumin) seeds have been used as traditional medicine since centuries (Ali & Blunden, 2003). Thymoquinone, the main phytoconstituent of black cumin seed, has been reported to have hepatoprotective, anti-inflammatory, anti-oxidant, cytotoxic and anti-cancer chemical properties (Khader & Eckl, 2014). There are a very few reports on understanding the mechanism of antiglycating and antiaggregation potentials of thymoquinone (Anwar et al., 2014). Therefore, thymoquinone was used in the present study to check its role in the prevention of glycation and glycation-induced processes like protein aggregation and glycoxidation. The results indicate significant role of thymoquinone in the prevention of accumulation of early as well as advanced glycation end products. Glycation-induced aggregation and DNA damage were also prevented in the presence of thymoquinone. Antioxidant potential of thymoquinone was also measured using DPPH assay. This study is an important step towards understanding the possible mechanism of thymoquinone in the prevention of glycation in the secondary complications of diabetes.

Materials and Methods

Materials

Bovine serum albumin, Agarose, methylglyoxal and thymoquinone were purchased from Sigma-Aldrich. Lysine and DPPH (2,2-diphenyl-1-picrylhydrazyl) were procured from HiMedia. pBR322 was purchased from Thermo Fisher. All other chemicals used were of high analytical grade.

Methods

Incubation of thymoquinone in vitro glycation system

10 mg/mL aqueous solution of BSA was incubated with glucose (100 mg/mL) with or without thymoquinone (10 µM and 20 µM) in 100 mM phosphate buffer (pH 7.4) at 37 °C for 28 days. The bacterial contamination during the prolonged incubation was prevented by adding 3 mM sodium azide.

Measurement of browning

The extent of browning was measured at 420 nm using Shimadzu UV 1800 spectrophotometer (Rondeau et al., 2007) and relative percentage of absorbance was used to plot the graph.

Estimation of fluorescent AGEs

The measurement of fluorescent intensity was carried out at excitation (370 nm) and emission (438 nm) wavelengths (Ali et al., 2017). Cary Eclipse Fluorescence spectrophotometer was used for the measurement of fluorescent AGEs.

Determination of protein aggregation index

The effect of thymoquinone was also checked on the protein aggregation by measuring the absorbance of glycated samples in the presence/absence of thymoquinone. The aggregation index was calculated by the following formula

$$\text{Aggregation index} = [A_{340} / (A_{280} - A_{340})] * 100$$

A_{280} and A_{340} - absorbance at 280 nm and 340 nm respectively (Pandey et al., 2018).

Congo red assay

Congo red dye bound to amyloid cross β-structure was checked by recording absorbance at 530 nm (Ali et al., 2017). The glycated sample (50 µL) was mixed with 50 µL of 100 µM Congo red dye and kept at 25 °C for 20 minutes. The volume was made sufficient with distilled water (1 mL) for spectrophotometric analysis at 530 nm.

In vitro glycation of plasmid DNA in the presence of thymoquinone

The effect of thymoquinone on the glycation-mediated DNA strand breakage was performed according to a previous method with minor modifications (Ali et al., 2014). The pBR322 plasmid (0.25 µg) in 100 mM potassium phosphate buffer (pH 7.4) was incubated with lysine (20 mM), MG (20 mM) and $FeCl_3$ (100 µM) in presence and absence of thymoquinone (10 µM and 20 µM). The reaction mixture of samples was incubated at 37 °C for two hours. pBR322 plasmid DNA (0.25 µg) in 100 mM potassium phosphate buffer (pH 7.4) without glycation system was used as control. The reaction was stopped by freezing the samples at -20 °C.

Agarose gel electrophoresis of glycated plasmid DNA sample

Ten microliters of samples were mixed with 2 µL of 6X gel loading dye and loaded on to 1% agarose gel. Electrophoresis was carried out initially at 90 V and once the samples left the well, voltage was decreased to 85 V. As soon as the dye band reached two-thirds of gel length, electrophoresis was terminated and gel was stained using ethidium bromide solution (final concentration 5 µg/mL) for 20 min in dark. Subsequently the gel was visualized under Gel-Doc and bands analysed with the help of control.

DPPH$^{\bullet}$ assay

The thymoquinone was screened for its antioxidant potential by DPPH radical scavenging assay with some minor modification (Lutterodt et al., 2010). 0-25 µM concentrations of thymoquinone (1 mM stock) and 100 µL DPPH$^{\bullet}$ (1 mM) were mixed and volume made up to 1 mL with methanol, then incubated at 37 °C for 30 minutes and absorbance was recorded at 517 nm.

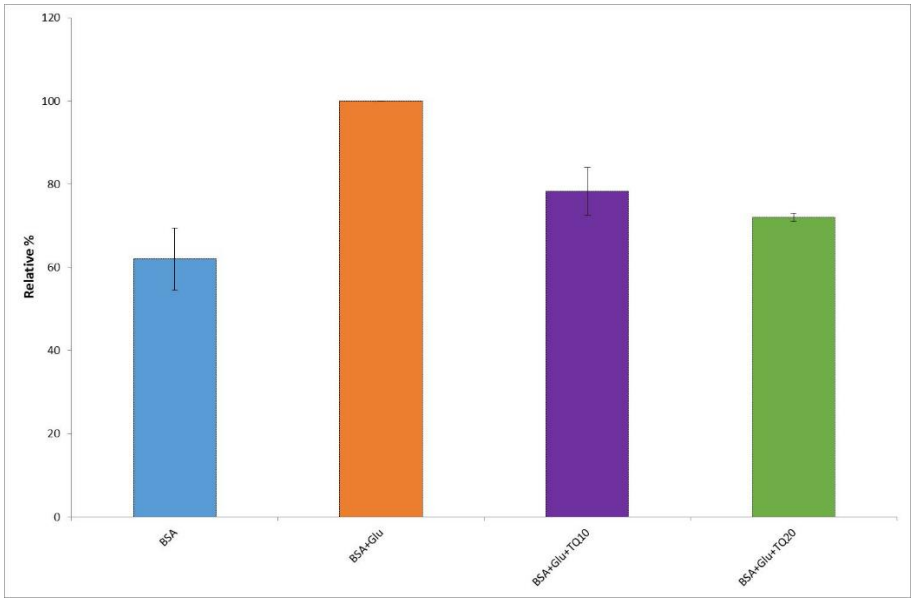
Results and Discussion

Results

Effect of *Nigella sativa* seed extracts on browning

BSA was glycated with glucose at 37 °C for 28 days in the presence and absence of thymoquinone. Initial indicator for glycation, browning, was measured spectrophotometrically at 420 nm. Thymoquinone caused inhibition of glycation at 10 µM (21.67%) and 20 µM (28.04%) of glycation as compared to glycated BSA (100%) (Figure 1). These results indicate that the decrease in browning in the presence of thymoquinone can be correlated with less formation of glycated products.

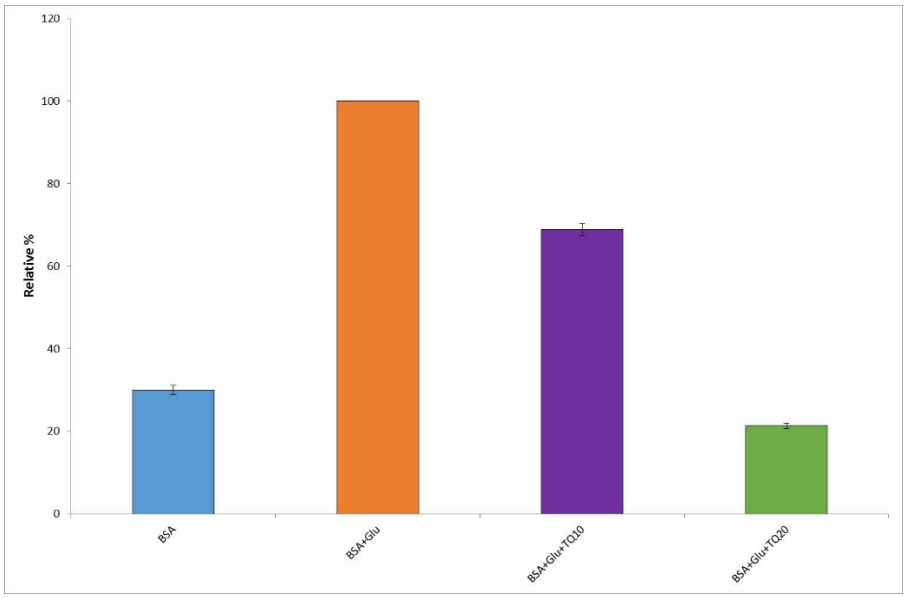
Figure 1. Measurement of Browning.



Estimation of fluorescent total AGEs in presence of thymoquinone

The extent of fluorescent AGE formation was checked by measuring the fluorescent intensity in glycated BSA with glucose as shown in Figure 2. The results showed that the addition of thymoquinone into the solution greatly reduced the formation of fluorescent AGEs by 31.13% (10 µM) and 78.64% (20 µM).

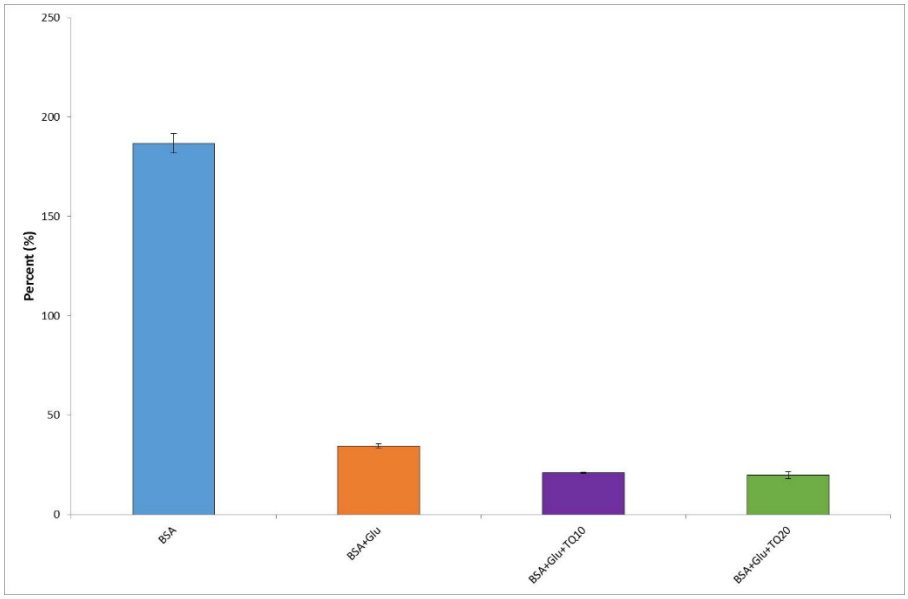
Figure 2. Measurement of Total Flourescent AGEs.



Effect of *Nigella sativa* seed extracts on protein aggregation Index

Aggregation of protein is the late stage of non- enzymatic glycation process in which there is formation of cluster when the carbonyl group is bound to protein. The aggregation index showed very significant reduction of amyloid cross-β structure in presence of thymoquinone in comparison of glycated protein (Figure 3).

Figure 3. Measurement of Aggregation index.



Measurement of amyloid cross-β structure in glycated BSA in presence of thymoquinone

The measurement of amyloid cross-β structure is one of the vital quantitative estimation of glycated protein and done by performing Congo red assay. Formation of amyloid cross-β structure in the glycated BSA approximately decreased by 19.98% and 41.94% at 10 µM and 20µM respectively (Figure 4).

Figure 4. Congo Red Assay for the measurement of protein aggregation.

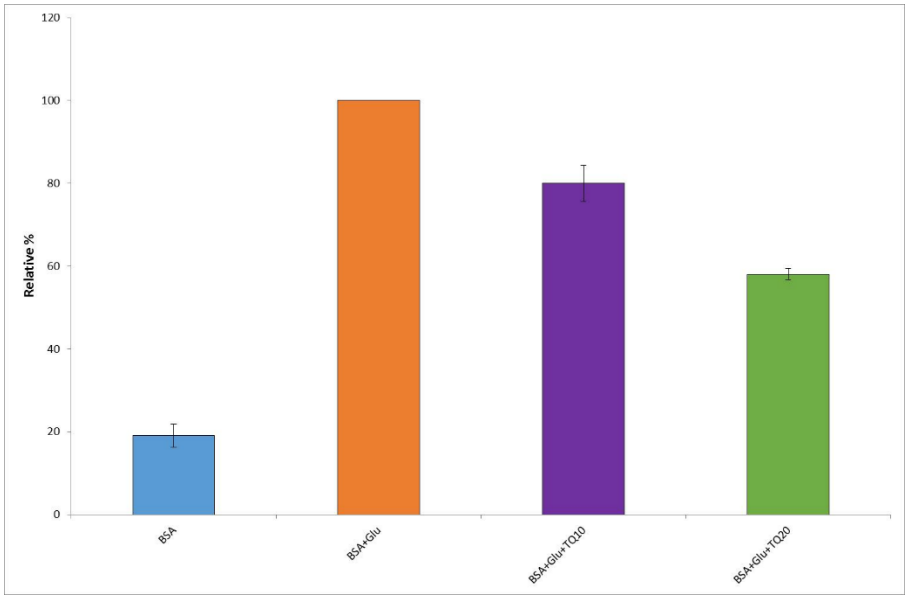
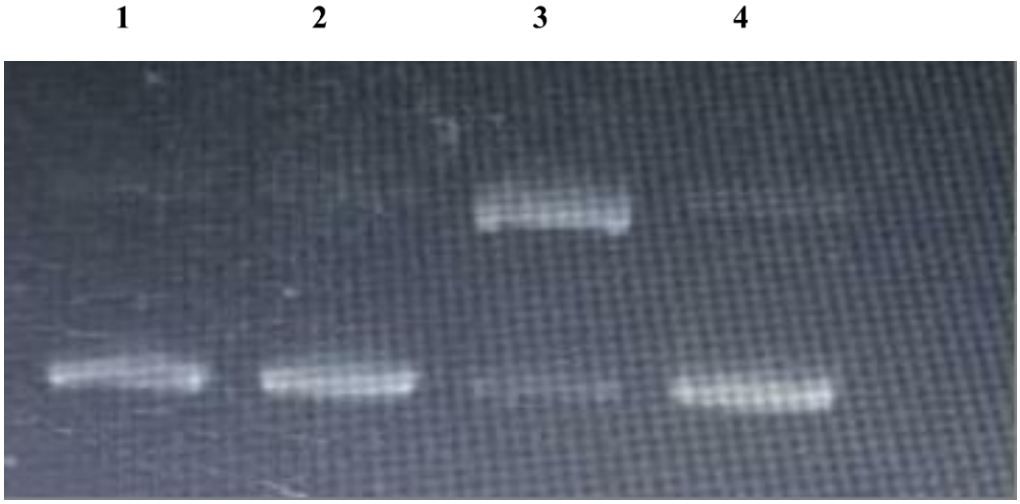


Figure 5. Glycoxidative damage of DNA.



LANE DESCRIPTION:

Lane 1- DNA alone

Lane 2- DNA + TQ (10 µM)

Lane 3- DNA + Lysine (20 mM) + MG (20 mM) + $FeCl_3$ (100 µM)

Lane 4- Lane 2 + TQ (10 µM)

Effect of thymoquinone on the glycation of DNA

Strand breakage was observed for DNA incubated with methyl glyoxal, lysine and ferric chloride for 2 hours at 37 °C (Figure 5, Lane 3) as compared to control (Figure 5, Lane 1). The thymoquinone (10 µM) caused the reversal of the strand breakage (Figure 5, Lane 4) and had no effect on its own (Figure 5, Lane 2).

DPPH• assay

It was found that thymoquinone caused reduction in free radical generation and inhibition increased from 8 % to 20 % in a concentration dependent manner (Table 1).

Table 1. DPPH• Assay.

Samples	Scavenging Activity (%) ± S.E.
5 µM TQ	8.27 ± 0.44
10 µM TQ	12.10 ± 0.91
15 µM TQ	15.71 ± 0.29
20 µM TQ	19.01 ± 0.95
25 µM TQ	20.99 ± 0.41

Conclusion

Advanced glycation end products have been implicated in many health complications. The major challenges scientists have faced as far as treatment of glycation related disorders is concerned is their detection and prevention. Many qualitative and quantitative methods have been developed from last few decades for the detection of the formation of glycation products and glycation induced processes (Banan & Ali, 2016). These methods include browning, total AGEs estimation, Congo red assay for amyloid cross β-structure, aggregation index and electrophoretic mobility of biomolecules. Traditionally glycated samples have been analyzed by different methods including spectrophotometric and electrophoretic techniques (Ali & Sharma, 2015). Recently advanced techniques like spectroflourimetry, HPLC and LC-MS have found application in the quantification of AGEs (Poulsen et al., 2013).

In last few decades focus has been shifted to utilize natural compounds for the prevention and cure of diseases. Black cumin seeds are sources of several natural compounds which cure many diseases (Najmi et al., 2012). Although there are many reports in literature on antidiabetic properties of black cumin seeds there are a very few reports on understanding the mechanism of antiglycating and antiaggregation potentials of black cumin seed extracts (Pandey et al., 2018; Zafer et al., 2013). In the present study thymoquinone was used to analyze its preventive role in the process of glycation and consequences of glycation induced processes like aggregation and glycoxidation. Measurement of extent of browning has been used as a conventional indicator of the process of glycation. It can be seen from the results obtained that thymoquinone reduced the extent of browning significantly. The amount of total fluorescent AGEs were checked using spectrofluorometer and thymoquinone was found to be very potent in inhibiting the formation of glycation products. Previously Khan et al. (2014) have also reported the antiglycating properties of thymoquinone.

Glycation also leads to formation of protein cross-linking and aggregates which have been implicated in neurodegenerative disorders (Ali et al., 2014). In the present study protein aggregation index and Congo red methods were used to measure the glycation-induced aggregation of the glycated BSA. The most significant reduction in the amyloid cross β-structure was observed for BSA samples glycated with glucose in the presence of thymoquinone. Spectroscopic analysis of glycated sample at the different wavelengths gives an idea of the extent of protein aggregation. A ratio of absorbance taken at 280 and 340 nm is used to calculate the aggregation index. The presence of thymoquinone reduced the aggregation index significantly. These results indicate the antiaggregation potential of thymoquinone.

The accumulation of AGEs leads to generation of reactive oxygen species *in vitro* and *in vivo*. Macromolecular structures like DNA and proteins are very prone to these free radicals. In the present study the effect of glycation-induced DNA damage was studied in the presence and absence of thymoquinone. Addition of metal ions catalysed the generation of free radicals and led to enhanced strand breakage of DNA. There was significant inhibition/reversal of DNA damaged by glycation in the presence of thymoquinone. Glycoxidative damage of DNA was prevented by the seed extract of *Nigella sativa* (Pandey et al., 2018). In another study Losso et al. (2011) have reported the application of thymoquinone in the prevention of AGE formation. Thymoquinone was also found to be good scavenger of oxidants (Solati et al. 2014). Earlier reports have shown the antiglycating potential of thymoquinone (Anwar et al., 2014; Khan et al., 2014) but there was a lack of information on the preventive role of this active constituent of *Nigella sativa* on glycation-induced processes.

It can be concluded from this study that the thymoquinone possess antiglycating, anti-aggregation and antioxidant potential. The thymoquinone can be used for preventing glycation-mediated secondary complications of diabetes, glycoxidative damage of DNA as well as protein aggregation mediated neurological disorders. Further studies need to be carried out to understand the mechanism of inhibition of glycation by thymoquinone.

ACKNOWLEDGEMENTS

The study was funded by Research Society for the Study of Diabetes in India (RSSDI/HQ/Grants/2017/342).

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Received: 02.18.2019
Accepted: 04.22.2019