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ORIGINAL ARTICLE

Inhibitory Roles of *Nigella sativa* Seed Extracts on *in vitro* Glycation and Aggregation

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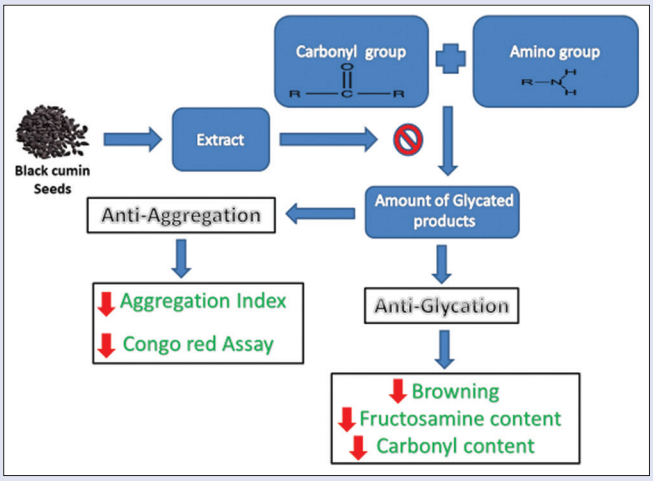
ABSTRACT

Background: Glycation is the multi-step process wherein the carbonyl group of glucose reacts with the amino group of other nitrogen-containing compounds like proteins and DNA. This interaction leads to the generation of harmful metabolites commonly known as advanced glycation end products (AGEs). These AGEs have been implicated in various diseases such as cataracts, diabetes, and Alzheimer's. **Objectives:** The aim of the present study was to check the effect of *Nigella sativa* seed extracts on glycation and aggregation and for this purpose, two proteins (bovine serum albumin [BSA] and hemoglobin) and two sugars (glucose, and ribose) were selected. **Materials and Methods:** The amount of glycation mediated products was measured by established methods such as browning, nitroblue tetrazolium (NBT) assay, carbonyl content, and aggregation assays in the presence or absence of extracts (1 mg/ml). The effect of extracts was also checked on glycated DNA by agarose gel electrophoresis. **Results:** Analysis of results indicates that extracts (1 mg/ml) significantly prevented the generation of early products up to 30.57% (BSA-Rib) and 64.90% (Hb-Glu). The AGEs formation was also suppressed in different glycation systems up to 60.76% (BSA-Glu), 30.79% (BSA-Rib) and 10.74% (Hb-Glu) in the presence of extracts. There was also a decrease in the level of glycation-mediated protein aggregation. Seed extracts were able to prevent the glycoxidative damage of DNA. **Conclusion:** It can be concluded that black cumin seeds have antiglycating and antiaggregation potential as well as antioxidant properties. Further characterization will help in the identification of active compounds which can be used for the prevention of secondary complications of diabetes and aggregation.

Key words: Advanced glycation end-products, aggregation, DNA damage, glycation, *Nigella sativa* (Black cumin)

SUMMARY

- *Nigella sativa* extracts played crucial role in the prevention of early as well as advanced glycation end products. The aqueous extract possessed higher antioxidant activity and was more potent in preventing the process of glycation and glycation-induced aggregation as well as glycoxidation. This study is significant in understanding the possible mechanism of *N. sativa* seeds in the prevention of glycation related complications observed during diabetes and its consequences.



Abbreviations used: AGEs: Advanced glycation end products; BSA: Bovine serum albumin; DPPH: 2,2-diphenyl-1-picrylhydrazyl; Glu: Glucose; Hb: Hemoglobin; HbA_{1c}: Glycated hemoglobin; Lys: Lysine; MG: Methylglyoxal; NBT: Nitro blue tetrazolium; NS-1: *Nigella sativa* seed extracts (methanol); NS-2: *Nigella sativa* seed extracts (aqueous); Rib: Ribose

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INTRODUCTION

The process of glycation starts with a covalent interaction between the reactive carbonyl and nitrogenous amino groups of sugars and proteins, respectively. This leads to the generation of Schiff's bases which get converted to Amadori products for example, hemoglobin A1c, ε-N-deoxylactulosyllysine, etc., The rearrangement of these metabolites leads to the generation of a group of nondeleterious compounds commonly called as advanced glycation end products (AGEs). In this process of glycation, glucose is the least reactive as compared to other sugar-like ribose and fructose.[1] Similarly, dicarbonyls (methyl glyoxal) have more reactivity than monocarbonyls in the formation of AGEs. The intermediates of the glycation process are also known to generate free radicals and cause decline in the antioxidant defence mechanisms which damages cellular organelles and enzymes.[2] Oxidative stress is triggered by high reactive oxygen species (ROS) levels while carbonyl stress occurs due to active dicarbonyl compounds (glyoxal and methylglyoxal). These increments occurring simultaneously in the body leads to the increased rate of AGEs formation. The biological reaction of glycation and oxidative

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stress are closely related and often called as the glycoxidation process. They have been shown to be involved in diabetes, neurodegenerative disorders, cardiovascular disease, and associated complications.[3]

In the last few decades, glycation has been noticed for its involvement in secondary complications of many diseases and disorders has brought the attention of researchers towards it. Several natural and synthesized drugs have been developed for inhibition of the glycation process or removal of formed early and AGEs. Aminoguanidine, synthesized drug, has been approved for treatment but reported for harmful effects on the body.[4]

Many natural compounds and plants metabolites have been tested for glycation prevention properties. Curcumin, garcinol, eugenol, etc., have been reported for antiglycation and antioxidant potentials.[5] The use of *Nigella sativa* (black cumin) seeds as traditional medicine have been seen for many centuries. The pharmacological properties of *N. sativa* seeds have been characterized and shown to possess antioxidant, antidiabetic antihypertensive and anticancer activities.[6] However, there are very few reports on the effect of *N. sativa* seeds on the process of glycation.[7-9] The mechanism of action of the extracts used for the prevention of glycation in the earlier reports is not very clear. In the previous reports available in the literature only serum proteins were analyzed for glucose/fructose-mediated glycation.[7,8] Apart from the serum proteins, other proteins like hemoglobin, DNA also get glycated.[8] Furthermore, only a few parameters like total AGEs have been analyzed in earlier reports. The main aim of the present study was to analyze the effect of *N. sativa* seed extracts on the glycation of proteins and DNA, glycation-induced aggregation and to identify the stage at which glycation is prevented (early, intermediate, or advanced). The *in vitro* glycation system consisted of bovine serum albumin (BSA) and hemoglobin (Hb) as model proteins and two sugars (glucose and ribose).

MATERIALS AND METHODS

Materials

Black cumin seeds were purchased from the local market of Mumbai and certified by the Institute of Herbal Science, Plant anatomy Research Centre, Chennai, India (Certificate no. PARC/2019/3911). Agarose, methylglyoxal, BSA, 2,2-diphenyl-1-picrylhydrazyl (DPPH) procured from Sigma-Aldrich. pBR322 was purchased from Thermo Fisher Scientific. All the other chemicals used were of the high analytical grade of S D Fine-Chem Limited and HiMedia Laboratories Pvt. Ltd.

Extraction of *Nigella sativa*

The *N. sativa* seeds were extracted using two methods because extraction solvent and technique greatly affect the extraction of plant metabolites.

1. Hot extraction-methanol was used as the solvent. 15 g of seeds were soaked in 60 ml of methanol, heated gradually at 50°C and filtered through Whatman paper number 42 at RT and in a weighed beaker kept for drying in thermostat at 50°C. The residue (130 mg) obtained was dissolved in 10 ml methanol (13 mg/ml). The *N. sativa* seed extracts (methanolic) was abbreviated as NS-1 extract
2. Soxhlet extraction– water was used as solvent. 15 g of seeds were soaked in 60 ml of methanol. Cold water was circulated through the condenser using the pump for 6 h. The residue (220 mg) obtained was dissolved in 10 ml water (22 mg/ml). The *N. sativa* seed extract (aqueous) was abbreviated as NS-2 extract.

Incubation of *Nigella sativa* seed extract with *in vitro* glycation system

BSA (10 mg/ml) and Hb (10 mg/ml) were incubated with two sugars (glucose/ribose-100 mg/ml) and with or without *N. sativa* seed extracts (1 mg/ml of NS-1 and NS-2) at 37°C for 28 days (BSA-Glu) and 7 days (BSA-Rib and Hb-Glu). 3 mM sodium azide and 100 mM phosphate buffer were also added for the prevention of bacterial contamination and to maintain pH 7.4 respectively. The samples were dialyzed extensively overnight in phosphate buffer (10 mM, pH 7.4) before using them for further analysis.

Methods for measurement of glycation products

Measurement of browning

Browning has been used as a simple colorimetric method for the assessment of the process of glycation. The incubated protein samples (1 ml) with both sugars in the presence and absence of extracts were analyzed for browning at 420 nm.[10]

Fructosamine content measurement by nitroblue tetrazolium method

The NBT method was employed for the measurement of fructosamine content.[10] The protein samples (10 µl) were incubated with 100 µl NBT (0.5 mM) at 37°C for 15 min. On completion of incubation, the reaction mixture was brought up to 1 ml with distilled water and absorbance measured at 530 nm.

Carbonyl content measurement by 2,4-Dinitrophenylhydrazine method

The carbonyl content was measured by 2,4-dinitrophenylhydrazine (DNPH) assay according to Meeprom *et al.*[11] with minor modifications. The glycated BSA and Hb proteins with/without *N. sativa* extracts (0.1 ml) and 0.4 ml of DNPH was mixed and kept in dark for 1 h. After the addition of 20% Trichloroacetic acid (0.5 ml), the mixture was kept in ice-cold condition for 5 min. The reaction mixture was centrifuged at 10000 rpm for 10 min. Then the pellet was washed with a 0.5 ml mixture of ethyl acetate and ethanol (1:1) thrice and centrifuged each time at 10,000 rpm for 10 min. Later, the pellet was resuspended in 6 M Urea and the volume of the sample raised to 1 ml with distilled water for recording absorbance at 370 nm.

Assessment of glycation-induced aggregation of proteins

Congo red assay

The analysis of amyloid cross β-structure by Congo red assay was performed according to previous publications.[12] The glycated BSA and Hb proteins with/without extracts (0.05 ml) and 0.05 ml of Congo red dye (100 µM) was mixed and incubated at RT for 20 min. The absorbance of measured at 530 nm.

Aggregation index

The absorbance of glycated protein samples was noted at 280 nm and 340 nm.[12] The following formula was used for the calculation of the aggregation index:

Aggregation index (%) = $A_{340}/(A_{280} - A_{340}) \times 100$

Effect of *Nigella sativa* seed extract on glycated DNA

pBR322 plasmid (0.25 µg) was incubated with lysine (20 mM), MG (20 mM) and $FeCl_3$ (100 µM) in the presence and absence of *N. sativa* seed extracts (NS-1 and NS-2-10 µg) at 37°C for 3 h. The Agarose gel electrophoresis was performed and analyzed as per the methods of Kumar and Ali.[12]

Antioxidant activity by *2,2-diphenyl-1-picrylhydrazyl* method

DPPH radical scavenging assay was employed with some minor modification as described by Sylvie *et al.*[13] The varying

concentrations (1 mg to 5 mg) of both extracts (NS-1 and NS-2) and 100 µl of DPPH (1 mM) were incubated at 37°C for 30 min. The volume of the reaction mixture was made up to 1 ml with distilled water for taking absorbance at 517 nm. The percentage inhibition by *N. sativa* seed extracts was calculated by formulae:

$$\% \text{ inhibition} = ([A_{control} - A_{sample}]/A_{control}) \times 100$$

Statistical analysis

The statistical analysis was carried out by applying one-way ANOVA in MS Excel version 2019. The results were represented as mean ± standard error (number of sets, $n = 3$) and considered statistically significant with $P < 0.05$.

RESULTS

Effect of *Nigella sativa* seed extracts on browning

The aqueous extract (NS-2) caused more decrease in the browning of the glycated sample (BSA; 76.33%) as compared to methanolic extract (NS-1; 33%) that can be observed in Figure 1. Similarly, when BSA was glycated with ribose in the presence and absence of *N. sativa* seed extract the intensity of browning decreased to 27.65% in NS-1 and 43.2% in NS-2 as compared to control. The *N. sativa* seed extracts reduced the browning of the glycated sample (Hb in the presence of glucose) by 48.27% in NS-1 and 27.3% in NS-2 compared to control [Figure 1].

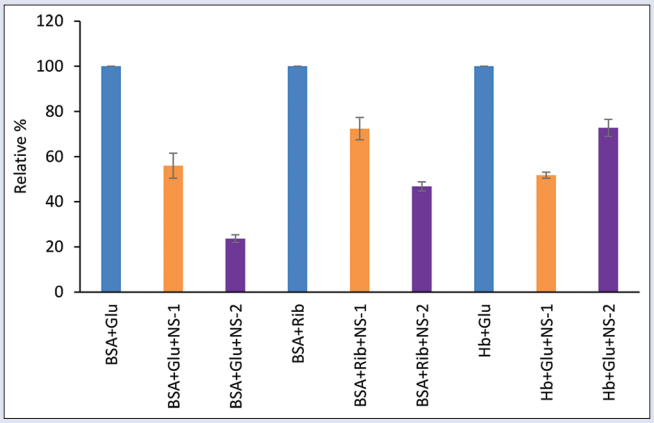

Figure 1: Measurement of browning; BSA-Glu, BSA-Rib and Hb-Glu (results expressed in relative% ± standard error ($n = 3$) and $P < 0.5$; BSA: Bovine serum albumin; Hb: Hemoglobin; Glu: Glucose; Rib: Ribose; NS-1 and NS-2: *Nigella sativa* seed extracts)

Effect of *Nigella sativa* seed extracts on the formation of fructosamine

After performing the NBT (Fructosamine) assay it was observed that fructosamine content in the BSA-Glu system was increased in presence of 18.56% in NS-1 and 16.58% in NS-2 [Figure 2] in extracts. The amount of fructosamine was suppressed in the presence of seed extracts NS-1 (7.94%) and NS-2 (30.57%) in the BSA-Rib system [Figure 2]. Hb was also incubated with glucose along NS-1 and NS-2 extracts at 37°C for 7 days. It was found that the presence of both black cumin extracts caused the reduction of the amount of fructosamine to about 44.83% in NS-1 and 64.9% in NS-2, respectively [Figure 2].

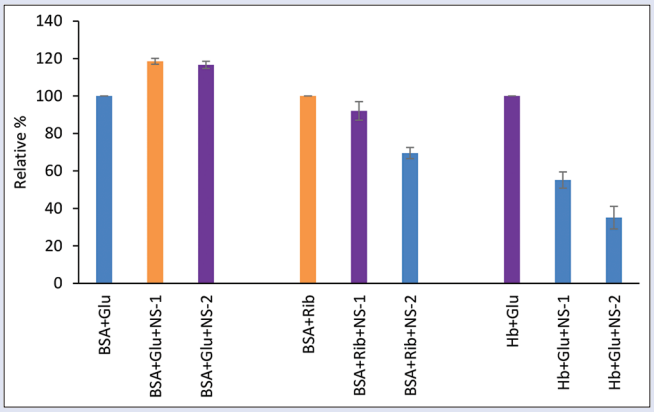

Figure 2: Measurement of fructosamine content: BSA-Glu, BSA-Rib and Hb-Glu (results expressed in relative% ± standard error ($n = 3$) and $P < 0.5$; BSA: Bovine serum albumin; Hb: Hemoglobin; Glu: Glucose; Rib: Ribose, NS-1 and NS-2: *Nigella sativa* seed extracts)

Effect of *Nigella sativa* seed extracts on carbonyl content

Carbonyl content decreased by 44.21% and 60.76% in NS-1 and NS-2 extracts respectively compared to glucose-glycated samples [Figure 3]. Similarly, in the glycated sample containing BSA + Rib the carbonyl content was decreased by 39.79% in NS-1 and 30.79% in NS-2 [Figure 3]. After incubating hemoglobin with glucose in the presence of NS-1 and NS-2 at 37°C for 7 days, the amount of carbonyl content was inhibited by about 60.02% by NS-1 and 10.74% in NS-2 respectively [Figure 3].

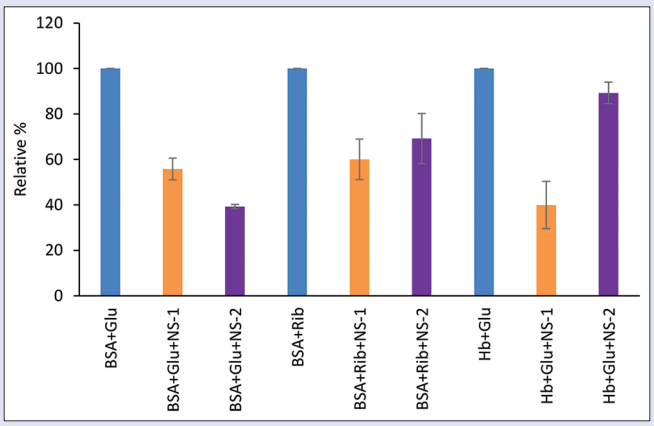

Figure 3: Measurement of carbonyl content: BSA-Glu, BSA-Rib and Hb-Glu (results expressed in relative% ± standard error ($n = 3$) and $P < 0.5$; BSA: Bovine serum albumin; Hb: Hemoglobin; Glu: Glucose; Rib: Ribose; NS-1 and NS-2: *Nigella sativa* seed extracts)

Protein aggregation index in presence of *Nigella sativa* seed extracts

The aggregation of glycation mediated protein has occurred when the carbonyl group of sugar binds to biomolecules of protein. After calculating the aggregation index there was less formation of amyloid cross-β structure in the presence of NS-1 and NS-2 when incubated with sugars and proteins [Table 1].

The aggregation index was measured for all the three glycation systems in the presence and absence of seed extracts. As shown in Table 1, the ribose glycation system showed the highest aggregation level. Both the extracts caused a significant decrease in the glycation-induced aggregation of glucose and ribose glycated proteins as well as aggregation of Hb.

Table 1: Aggregation index of bovine serum albumin-glucose/ribose and hemoglobin-glucose in the presence and absence of seed extracts

Samples	Aggregation index±SE	Samples	Aggregation index±SE	Samples	Aggregation index±SE
BSA + Glu	70.00±3.41	BSA + Rib	91.14±5.19	Hb + Glu	84.18±2.04
BSA + Glu + NS-1	20.97±0.97	BSA + Rib + NS-1	46.56±0.04	Hb + Glu + NS-1	72.71±2.24
BSA + Glu + NS-2	13.04±1.56	BSA + Rib + NS-2	61.70±0.82	Hb + Glu + NS-2	59.29±1.02

P<0.5. BSA: Bovine serum albumin (10 mg/ml); Hb: Hemoglobin (10 mg/ml); Glu: Glucose (100 mg/ml); Rib: Ribose (100 mg/ml); NS-1 and NS-2: *Nigella sativa* seed extracts (1 mg/ml); SE: Standard error (*n*=3)

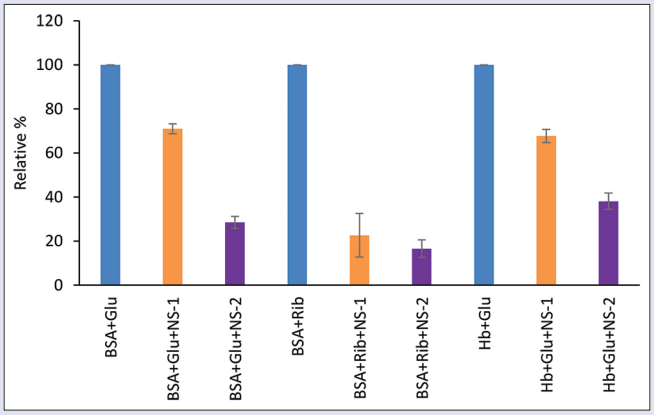

Figure 4: Measurement of amyloid cross-β structure: BSA-Glu, BSA-Rib and Hb-Glu (results expressed in relative% ± standard error [*n* = 3] and *P* < 0.5; BSA: Bovine serum albumin' Hb: Hemoglobin; Glu: Glucose; Rib: Ribose, NS-1 and NS-2: *Nigella sativa* seed extracts)

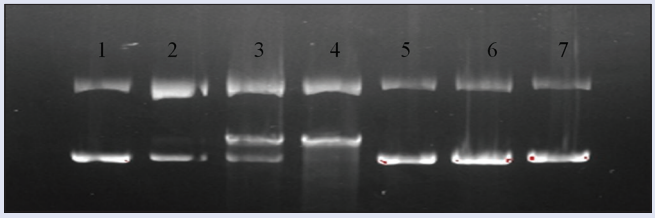

Figure 5: Effect of *Nigella sativa* extracts on glycated DNA (lane description: Lane 1-DNA alone, Lane 2-DNA + Lysine [20 mM] + MG [20 mM] + $FeCl_3$ [100 µM], Lane 3-DNA + Methanol, Lane 4-DNA + NS-1 [10 µg/µl], Lane 5-Lane2 + NS-1 [10 µg/µl], Lane 6-DNA + NS-2 [10 µg/µl] and Lane 7-Lane2 + NS-2 [10 µg/µl]

Measurement of amyloid cross-β structure in glycated bovine serum albumin/Hb in presence of *Nigella sativa* seed extracts

The measurement of amyloid cross-β structure is one of the vital quantitative estimations of glycated protein and is done by performing Congo red assay. The amyloid cross-β structure formation in the glycated model protein (BSA) approximately decreased by 29.03% and 71.49% in the presence of NS-1 and NS-2, respectively [Figure 4]. Similarly, they were analysed in the glycated BSA with ribose and extracts and decreased by 77.37% and 16.13% on the addition of methanolic and aqueous extract, respectively [Figure 4]. In the glycated hemoglobin (HbA_{1c}) which was incubated with glucose, the amount of amyloid cross-β structure was also measured and was found decreased in the presence of NS-1 and NS-2 about 33.3% and 61.93% [Figure 4].

Effect of *Nigella sativa* extracts on glycated DNA

Incubation of DNA in the presence of ferric ions (Fe^{3+}) in the glycation system enhanced strand breakage of DNA by converting the supercoiled form into circular form. *N. sativa* seed extract (NS-2-10 µg/µl) reversed the damage of DNA caused by MG, lysine and $FeCl_3$ [Figure 5: Lane 6 – lane 7].

Antioxidant activity of crude *Nigella sativa* seed extracts

It was found that as compared to NS-2, the NS-1 has shown a significant reduction in the percentage inhibition at each concentration [Table 2].

DISCUSSION

In the present study, extracts of black cumin seed were checked for their antiglycating potential in *in vitro* glycation of model proteins (BSA and hemoglobin) by sugars (Glu/Rib). For this purpose, physiologically important two proteins and two sugars were used to prepare *in vitro* glycation systems (BSA-Glu, BSA-Rib, and Hb-Glu). In all the glycation systems, an increase in the intensity of browning was observed as compared to controls. However, the presence of seed extracts caused inhibition in the extent of browning. The aqueous extract was more potent in reducing the extent of browning. It has been suggested earlier that the extent of browning of BSA mediated by fructose was decreased in the presence of seed extracts.[8]

Fructosamine assay is a very sensitive method to measure early glycation products. The glycation system showed a significant increase in these products. Both the aqueous and methanolic extracts did not decrease the amount of early glycation products in the BSA-Glucose system. On the other hand, there was a slight decrease in the fructosamine by the methanolic extract in the BSA-Ribose system. This could be possibly due to the differences in the amount of early glycation products generated in both the glycation systems. These products are generated less in the presence of glucose as compared to ribose. Pandey *et al.*[8] have reported a significant reduction by the black cumin seed extracts in the fructosamine content when fructose was used as the glycating agent. The most significant reduction in the amount of early glycation product was observed for the Hb-glucose system. HbA_{1c} has been used as marker for detection of diabetes.[14]

The carbonyls are generated as a result of the modification of early glycation products. The number of carbonyls increases with time and the concentration of glycating agents.[10] The most significant reduction was observed when methanolic extract was incubated with the BSA-Glucose system. Interestingly, the extent of decrease in carbonyl content was more as compared to fructosamine content in all the systems. These results are comparable with earlier reports.[8] Mehmood *et al.*[7] have also reported the role of black cumin seeds in the prevention of accumulation of total AGEs.

Aggregation of proteins is one of the mechanisms by which glycation brings about conformational change in the proteins. The aggregation can be measured by several methods, namely Congo red, aggregation index, and Thioflavin T. It can be observed in the present study that glycation by both glucose and ribose caused severe aggregation of BSA and Hb. Both aqueous and methanolic extracts were able to prevent glycation-induced aggregation. The reversal of aggregation of BSA

Table 2: 2,2-diphenyl-1-picrylhydrazyl assay

Nigella sativa seed sample (concentration) (mg/ml)	Percentage inhibition±SE (NS-1)	Percentage inhibition±SE (NS-2)
1	41.47±4.33	35.21±7.84
2	52.58±0.75	46.71±4.54
3	72.93±5.69	39.02±2.45
4	88.34±4.48	35.48±7.44
5	92.27±0.70	44.37±2.35

$P<0.5$. NS-1 and NS-2: *Nigella sativa* seed extracts; SE: Standard error ($n=3$)

caused by fructose on the addition of *N. sativa* seed extracts has been reported recently.[8]

pBR322 is a sensitive indicator of single strand breaks arising due to damage by glycation and free radicals. The present study was performed to estimate the damage to DNA caused by late stage of glycation model system (MG and lysine). There was reported that free radicals mediated damage to DNA strand and the presence of ferric ion (Fe^{3+}) promoted the damage.[15] The glycation system (lysine, MG, $FeCl_3$) along with *N. sativa* seed extracts was found reversal of DNA damage. Aqueous extract was more potent in reversing this damage as compared to methanolic extract. There are several reports which have shown the prevention of glycoxidative damage of DNA by natural compounds.[8,12] The mechanism of action of these extracts is through the suppression of the generation of free radicals.

The antioxidant capacity was also analysed by DPPH assay to establish the relationship between antioxidant properties of plant extract, the glycation-induced free radical generation and glycoxidative DNA damage.[13] The inhibition of free radical generation increased with increase in the amount of extract. The aqueous extract was found to be possessing much more antioxidant activity as compared to methanolic extract.

The results presented in this study indicate that *N. sativa* seed extracts caused a significant reduction in glycation products as indicated by the decrease in browning, fructosamine content and carbonyl content. These extracts also reversed the structural alteration of protein (aggregation) and DNA (glycoxidative damage) caused by the end products of glycation. However, the solvent used for the preparation of seed extract impacted the inhibitory potential of *N. sativa*. There are many bioactive compounds in the black cumin seeds and one of the major active compounds is thymoquinone. These compounds may act either by preventing the formation of glycation products at any of the stages (early or late) or as potent antioxidants and/or both. In a recent report, thymoquinone was shown to have antiglycating and antiaggregation potential.[12]

CONCLUSION

In the present study, we have compared the antiglycating potential of black cumin seed extract on two physiologically important proteins (BSA and Hb) and two sugars with different reactivity. The results presented in this study show that the black cumin seed extracts (aqueous and methanolic) possess substantial antiglycating potential. The inhibitory effect was more prominent at the late glycation stage. For most of the measured parameters the aqueous extract was found to be more potent in preventing the accumulation of glycation products as compared to methanolic extracts. Interestingly the antioxidant potential of the aqueous extract was more than two-fold than the methanolic extract. It can be concluded that the observed inhibitory effect of this aqueous extract is probably due to its high free radical scavenging capacity. The seed extract can be used for preventing glycation-mediated secondary complications of diabetes, glycoxidative damage of DNA as well as protein aggregation mediated neurological disorders. The exact mechanism of the inhibition by seed extract and active compounds involved in the process need to be characterized with further studies.

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Conflicts of interest

There are no conflicts of interest.

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