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Monosaccharide-Mediated Glycoxidation of Bovine Serum Albumin and Its Prevention by *Nigella sativa*

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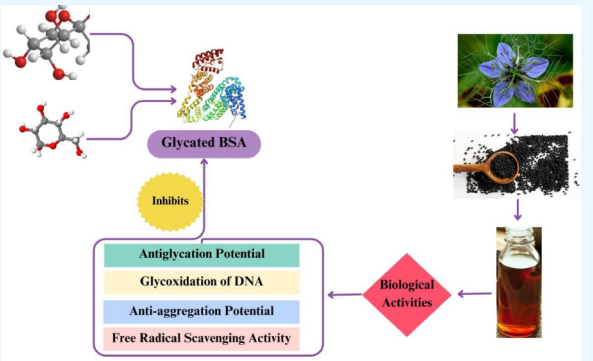
ABSTRACT: The substantial rise in metabolic illnesses that has occurred in both developed and developing countries over the last three decades has been linked to an increase in sugar-added foods and sweetened beverage intake. The significance of advanced glycation end products (AGEs) in the pathophysiology of metabolic diseases related to modern nutrition is an emerging issue. Spices and herbs can potentially be potent AGE production inhibitors due to their high polyphenol content. The inhibitory activity of an aqueous extract of *Nigella sativa* seeds (NS) on glucose- and fructose-mediated glycation of bovine serum albumin (BSA) was investigated. The glycation of proteins and its prevention using NS were assessed using spectrophotometry, spectrofluorometrics, and electrophoretic techniques. Additionally, the NBT assay, DNPH assay, Ellman assay, and thioflavin T assay were used to observe the biochemical alterations caused by glycated BSA. Molecular docking was employed to dock the BSA active site residues with inhibitors. Our data showed that NS protects against glucose- and fructose-mediated glycation and aggregation in vitro by inhibiting the formation of fructosamine, protein carbonyl content, free sulfhydryl groups, and fluorescent AGEs. Furthermore, NS also inhibited the production of β-cross-amyloid aggregates in proteins. It was interesting to note that the inhibition was found to be significantly higher in the Glu-BSA system, although the glycation product formed in the Fru-BSA system was higher compared to the Glu-induced protein system. It can be concluded that, by inhibiting AGE production, oxidation, and aggregation of the protein, NS may be an effective antiglycation drug for the prevention of diabetes complications.



INTRODUCTION

In the past few decades, nonenzymatic processes involving carbohydrates have received a lot of interest in both in vitro and in vivo conditions.[1] This might be explained by the positive and negative effects that carbohydrates have in various experimental paradigms.[2] Both effects can be attributed to processes such as free radical modification, glycoxidation (a chemical process involving the oxidation of sugars, specifically through reactions with proteins or lipids), or autoxidation of carbohydrates and their derivatives. It has been well studied how reducing monosaccharides affect the production of reactive oxygen species (ROS) and reactive carbonyl species (RCS).[3−5] While glucose has been extensively studied due to its abundance and its role in various physiological processes, fructose has received relatively less attention in the field of non-enzymatic carbohydrate reactions.[6,7] Fructose, like glucose, is a reducing monosaccharide that can undergo nonenzymatic processes, leading to the generation of ROS and RCS.[8] Furthermore, there is disagreement in the literature about the reactivities of fructose and glucose. Although there is some evidence that glucose reacts more quickly than fructose in nonenzymatic glycosylation reactions in vitro, numerous studies have reported the opposite, suggesting that fructose is more reactive than glucose.[9−11] The conflicting findings make it difficult to draw definitive conclusions.

Additionally, fructation, also referred to as glycation in the case of glucose or fructose, is a process in which the reducing free C═O group of fructose, similar to that of glucose, may interact nonenzymatically with the $-NH_3$ group of biological molecules.[12] To assess glucose and fructose involvement in the formation of glycoxidation products and their prevention, an aqueous extract prepared from *Nigella sativa* seeds has been used as an in vitro model. Antiglycation drugs have drawn a lot of attention as a potential treatment for diabetic problems, although medications like aminoguanidine have exhibited substantial side effects.[13] As a result, considerable effort has been put into finding medicinal plants with the ability to both

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effectively suppress AGE production and possess antioxidant characteristics.[14−16]

Traditional medicines, such as traditional Chinese medicine, Ayurveda, and Unani medicine, are still used in many parts of the world as they contain a variety of distinct advantages, including extensive clinical experience, a wide range of chemical constituents, and biological activity.[17] Numerous aromatic, medicinal, spicy, and other plant species contain chemical elements, such as alkaloids, tannins, terpenoids, flavonoids, and steroids, that have antioxidant capabilities and show these beneficial effects. Black cumin seeds, also known as *Nigella sativa* L., are of great commercial significance, particularly to the culinary, pharmaceutical, and cosmetics industries.[18]

N. sativa is a significant medicinal plant in the Ranunculaceae family, with numerous health benefits, and has demonstrated therapeutic actions against several diseases. Since ancient times, many ailments have been treated using whole seeds, extracts, and oils.[19] Combining the *N. sativa* seed extract with conventional antiglycation agents could offer synergistic effects in preventing protein carbonylation and AGE formation. *N. sativa*, rich in thymoquinone, provides strong antioxidant and anti-inflammatory benefits.[20] Conventional antiglycation agents, such as aminoguanidine or pyridoxamine, directly inhibit glycation processes. Together, they may enhance overall antioxidant defense, more effectively reduce oxidative stress and AGE formation, and offer broader health benefits. This combination could potentially allow for lower doses of each agent, minimizing side effects and maximizing therapeutic efficacy. The complementary mechanisms of action could lead to improved prevention of AGE-related damage.[21]

N. sativa contains several active compounds, but thymoquinone has been shown in numerous trials to have anticancer, antidiabetic, and hepatoprotective properties. Therefore, the present investigation examined the effect of the aqueous extract of the *N. sativa* seeds on glucose- and fructose-mediated glycation, oxidation, and aggregation of bovine serum albumin (BSA). Molecular docking has been used to study the binding affinity of TQ with BSA.

MATERIALS AND METHODS

Plant Material and Chemicals. The fresh seeds of *N. sativa* were provided by Dr. A. Sattar, Mumbai, and have been authenticated by the Institute of Herbal Science, Plant Anatomy Research Centre, Chennai, India (Registration no. PARC/2019/3911). Bovine serum albumin (BSA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenylhydrazine (DNPH), glucose, fructose, L-cysteine, nitroblue tetrazolium (NBT), and thioflavin T (ThT) were obtained from Sigma-Aldrich (India). Guanidine hydrochloride and trichloroacetic acid (TCA) were purchased from Merck. All of the other chemicals that were used were of analytical grade. Initially, the seeds were washed three times with distilled water and then dried at 37 °C on blotting paper in the laboratory to eliminate moisture. Next, the seeds were ground into a fine powder and subjected to extraction using Soxhlet apparatus with water as the solvent. Following extraction, the solvent was evaporated using a thermostat to concentrate the extract.

High-Performance Liquid Chromatography Analysis of *N. sativa* Seed Extract. A reversed-phase high-performance liquid chromatography (RP-HPLC) method, known for its simplicity, sensitivity, and precision, was employed for the characterization of the aqueous extract of *N. sativa* seeds using a Shimadzu high-performance liquid chromatography instrument.[22] Reverse-phase separations were carried out on the C-18 (25 cm × 4.6 mm; diameter 5 μm) column. Approximately 1% (v/v) aqueous acetic acid solution (solvent A) and acetonitrile (solvent B) were used as the mobile phases in an isocratic system with a flow rate of 0.5 mL/min. The column was thermostatically controlled at 25 °C, and the injection volume was maintained at 20 μL with 254 nm as the detection wavelength. The peaks corresponding to thymoquinone were identified by comparing the chromatogram of the extract with that of thymoquinone. The amount of thymoquinone present in the sample was calculated as follows: (peak area of *N. sativa* seed extract/peak area of standard)/(concentration of seed extract/concentration of standard) × 100.

Antidiabetic Potential of *N. sativa* Seed Extract. *α-Amylase Inhibition Assay.* Inhibition of α-amylase activity was determined using dinitrosalicylic acid as described before.[23] Acarbose (0−1 mg/mL) was used as a standard. The percentage of inhibition of enzyme activity was calculated as % Inhibition = $[(A_{control} - A_{treatment})/A_{control}] \times 100$, where $A_{control}$ is the absorbance in the control sample at 540 nm and $A_{treatment}$ is the absorbance in treatment with the extract at 540 nm.

α-Glucosidase Inhibition Assay. Inhibition of α-glucosidase activity was determined using *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) as described before.[23] Acarbose (0−1 mg/mL) was used as a standard. The percentage of inhibition of enzyme activity was calculated as % Inhibition = $[(A_{control} - A_{treatment})/A_{control}] \times 100$, where $A_{control}$ is the absorbance at 405 nm in the control sample, and $A_{treatment}$ is the absorbance at 405 nm in treatment with the extract.

Incubation Scheme to Evaluate Sugar-Induced In Vitro Glycation of BSA. The glycated BSA was formed using a previous approach with slight modifications.[24] 10 mg/mL of BSA was incubated with 100 mg/mL of glucose and fructose in 100 mM PBS at a pH of 7.4 and 0.02% sodium azide (NaN_3) for 28 days at 37 °C with or without the aqueous extract of *N. sativa* seeds (100 μg/mL).

Assessment of Browning. The degree of browning was assessed at 420 nm.[25] The % inhibition was measured using the following equation: % Inhibition = [(absorbance of Glu-BSA/Fru-BSA − absorbance of native/NS-treated BSA)/absorbance of Glu-BSA/Fru-BSA] × 100.

Estimation of Fructosamine Content in Glycated BSA. The keto-amine moieties created during protein glycation were identified using the NBT reduction assay.[26] As previously mentioned, the absorbance of samples was measured at 530 nm, and the amount of ketoamine in each sample (nmol/mg of protein) was calculated using an extinction coefficient value of 12640 M^{-1} cm^{-1}.

Estimation of Fluorescent AGE Formation in Glycated BSA. The presence of fluorescent AGEs in the incubated samples was estimated by using fluorescence spectroscopy on a Cary Eclipse fluorescence spectrophotometer. The fluorescence intensities (F.I.) of incubated samples were measured at excitation and emission wavelengths of 380 and 440 nm, respectively.[27] The percentage change in F.I. was computed using the following equation: % change in F.I. = [(F.I. of Glu-BSA/Fru-BSA − F.I. of native/NS-treated BSA)/F.I. of Glu-BSA/Fru-BSA] × 100.

Estimation of Protein-Bound Carbonyl Groups in Glycated BSA. The carbonyl group in all the incubated

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samples was assayed and evaluated using the method described by Levine and colleagues, with a few minor modifications.[28] The absorbance was taken at 370 nm, and the amount of carbonyl content in each sample (nmol/mg of protein) was calculated using an extinction coefficient value of 22000 M^{-1} cm^{-1}.

Estimation of Protein-Bound Sulfhydryl Groups in Glycated BSA. Ellman's assay was used to detect the free thiols in all the samples.[29] The absorbance of the incubated samples was measured at 410 nm. From the standard curve of L-cysteine, the amount of free sulfhydryl groups (nmol/mg of protein) was determined.

Thioflavin T (ThT)-Specific Fluorescence Detection. The production of amyloid fibrils was assessed in each of the incubated samples. At 440 nm, the samples were excited and emission was observed between 450 and 600 nm. The fluorescence intensities of incubated samples were measured at 490 nm using the same equation mentioned in the last section, and the findings were expressed as arbitrary units (a.u.).[30]

SDS-PAGE. Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to examine the impact of NS on the glycation system.[31] BSA alone, BSA + glucose, BSA + fructose, BSA + NS, BSA + glucose + NS, and BSA + fructose + NS were incubated at 37 °C for 4 weeks. At 80 V, samples were electrophoresed after being loaded onto gels with a tracking dye. Gels were dyed with a bromophenol blue dye for 60 min, after which they were destained according to established protocols. The staining solution typically contains 0.1% Coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid. The destaining solution typically contains 40% methanol, 10% acetic acid, and 50% distilled water.

In Vitro Glycation of Plasmid DNA. The monosaccharide-mediated DNA damage was investigated using the previous approach.[29] The pBR322 (0.25 μg), lysine (20 mM), glucose/fructose (250 mM), and $FeCl_3$ (100 μM) were incubated in the presence or absence of extract in potassium phosphate buffer (100 mM; pH 7.4). The reaction mixtures were incubated at 37 °C for 3 h. Samples were examined by using 1% agarose gel electrophoresis and visualized in Gel-Doc after incubation.

Molecular Docking. *Template Search and Energy Minimization.* The protein sequence of bovine serum albumin was considered for a BLAST search[32] and the listed template with better identity, and query coverage was considered for structure validation and docking. The listed template with a better resolution was downloaded from the Protein Data Bank[33] and considered for energy minimization using GROMACS software.[34] The ligand, thymoquinone, was downloaded from the Protein Data Bank with a PDB ID: 7SJG[35] and considered for conformer generation using the FROG2 online server.[36]

Protein–Ligand Docking. The energy-minimized bovine serum albumin and thymoquinone were considered for protein–ligand docking using AutoDock 1.5.6 software.[37] Prior to docking, the Kollman and Gasteiger charges were added to the protein and the ligand, respectively. The grid for thymoquinone was placed based on the active site details obtained from the literature review, wherein thymoquinone was docked at binding site 1.[38] The grid size for thymoquinone was maintained at 46, 60, and 50 Å for *x*, *y*, and *z*, respectively. The grid center was also set to −5.77, −8.2, and −10.41 Å for *x*, *y*, and *z*, respectively. The AutoGrid 4.0 and AutoDock 4.0 programs were used to generate grid maps, and 10 conformers were generated using the Lamarckian genetic algorithm (LGA) for the compound.

Statistical Analysis. ANOVA was used to examine the statistical significance using GraphPad Prism for Windows (version 8.01, GraphPad Software, San Diego, USA). The samples were obtained in triplicate, and the data were represented as mean ± SD.

RESULTS AND DISCUSSION

The current study evaluated the antidiabetic and antigication potential of *N. sativa* seed extract to treat symptoms of diabetes and its complications. Our prior report[21] has examined the antioxidant capacity of the extract. The ability of the extract to impede the glycation reaction has been assessed at three levels: 1) early and late stages of the glycation reaction, 2) oxidation of albumin, and 3) aggregation of amyloid.

HPLC Analysis. For the standardization of the plant extract, HPLC is a sensitive and accurate tool that is widely used for the quality assessment of plant extracts and their derived product/formulation. HPLC chromatograms for the aqueous extract of *N. sativa* seeds and standard thymoquinone were obtained and are shown in Figure 1. The thymoquinone peak

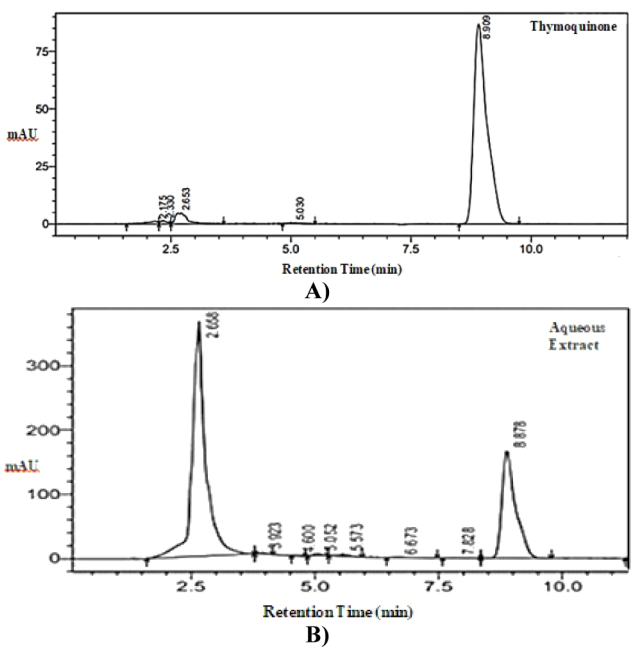

Figure 1. HPLC chromatogram of A) standard thymoquinone and B) the aqueous extract of *N. sativa* seeds.

from the aqueous extract was identified by comparing its single spot at R_f = 8.878 with those obtained by chromatography of the standard thymoquinone under the same conditions (R_f = 8.909). The thymoquinone content in the aqueous extract of *N. sativa* was quantified and found to be 480 μg/mL. These are impressive quantities compared to the existing data. Previously, reported levels of thymoquinone were 5 μg/mL or less.[39] The presence of the observed thymoquinone content in the aqueous extract of *Nigella sativa* seeds enhances the extract's ability to combat oxidative damage more effectively. Compared to other natural antioxidants like vitamin C or E, thymoquinone has shown strong free radical scavenging

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abilities, potentially providing a unique or complementary antioxidant profile.[40]

Antidiabetic Potential of *N. sativa* Seed Extract. The ability of the extract to inhibit α-amylase and α-glucosidase enzyme activity was determined at different concentrations between 200 and 1000 μg/mL. The extract showed α-amylase and α- glucosidase inhibition activity in a dose-dependent manner (Table 1). The inhibition of α-amylase activity was

Table 1. Percentage Inhibition of α-Amylase and α-Glucosidase Enzymes in the Presence of *N. sativa* Seed Extract.[a]

α-Amylase Enzyme Inhibition	
Sample	% Inhibition
acarbose	46.93 ± 0.098
aqueous extract of *N. sativa*	41.88 ± 0.035
α-Glucosidase Enzyme Inhibition	
acarbose	49.38 ± 0.009
aqueous extract of *N. sativa*	45.81 ± 0.027

[a]Results are presented as mean ± SD.

observed in the range of 19.35 ± 0.017% to 41.88 ± 0.035% in the presence of the extract and in the range of 22.01 ± 0.035% to 46.93 ± 0.098% with acarbose. The inhibition of α-glucosidase activity was observed in the range of 19.88 ± 0.054% to 45.81 ± 0.027% in the presence of the extract and in the range of 23.91 ± 0.083% to 45.81 ± 0.027% with acarbose. In a previous study, it was reported that the acetone fractions showed a slightly higher inhibition, 21.44 ± 1.54% to 72.26 ± 1.42%, as compared to hexane fractions, 14.60 ± 1.12% to 33.37 ± 0.31%.[41] This significant antidiabetic potential of NS compared to the antidiabetic drug acarbose may suggest the use of plant-originated natural antidiabetic agents such as those present in *N. sativa*.

Effects of the Extract on Protein Glycation. *Assessment of Browning.* Browning intensity is the initial indicator for glycation, and the degree of browning was measured at 420 nm.[23] The results indicate that *N. sativa* seed extract inhibited browning in a time-dependent manner in both Glu-BSA and Fru-BSA systems (Figure 2). The least browning was seen in BSA that was not incubated with Glu/Fru, which may be due

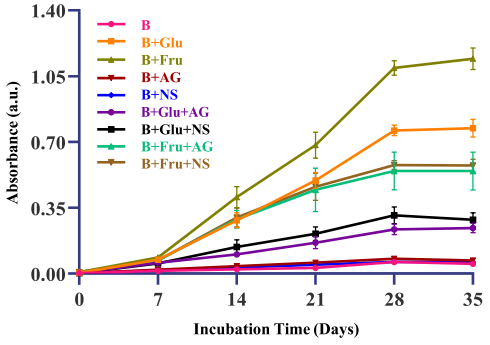

Figure 2. Browning measurement of the incubated samples that contained native BSA (B), BSA + glucose (B + Glu), BSA + fructose (B + Fru), BSA + aminoguanidine (B + AG), BSA + *N. sativa* extract (B + NS), BSA + glucose + aminoguanidine (B + Glu + AG), BSA + glucose + *N. sativa* extract (B + Glu + NS), BSA + fructose + aminoguanidine (B + Fru + AG), and BSA + fructose + *N. sativa* extract (B + Fru + NS).

to time-dependent internal structural modifications. It is noteworthy that the browning of BSA incubated with Glu/Fru (control) was hypothetically considered to be 100% (maximum browning). The percentage inhibition of browning by NS (100 μg/mL) was in the range of 19.78−40.59% from Day 7 to Day 35 for the Glu-BSA system and in the range of 14.63−31.26% in the Fru-BSA system from Day 7 to Day 35. Furthermore, AG reduced the browning in the range of 23.66 to 37.41% in the Glu-BSA system and 19.38 to 52.33% in the Fru-BSA system for the same period of incubation.

Estimation of Fructosamine Content. For the optimal generation of Amadori products, a nitroblue tetrazolium reduction (NBT) experiment was performed on BSA modified by Glu/Fru. The fructosamine content of samples during 4 weeks significantly increased throughout the incubation period (Figure 3). At week 4, the amount of ketoamine in BSA alone

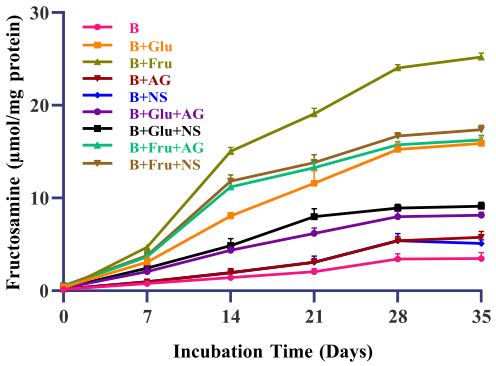

Figure 3. Fructosamine content measurement of the incubated samples that contained native BSA (B), BSA + glucose (B + Glu), BSA + fructose (B + Fru), BSA + aminoguanidine (B + AG), BSA + *N. sativa* extract (B + NS), BSA + glucose + aminoguanidine (B + Glu + AG), BSA + glucose + *N. sativa* extract (B + Glu + NS), BSA + fructose + aminoguanidine (B + Fru + AG), and BSA + fructose + *N. sativa* extract (B + Fru + NS).

was 14.36 ± 0.89 μmol/mg of protein, whereas the amount in the Glu-BSA and Fru-BSA systems was found to be 31.35 ± 1.67 and 38.38 ± 1.47 μmol/mg of protein, respectively. On the other hand, treatment with NS in the Glu-BSA and Fru-BSA systems significantly decreased the amount of ketoamine to 24.06 ± 1.03 and 27.03 ± 1.34 μmol/mg of protein, respectively (Figure 3). The presence of AG suppressed the fructosamine content to 7.98 μmol/mg of protein in the Glu-BSA system and 16.24 ± 0.50 μmol/mg of protein in the Fru-BSA system on Day 28. Comparing the percentages of reduction, NS inhibited more glucose-glycated BSA than fructose-glycated BSA, while the formation of glycation products was more in the fructose-glycated BSA system.

Detection of Fluorescent AGE Formation in Glycated BSA. A spectrofluorimetry technique was used to investigate changes in the thermodynamic stability of BSA in the presence of *Nigella sativa* extract. The formation of fluorescent AGEs in Glu/Fru-mediated protein glycation was monitored after 4 weeks of incubation. Figure 4 shows the effects of NS (100 μg/mL) on the formation of fluorescent AGEs after 28 days of incubation. The fluorescence investigation showed a substantial difference between the emission intensities of native and glycated proteins. When glycated BSA was exposed to 100 μg/mL of NS, the treatment decreased the intrinsic FI by 54.55% in the Glu-BSA system and 46% in the Fru-BSA system

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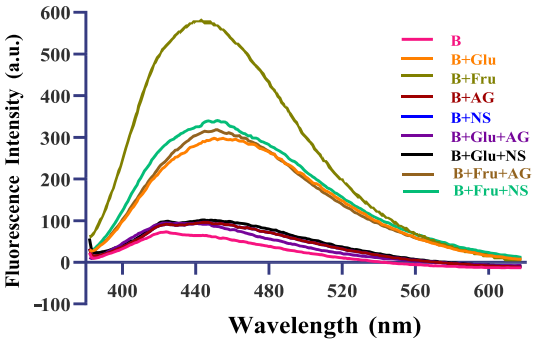

Figure 4. Fluorescence emission spectra of the incubated samples that contained native BSA (B), BSA + glucose (B + Glu), BSA + fructose (B + Fru), BSA + aminoguanidine (B + AG), BSA + *N. sativa* extract (B + NS), BSA + glucose + aminoguanidine (B + Glu + AG), BSA + glucose + *N. sativa* extract (B + Glu + NS), BSA + fructose + aminoguanidine (B + Fru + AG), and BSA + fructose + *N. sativa* extract (B + Fru + NS).

on Day 28, respectively. The findings show that fructose has a strong glycation activity compared to glucose, but the prevention of total fluorescent AGE formation was found to be greater in the Glu-BSA system. In the meantime, AG inhibited AGE formation by 93.45 ± 0.45% in the Glu-BSA system and 93.45 ± 0.45% in the Fru-BSA system.

Effects of *N. sativa* on Glycation-Induced Protein Oxidation. *Estimation of Carbonyl Groups Attached to Proteins.* As shown in Figure 5, the highest percentage of

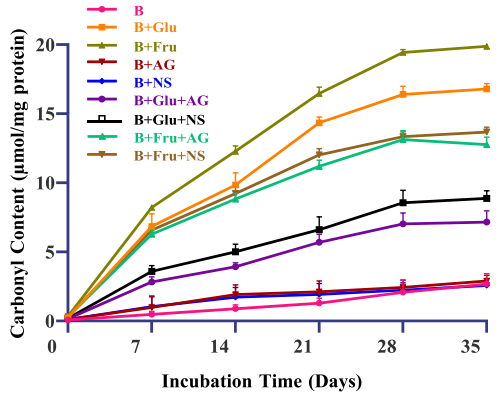

Figure 5. Carbonyl content measurement of the incubated samples that contained native BSA (B), BSA + glucose (B + Glu), BSA + fructose (B + Fru), BSA + aminoguanidine (B + AG), BSA + *N. sativa* extract (B + NS), BSA + glucose + aminoguanidine (B + Glu + AG), BSA + glucose + *N. sativa* extract (B + Glu + NS), BSA + fructose + aminoguanidine (B + Fru+AG), and BSA + fructose + *N. sativa* extract (B + Fru + NS).

carbonyl groups was detected in glycated BSA (positive control). However, the addition of the *N. sativa* seed extract to the glycated system significantly reduced carbonyl group formation, indicating its antioxidant activities. Carbonyls react with dinitrophenylhydrazine to generate 2,4-dinitrophenylhydrazone, which is detected at 370 nm.[42] The carbonyl concentration of BSA alone was determined to be 2.02 ± 0.26 mmol/mg of protein. Both glucose and fructose caused protein stress, resulting in a two-fold to 5-fold increase in carbonyl content. The formation of carbonyl content in the Glu-BSA system increased from 0.24 ± 0.0027 to 15.10 ± 0.68 μmol/mg of protein from Day 0 to Day 35, respectively (Figure 5). Aminoguanidine suppressed carbonyl content formation from 0.21 ± 0.41 μmol/mg of protein to 7.01 μmol/mg of protein from Day 7 to Day 28 compared to glycated BSA. After 4 weeks of incubation, NS reduced the concentration of protein carbonyl to 4.04 ± 0.52 in the Glu-BSA system and 6.54 ± 0.99 in the Fru-BSA system, respectively, as compared to control. This reduction suggests that the extract's antioxidant properties effectively scavenge reactive oxygen species (ROS) and prevent oxidative damage to proteins. By reducing carbonyl formation, *Nigella sativa* helps preserve the structural integrity and function of proteins, thus mitigating glycation-related protein damage.

Estimation of Sulfhydryl Groups Attached to Proteins. Protein modifications caused by sugars are usually accompanied by oxidative stress, which causes several metabolic alterations.[43] Oxidative stress can also cause a change in the redox state of proteins, and sulfhydryl group evaluation using Ellman's reagent is a reliable indicator. When BSA was incubated with Glu/Fru, the level of thiol groups continuously decreased throughout the experimental period (Table 2). The

Table 2. Sulfhydryl Group Measurement of the Incubated Samples that Contained Native BSA (B), BSA + Glucose (B + Glu), BSA + Fructose (B + Fru), BSA + Aminoguanidine (B + AG), BSA + *N. sativa* Extract (B + NS), BSA + Glucose + Aminoguanidine (B + Glu + AG), BSA + Glucose + *N. sativa* Extract (B + Glu + NS), BSA + Fructose + Aminoguanidine (B + Fru + AG), and BSA + Fructose + *N. sativa* Extract (B + Fru + NS).[a]

	free sulfhydryl group content
B	0.14 ± 0.010
B + G	0.059 ± 0.008
B + F	0.040 ± 0.006
B + AG	0.135 ± 0.009
B + NS	0.132 ± 0.007
B + G + AG	0.093 ± 0.017
B + G + NS	0.091 ± 0.003
B + F + AG	0.082 ± 0.009
B + F + NS	0.077 ± 0.016

[a]Results are presented as mean ± SD.

free sulfhydryl content of BSA was found to be 0.14 ± 0.009 mM, which gets reduced to 0.059 ± 0.008 and 0.040 ± 0.006 mM in the Glu-BSA and Fru-BSA systems, respectively. However, NS significantly reduced the depletion of protein sulfhydryl groups and helped the glycated system reach the concentrations of 0.091 ± 0.003 and 0.077 ± 0.016 mM in the Glu-BSA and Fru-BSA systems, respectively, after 4 weeks of incubation. AG also significantly prevented the depletion of protein sulfhydryl groups, and the amount of free sulfhydryl groups found was 0.093 ± 0.017 mM in Glu-BSA and 0.082 ± 0.009 mM in Fru-BSA systems. NS has been shown to preserve sulfhydryl groups on BSA and other proteins. This preservation indicates the extract's ability to maintain protein thiol status and prevent oxidative modifications that could lead to protein dysfunction and aggregation.

Effect of N. sativa on β-Amyloid Aggregation. Measurement of the Protein Aggregation Index. Aggregation of the protein is one of the significant indicators of glycation because carbonyl and thiol groups bound to proteins induce the formation of the cluster. The impact of the aqueous extract of *Nigella sativa* seeds (NS) on the formation of β-amyloid

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Table 3. Protein Aggregation Index of the Incubated Samples

Samples	Protein Aggregation Index ± SE %				
	Day 7	Day 14	Day 21	Day 28	Day 35
B	2.13 ± 0.89	2.42 ± 0.48	2.52 ± 0.34	2.64 ± 0.17	2.78 ± 0.41
B + G	7.87 ± 0.54	15.99 ± 0.55	22.83 ± 0.67	27.41 ± 0.30	28.83 ± 0.19
B + AG	2.26 ± 0.33	2.45 ± 0.88	2.59 ± 0.73	2.78 ± 0.85	2.86 ± 0.72
B + Glu + AG	4.11 ± 0.47	4.10 ± 0.39	4.24 ± 0.43	4.38 ± 0.67	4.45 ± 0.49
B + NS	2.52 ± 0.65	2.63 ± 0.48	2.94 ± 0.28	3.13 ± 0.49	3.21 ± 0.92
B + Glu + NS	3.91 ±0.30	4.89 ± 0.51	5.11 ± 0.31	5.67± 0.49	5.82 ± 0.51
B + F	26.78 ± 0.46	33.23 ± 0.25	38.78 ± 0.79	41.18 ± 0.32	42.09 ± 0.92
B + Fru + AG	4.45 ± 0.21	6.56 ± 0.23	8.34 ± 0.72	10.37 ± 0.78	11.88 ± 0.91
B + Fru + NS	4.93 ± 0.63	7.43 ± 0.36	9.19 ± 0.85	11.23 ± 0.53	12.97 ± 0.84

structures during glycation was assessed using the protein aggregation index (%), as shown in Table 3. This index categorizes protein aggregation as follows: no aggregate (0−2%), moderate (2−5%), and significant aggregation (>5%).[33] Aminoguanidine (AG) led to a reduction in the protein aggregation index (PAI) within the range of 4.11 ± 0.47% to 4.45 ± 0.49% in the Glu-BSA system and from 4.45 ± 0.21% to 11.88 ± 0.91 in the Fru-BSA system, respectively, from Day 7 to Day 35. Moreover, in the presence of NS at a concentration of 100 μg/mL, the PAI for the Glu-BSA system ranged from 3.91 ± 0.30% to 5.82 ± 0.29%, and the PAI for the Fru-BSA system ranged from 4.93 ± 0.63% to 12.97 ± 0.84%.

Thioflavin T (ThT)-Specific Fluorescence Detection. A morphological shift in glycated proteins affects amyloid fibrils and the cross β-structure. The fluorescent dye ThT was used to monitor these changes (Figure 6). When glycated BSA was

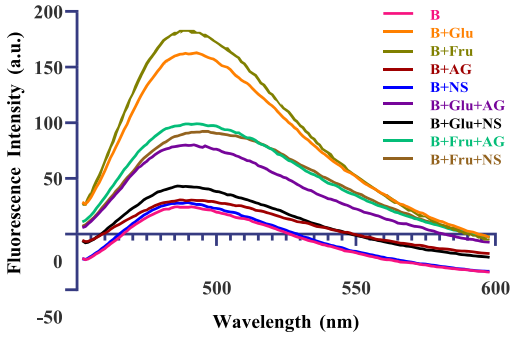

Figure 6. ThT Fluorsecnce spectra of the incubated samples that contained native BSA (B), BSA + glucose (B + Glu), BSA + fructose (B + Fru), BSA + aminoguanidine (B + AG), BSA + *N. sativa*extract (B + NS), BSA + glucose + aminoguanidine (B + Glu + AG), BSA + glucose + *N. sativa* extract (B + Glu + NS), BSA + fructose + aminoguanidine (B + Fru + AG), and BSA + fructose + *N. sativa* extract (B + Fru + NS).

incubated with NS, a substantial decrease in the β-structure was detected compared to the standard. The percentage decrease in fluorescence intensities of glycated BSA in the presence of NS was found to be 56.25% in the Glu-BSA system and 48.65% in the Fru-BSA system on Day 28. In the meantime, AG (10 mM) also reduced the formation of cross amyloid β-structure in Glu-BSA (61.87%) and Fru-BSA (51.33%) systems after 4 weeks of incubation. As a result, the present study demonstrated the ability of NS to reduce the level of the β-amyloid cross structure in BSA mediated by monosaccharides.

SDS-PAGE. The glycated samples were fractionated by SDS-PAGE after 28 days of incubation at 37 °C (Figure 7) to detect protein glycation. Glycosylated aggregates represent a hallmark of the advanced phases of nonenzymatic glycation [35]. Incorporating the NS extract into the glycated system effectively restrained the formation of high molecular weight glycosylated aggregates. The SDS gel pattern in the glycated system (Lane 2) of the gels demonstrated increased cross-linking and protein buildup. High molecular weight protein chains were found to be relatively abundant due to the increased components. As seen in Figure 7, NS (Lane 4) inhibited the production of monosaccharide-induced aggregation and modifications to its properties. Increased components revealed the relative abundance of high molecular weight protein chains. However, the formation of aggregates is more in the Fru-BSA system (Figure 7C; Lane 2) as compared to the Glu-BSA system (Figure 7A,B; Lane 2). NS (Lane 4 of Figure 7A and Lane 6 of Figure 7C) and AG (Lane 4 of Figure 7B and Lane 1 of 7C) prevented sugar-induced aggregation formation and changes in its characteristics. In both, the structural alterations indicated a reduction in cross-linking. The band positions of BSA in the presence of NS and AG were similar to band migration patterns and intensity of bands compared to native BSA (Lane 1 of Figure 7A−C).

In Vitro Glycation of DNA. Glycation of DNA primarily occurs through a series of reactions that involve the attachment of sugar molecules to DNA bases (adenine, cytosine, guanine, and thymine) and the subsequent formation of various cross-links and adducts. These modifications can lead to an altered DNA structure and function, potentially affecting gene expression, replication, and repair mechanisms. Figure 8 shows the effects of AG and NS on DNA damage mediated by the glycation of glucose and fructose with lysine in the presence of Fe^{3+}. The pBR322 alone displayed two bands: band I indicates the linear form, and band II indicates the supercoiled form (Lane 1). Adding glucose/fructose, lysine, and $FeCl_3$ to pBR322 increased the breakage of DNA strands by increasing the intensity of the open circular form of bands (Lane 2). However, the addition of glucose/fructose, lysine, and $FeCl_3$ in the presence of NS/AG/TQ to pBR322 did not result in DNA cleavage; i.e., pBR322 remained in its supercoiled form (Lanes 4 and 6).

Molecular Docking. The BLAST search for the query sequence bovine serum albumin listed PDB ID: 4JK4 (10) with an identity score of 100%. This 3D structure was subjected to energy minimization and considered for docking with the ligand thymoquinone.[44] After docking bovine serum albumin with thymoquinone, ten poses were generated, and the pose with the best binding affinity and lowest inhibitory

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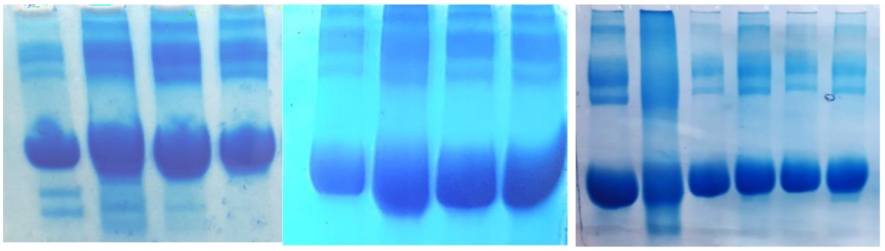

Figure 7. SDS-PAGE analysis of the effect of NS on the aggregation of protein. A) Lane 1: BSA alone; Lane 2: BSA + Glu; Lane 3: BSA + NS; Lane 4: BSA + Glu + NS. B) Lane 1: BSA alone; Lane 2: BSA + Glu; Lane 3: BSA + AG; Lane 4: BSA + Glu + AG. C) Lane 1: BSA alone; Lane 2: BSA + Fru; Lane 3: BSA + NS; Lane 4: BSA + Fru + NS; Lane 5: BSA + AG; Lane 6: BSA + Fru + AG.

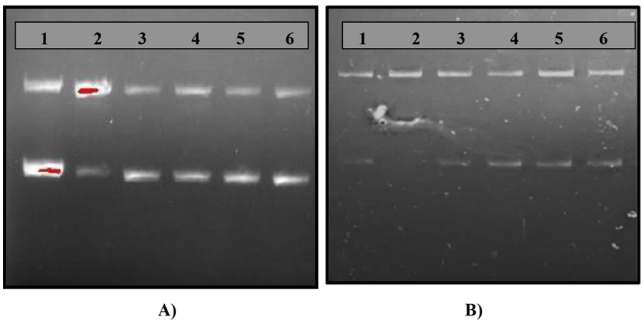

Figure 8. In vitro glycation of DNA. A) Lane 1: DNA; Lane 2: DNA + Lysine + Glucose + $FeCl_3$ (GS); Lane 3; DNA + AG; Lane 4: DNA + GS + AG; Lane 5: DNA + NS; Lane 6: DNA + GS + NS; B) Lane 1: DNA; Lane 2: DNA + Lysine + fructose + $FeCl_3$ (GS); Lane 3; DNA + AG; Lane 4: DNA + GS + AG; Lane 5: DNA + NS; Lane 6: DNA + GS + NS.

constant value was selected. As per the docking report, the binding affinity between thymoquinone and bovine serum albumin is −6.23 kcal/mol, and their inhibitory constant value was 27.12 μM. Positively charged residues like Arg217 and Arg256 showed hydrogen bonds with thymoquinone (Figure 9).

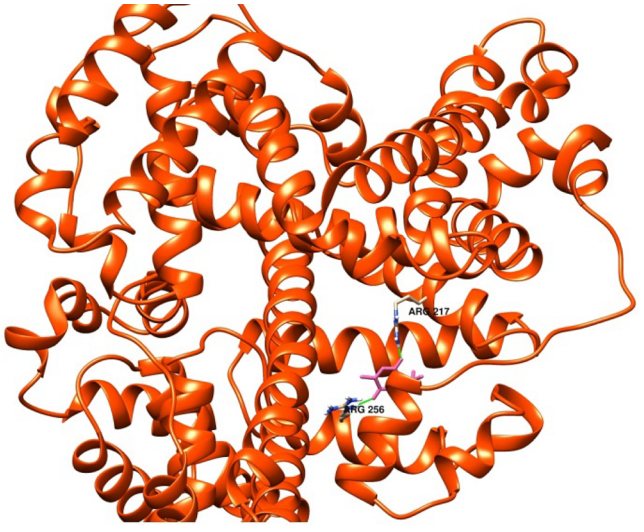

Figure 9. Molecular docking studies of BSA and thymoquinone. The protein and the ligand are represented in ribbon (orange) and wireframe formats, respectively.

DISCUSSION

The study of proteins containing early stage glycation products, or AGEs, has gained attention in scientific research due to their negative effects on protein function, oxidative stress, and tissue damage in aging and certain disorders, particularly diabetes.[45] *Nigella sativa* seeds, with a variety of advantageous biological properties, have been investigated for their antiglycation activity using in vitro experimental methods. The models utilized in this work involved glucose- and fructose-induced glycation of BSA, which is a common method for in vitro glycation and is thought to produce glycated BSA as the main byproduct. In this investigation, BSA-glucose and BSA-fructose incubation would result in heterogeneous groups of AGEs; however, we only emphasized the fluorescent AGEs (instead of the nonfluorescent AGEs), although nonfluorescent AGEs like CML and MG-H1 are important from a clinical perspective.[46] The use of fluorescent AGEs to identify diabetes and cardiovascular problems has been suggested in numerous papers in the literature.[47−49] The fact that the increase in browning intensity was insignificant between Days 28 and 35 suggests that browning in the glycation process reached its maximum in 4 weeks. The results indicate that the decrease in browning in the presence of the *N. sativa* seed extract can be correlated with less formation of glycated brown products. These results agreed with the findings of Pandey et al. (2018), who reported that the aqueous extract (1 mg/mL) caused 47.96% inhibition of the browning of BSA by fructose.[50]

After 4 weeks of incubation, monosaccharide-induced glycated BSA formed much more fructosamine than non-glycated BSA; however, fructosamine was found to be significantly lower in the Glu-BSA system than in the Fru-BSA system. Fructosamine generation was dramatically reduced by the addition of NS. NS reduced fructosamine generation in the Fru-BSA system by 13.84 + 0.87% and in the Glu-BSA system by 23.25 + 1.03%. We found that the glycation rate of fructose was higher than that of glucose. The outcome is consistent with what Suarez and colleagues have observed. They discovered that the rate of glycated BSA formation induced by fructose (0.5 M) was approximately ten times higher compared to glucose 0.5 M over a 32-day incubation period.[51]

The findings of the current study showed that a reduction in fructosamine levels in the presence of NS was linked to a decrease in the generation of AGEs, indicating that NS has an inhibiting effect on the formation of Amadori products and their conversion into AGEs. We investigated how NS affected the emergence of total AGEs using fluorescence attributes. NS effectively prevented the production of AGEs. Meerwaldt et al. (2008) developed a method to quantify AGE accumulation in

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diabetes patients by measuring the fluorescence of AGEs in the skin.[52] This approach allows for a noninvasive assessment of AGE levels and provides valuable information about the glycation effects in patients with diabetes. Fluorescent AGEs have also been investigated as markers for assessing the risk of acute coronary syndrome, a condition characterized by the blockage of blood flow to the heart,[53] and Yamashita et al. (2020) proposed the use of fluorescent skin AGEs as a diagnostic marker for neuropsychiatric illnesses.[54]

We looked at the impact of NS on oxidative markers in an effort to determine the route of action. A key factor in the production of AGEs is the oxidation reactions. There are two ways that this process can happen: 1) through the oxidation of Amadori products, resulting in the generation of reactive protein enediols and protein dicarbonyls. These reactive intermediates can then react with proteins to form AGEs, which are associated with various pathological conditions.; 2) through the autoxidation of free sugars that generates reactive dicarbonyl compounds, such as glyoxal and methylglyoxal, which are highly reactive and can readily react with proteins. The reaction between these dicarbonyl and proteins leads to the formation of ketamine, another type of AGE.[55] NS also reduced the level of protein carbonyl by 28.51 ± 0.52% in Glu-BSA and 22.94 ± 0.99% in Fru-BSA, respectively. The loss of free thiols in albumin also occurs in conjunction with the formation of carbonyl proteins by oxidation. Carbonyl levels were noticeably lower, and there were more free thiol groups in the presence of NS. In another investigation, researchers compared the time courses of glycation of sugars at the same concentrations. According to their studies, the glycation capability of sugars is listed in the following order: ribose > fructose > glucose.[56] Our findings agree with those of several studies that investigated the glycation capacities of various sugars at the same concentrations.

The reduction of the aggregation index in the presence of *N. sativa* seed extract showed a very significant reduction of amyloid cross β-structures compared to the glycated protein, proving the therapeutic potential of the extract against glycation-induced structural alterations in proteins. The reduction of the aggregation index by *N. sativa* seed extract confirms its antiaggregation potential. ThT fluorescence and SDS-PAGE analysis revealed that fructose- and glucose-mediated glycation considerably changed the secondary structures, with the Fru-BSA system producing more β-sheet structures than Glu-BSA, which were essential for the aggregation of proteins. It has been concluded that NS markedly decreased the level of thioflavin T fluorescence, which is a marker of amyloid-β aggregation and fibril formation in the amyloid-β peptide model. The observed reduction in protein aggregation with *Nigella sativa* seed extract can likely be attributed to its antioxidant and anti-inflammatory effects, which help mitigate oxidative stress and may influence pathways related to protein folding and degradation. This, in turn, helps to prevent protein damage associated with glycation. Therefore, it can be speculated that *N. sativa* may help decrease the risks of debilitating neurodegenerative disorders. It is worth noting that a previous study involving *Bunium persicum* hydroalcoholic extract demonstrated a concentration-dependent reduction in amyloid cross-β structures, with reductions of 10.5%, 10.8%, and 29.7% at concentrations of 10, 15, and 30 μg/mL, respectively, similar to the effects observed with *Nigella sativa* extract.[57]

The final part of the study dealt with determining the perturbation of DNA using the pBR322 model and further prevention of strand breaks using an extract. *N. sativa* extract was found to be coherent, decreasing linearity, and restoring the DNA back to its supercoiled form. The formation of AGEs, including those resulting from glycoxidation of DNA, has been implicated in various age-related diseases, such as diabetes, neurodegenerative disorders, cardiovascular diseases, and cancer.[58]

These findings suggest that fructose glycation modifies the microenvironment of BSA and enhances the level of protein aggregation. The following inference can be made in light of these findings: "fructose may play a key role in diabetic complications alongside glucose in the leading role." One of the most significant factors in the pathophysiology of chronic diabetes is the formation of AGEs. In our research, fructose produced significantly more AGEs than glucose when it was incubated with BSA. These findings show that fructose is a major factor in problems associated with diabetes.

It has to be noted that this study has several limitations, including the use of high sugar concentrations to elevate the glycation process. Potential interactions between *Nigella sativa* and existing medications or treatments for metabolic disorders should be carefully considered in future clinical studies. It is still unclear exactly how the compounds in the extract get metabolized in vivo and the plethora of factors orchestrating this process, such as the amount and extent of glycemia. However, a comprehensive study of the extract could help determine the bioactive compound involved in contributing to the antiglycating properties of *N. sativa*.

CONCLUSION

According to our findings, *Nigella sativa* seeds (NS) exhibit protective effects against glucose- and fructose-mediated glycation in vitro. It helps in lowering fructosamine levels, inhibiting the development of fluorescent advanced glycation end-products (AGEs), reducing protein aggregation, decreasing carbonyl content in proteins, and preventing sulfhydryl group modification in BSA. These positive effects of NS suggest that it could potentially be utilized for the prevention or management of AGE-mediated diseases, especially in individuals who are at risk of developing complications related to diabetes. However, while the in vitro findings are promising, it is important to investigate how the components of NS are metabolized in the body (in vivo) and various factors that influence this process. The precise mechanisms by which NS exerts its effects on living organisms remain unknown. Factors such as the level and severity of glycemia (glucose levels in the blood) may play a role in the antiglycation properties of NS. To gain a better understanding of the potential benefits of NS in preventing glycation-related diseases, further research using animal models is necessary. Animal studies can provide valuable insights into the effects of NS on glycation processes within a living system, allowing for a more comprehensive assessment of its antiglycation properties.

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