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RESEARCH ARTICLE

Fenfuro®-mediated arrest in the formation of protein-methyl glyoxal adducts: a new dimension in the anti-hyperglycemic potential of a novel fenugreek seed extract

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ABSTRACT

The fenugreek plant (*Trigonella foenum-graecum*) is traditionally known for its anti-diabetic properties owing to its high content of furostanolic saponins, which can synergistically treat many human ailments. Non-enzymatic protein glycation leading to the formation of Advanced Glycation End products (AGE) is a common pathophysiology observed in diabetic or prediabetic individuals, which can initiate the development of neurodegenerative disorders. A potent cellular source of glycation is Methyl Glyoxal, a highly reactive dicarbonyl formed as a glycolytic byproduct. We demonstrate the *in vitro* glycation arresting potential of Fenfuro®, a novel patented formulation of Fenugreek seed extract with clinically proven anti-diabetic properties, in Methyl-Glyoxal (MGO) adducts of three abundant amyloidogenic cellular proteins, alpha-synuclein, Serum albumin, and Lysozyme. A 0.25% w/v Fenfuro® was able to effectively arrest glycation by more than 50% in all three proteins, as evidenced by AGE fluorescence. Glycation-induced amyloid formation was also arrested by more than 36%, 14% and 15% for BSA, Alpha-synuclein and Lysozyme respectively. An increase in MW by attachment of MGO was also partially prevented by Fenfuro® as confirmed by SDS-PAGE analysis. Glycation resulted in enhanced aggregation of the three proteins as revealed by Native PAGE and Dynamic Light Scattering. However, in the presence of Fenfuro®, aggregation was arrested substantially, and the normal size distribution was restored. The results cumulatively indicated the lesser explored potential of direct inhibition of glycation by fenugreek seed in addition to its proven role in alleviating insulin resistance. Fenfuro® boosts its therapeutic potential as an effective phytotherapeutic to arrest Type 2 diabetes.

HIGHLIGHTS

1. Fenfuro® is a novel patented formulation of Fenugreek seed extract with more than 45% furostanolic saponins and anti-diabetic property free from any side effect as established through clinical study.
2. In the present study, the role of Fenfuro® in arresting *in vitro* AGE formation and glycation-induced amyloid formation has been demonstrated with the help of three amyloidogenic proteins, namely Human Lysozyme, Human alpha-synuclein and Bovine Serum Albumin using Methyl Glyoxal as the glycating agent.
3. A 0.25% (w/v) ethanolic solution of Fenfuro® resulted in more than 50% arrest in glycation with simultaneous prevention of aggregation as demonstrated by native PAGE, DLS and inhibition of development of Thio-T positive amyloid like entities.
4. The studies collectively aim toward the development of a safe therapeutic method for arresting protein glycation through direct physical intervention.

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Advanced glycation end product; fenugreek seed extract; amyloidogenic protein; methyl glyoxal adduct; antidiabetic phytotherapeutic

Introduction

Prolonged persistence of sugar molecules in the extracellular milieu leads to hyperglycemia or elevated blood sugar levels, a clinical marker for diabetes or prediabetes. However, in addition to diabetic complications, an often ignored but intricately related pathophysiology occurs from the accumulation of Advanced Glycation End Product (AGE) adducts, resulting from the attachment of sugars to free amino groups of macromolecules including proteins and nucleic acids *via* Schiff base formation. AGE adducts present a twofold challenge for the cellular defense: a) they are often sequestered by specific membrane receptors termed as Receptors for AGE or RAGE expressed ubiquitously by many cell types, especially macrophages, which leads to induction of inflammatory response,

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oxidative response, or apoptosis *via* multiple signaling pathways (Bhattacharya et al. 2023). Alongside, these adducts also induce protein cross-linking, accounting for additional pathophysiologies such as arterial wall thickening associated cardiovascular ailments, diabetic neuropathy, diabetic nephropathy (Vasan et al. 2003), and other acute inflammation-associated disorders (Banik et al. 2020) b) AGE adducts of several serum abundant proteins such as albumin, lysozyme, insulin etc. resemble amyloid-like plaques (Iannuzzi et al. 2014; Das et al. 2020; 2022) with associated cytotoxicity (Wei et al. 2009) which can eventually result into onset or aggravation of neurodegenerative syndromes (Brás et al. 2019; Li et al. 2012). Since carbohydrates represent the staple diet of the global population, the formation of AGE is generally unavoidable. However, such adducts generally do not accumulate beyond critical concentrations to lead to amyloid-like deposits or initiate other cellular complications. Instead, the major source of AGE adducts in the body comes from other lifestyle habits such as high free sugar diet (mostly in the form of fast foods and carbonated sweetened beverages), consumption of processed food, and smoking (Rungratanawanich et al. 2021). Therefore, chronic hyperglycemia may account for a significant reason for cognitive decline in an individual, as has been observed in numerous clinical cases over the years (Umegaki 2012; Madhusudhanan et al. 2020; Gupta et al. 2023).

A fenugreek seed extract has been a popular phytotherapeutic for curbing blood sugar levels for ages (Shabil et al. 2023). Therefore, it has been widely used as an effective alternative medicine for the treatment of T2DM and other associated ailments (Verma et al. 2016; Kim et al. 2023; Hota et al. 2024). There have been numerous theories and hypotheses to explain the antihyperglycemic effect of fenugreek; a popular theory states that fenugreek contains soluble fibers such as glucomannan, which slows down absorption of sugars in the intestine, thus arresting the quick rise in blood sugar levels (Mirzababaei et al. 2022). In addition, alkaloids such as fenugrecin and trigonelline have been implicated in enhancing insulin sensitivity by augmenting the function of β-cells (Subramanian and Prasath 2014). Another abundant molecule found in Fenugreek, 4-hydroxyisoleucine, has also been reported to increase the pancreatic release of insulin (Avalos-Soriano et al. 2016). An active compound isolated from Fenugreek seed extract termed N55 has been reported to enhance Glucagon Like Peptide-1 (GLP-1) hormone activity (King et al. 2015) and stimulate tyrosine phosphorylation of the insulin receptor, insulin receptor substrate 1 (IRS-1), as well as the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Vijayakumar et al. 2005). Almost all of the mechanistic insights obtained thus far on antidiabetic properties of fenugreek have been synergistic and centered on cellular signaling pathways to increase insulin sensitivity. However, studies on direct inhibition of protein glycation by fenugreek have been rare. A novel proprietary form of Fenugreek seed extract, Fenfuro®, was prepared in our laboratory, which contained more than 45% furostanolic saponins. The clinical efficacy of this compound against Type 2 diabetes was successfully demonstrated in a randomized, double-blind, placebo-controlled study (Hota et al. 2024) where supplementation of 500 mg of the formulation twice daily along with Metformin and/or sulphonylurea for a period of 12 weeks resulted in more than 33% decrease in postprandial glucose with significant reduction in fasting glucose. Concomitantly, the insulin sensitivity of the affected subjects also improved considerably, as revealed by a drop in baseline HOMA index from 4.27 to 3.76. The formulation was also applied successfully for the treatment of Polycystic Ovarian Syndrome (Sankhwar et al. 2023; Singh et al. 2023). However, a mechanistic study to explore the mode of action of this active constituent hasn't yet been carried out.

In the present studies, we demonstrate the direct effect of 0.25% (w/v) ethanolic solution of a patented fenugreek extract, Fenfuro®, in the partial arrest of Methyl Glyoxal mediated glycation of three abundant serum proteins, a) Bovine Serum Albumin (BSA), an abundant serum transporter protein (Watanabe et al. 1989) b) lysozyme, a small antibacterial protein found abundantly in mucosal secretions (Ferraboschi et al. 2021) and alpha-synuclein, a synaptic chaperone mediating folding and proper secretion of neurotransmitters across synaptic junctions (Stefanis 2012). Both BSA and lysozyme are globular proteins that can undergo reversible self-aggregation to transform into beta-pleated sheets typical of amyloidogenic transition (Holm et al. 2007; Catalini et al. 2021), whereas alpha-synuclein departs from its physiological alpha-helical conformation at the slightest cellular cue to result in amyloid fibrils (Ohgita et al. 2022). Methyl Glyoxal is a highly reactive dicarbonyl byproduct of the glycolytic pathway and hence represents one of the most potent physiologically active glycating agents (Allaman et al. 2015). Glycation is known to adversely affect both the structure and function of all these proteins. Ribosylation of BSA resulted in the formation of Thio-T positive globular amyloids (Das et al. 2020), whereas attachment of Methyl Glyoxal resulted in both structural and functional alteration of lysozyme (Muraoka et al. 2022). Glycation is also known to potentiate the fibrillation of alpha-synuclein by increasing the propensity toward adoption of beta fibrillar structures (Vicente Miranda et al. 2016). Through this *in vitro* study, we wanted to investigate whether fenugreek has the potential to directly inhibit protein glycation.

Materials and methods

Purification of alpha-synuclein

Expression and purification of recombinant human α-synuclein was carried out in Escherichia coli BL21 (DE3) strain transformed with pRK172 α-Synuclein WT plasmid using 1 mM isopropyl thiogalactoside (IPTG) as inducer as described previously (Mahapatra et al. 2020).

In vitro glycation of proteins in the presence and absence of fenfuro®

In vitro, glycation of the three model proteins Bovine Serum Albumin (Sigma, A7030), Human Lysozyme (Sigma, L1667), and Alpha-synuclein (recombinant human, expressed in *E.coli*) was performed with Methyl Glyoxal (MGO) by the method of

Muraoka (Muraoka et al. 2022). Briefly, 200–300 µM of the respective proteins with 50 mM MGO in 200 mM sodium phosphate buffer, pH 7.4 along with 0.02% sodium azide were incubated in the dark at 37 °C for 96 h in presence or absence of a 2.5 mg/ml ethanolic (30% v/v in millipore water) solution of the fenugreek seed extract, freshly prepared at a stock concentration of 50 mg/ml and subsequently filter sterilized. The concentration of Fenfuro® used in the study, as well as the time of glycation, was optimized according to the maximal inhibition in glycation without any major structural perturbation of the protein structures. Glycation was stopped after 10 days for BSA and 4 days for each of alpha-synuclein and lysozyme by dilution in five volumes of 0.2 M sodium acetate buffer, pH 5, with subsequent incubation at 37 °C for 2 h to release glycosylamine adducts. Thereafter, the samples were dialyzed O/N extensively against 0.1 M sodium phosphate buffer, pH 7.4, at 4 °C to get rid of the free unreacted MGO molecules.

AGE-fluorescence

In vitro glycation was confirmed by the AGE fluorescence assay as described previously on a Perkin-Elmer LS55 spectrofluorometer (Das et al. 2020). About 10 µM protein samples were excited with 370 nm, and corresponding emission spectra were obtained between 390–500 nm. Both excitation and emission bandwidths were set at 5 nm.

Thioflavin - T fluorescence

The formation of amyloid fibril was probed by Thio-T, the amyloid-specific fluorophore binding assay (Xue et al. 2017). A 2.5 mM stock solution of Thio-T was freshly prepared in a 10 mM phosphate buffer with 150 mM NaCl, pH 7.0. Subsequently, it was filtered through a 0.22 µm syringe filter (Millipore). The stock solution was diluted about 50 fold in respective protein solutions (2–5 µM) and incubated in the dark for about 1 min before analysis. The fluorescence intensities of the samples were measured on a Perkin-Elmer LS55 spectrofluorometer by excitation at 440 nm (slit width 5 nm) and emission at 482 nm (slit width 10 nm) at medium scan speed.

SDS PAGE and Native PAGE analysis

The molecular weight shifts and migration profiles of the three proteins were analyzed by 12% SDS PAGE according to standard protocols. In order to understand the glycation inflicted changes in their aggregation pattern, a 7% Native PAGE was also run alongside with 10 µg protein samples according to the method as described before (Das et al. 2020). All gels were stained with Coomassie Brilliant Blue Dye solution for visualization.

Dynamic light scattering analyses

Dynamic light scattering measurements were performed with the three proteins in the concentration range of 50–100 µM for both lysozyme and alpha-synuclein and between 5–10 µM for BSA chosen as per the response and sensitivity of the instrument. Data were obtained in a Nano zeta-sizer (Malvern Instruments) DLS instrument at a fixed 90-degree scattering angle using an Nd-doped solid-state laser of 632.5 nm with 100 s of integration time; the intensity correlation function was obtained by acquiring data between 5 and 1,000 ms in 200 channels. The samples were passed through 0.22-micron syringe filters before analysis.

Results and discussions

Fenfuro® mediated arrest of in vitro glycation

The attachment of Methyl Glyoxal (MGO) to the three proteins was confirmed by AGE-specific fluorescence (Figure 1). In the case of all three proteins incubated with MGO for definite time periods, emission spectra at around 440 nm – 445 nm confirmed the formation of protein-MGO adducts or AGE molecules. However, AGE fluorescence was arrested by about 47%, 83%, and 71%, respectively, for BSA (Figure 1A), Lysozyme (Figure 1B), and Alpha-synuclein (Figure 1C) in the presence of 0.25% Fenfuro® solution. The corresponding control proteins incubated in the presence of only 0.25% Fenfuro® didn't show any AGE formation. The attachment of sugars to proteins causes an increase in their MW. Glycation-induced increase in molecular weights were tracked by SDS PAGE. The MW of human lysozyme (Figure 2A), as reported to be

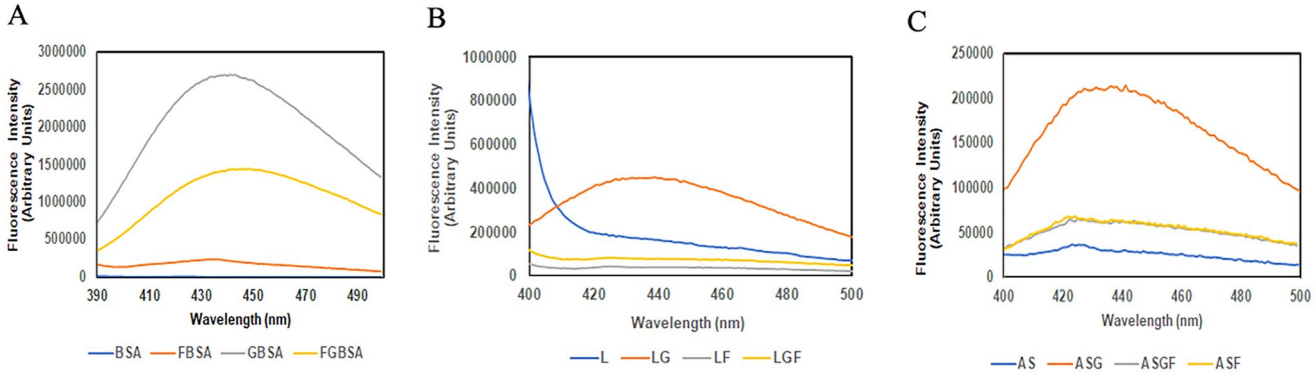

Figure 1. AGE fluorescence study to analyze protein-MGO adduct formation in case of A) BSA B) lysozyme and C) alpha-synuclein in presence and absence of Fenfuro®. the prefixes used before the three proteins stand respectively for F-in presence of 0.25% Fenfuro®, G- glycated in presence of MGO and FG- glycated in presence of 0.25% Fenfuro®. the same trend was obtained in all the three replicates of individual glycation reactions.

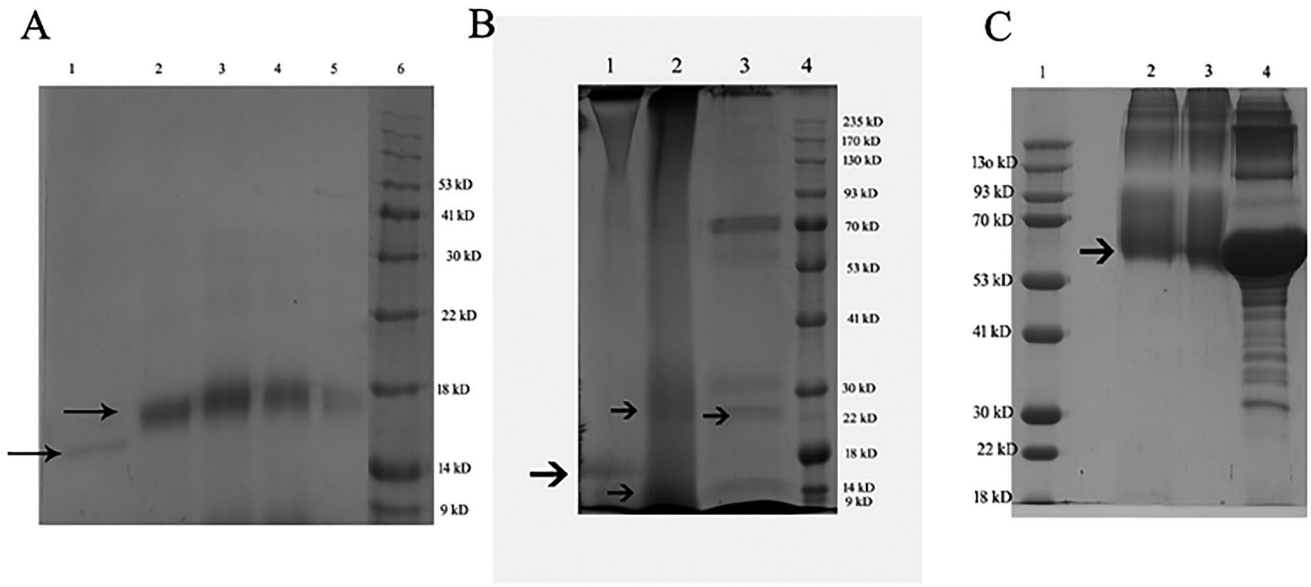

Figure 2. Analysis of glycation in presence and absence of Fenfuro® by SDS-PAGE A) lysozyme lane 1- control protein, lane 2 - lysozyme + MGO + FG, lanes 3,4,5 – lysozyme + MGO, lane 6- MW marker B) alpha-synuclein lane 1 – control protein, lane 2 - alpha-synuclein + MGO + FG, lane 3 – alpha-synuclein + MGO, lane 4 – MW marker C) BSA lane 1 – MW marker, lane 2 – control protein, lane 3 – BSA + MGO + FG, lane 4 – BSA + MGO. Same migration profiles were obtained with samples prepared from three independent sets of glycation.

around 14.3 kD (Proctor and Cunningham, 1988) (lane 1), was shifted to approximately 17 kD (lane 3). In the case of lysozyme incubated with both MGO and Fenfuro®, the shift in MW was visibly lesser (lane 2). Similar observations were noted with respect to alpha-synuclein (Figure 2B). In addition to the monomeric alpha-synuclein, dimers or trimers are often seen in SDS PAGE due to the strong SDS recalcitrant aggregational behavior of the protein, as has been reported frequently (Cappai et al. 2005; Roostaee et al. 2013). Moreover, the protein starts oligomerization even at room temperature, thus making it mandatory to thaw frozen alpha-synuclein aliquots immediately before use. In addition to an increase in MW due to the attachment of MGO residues, glycation most probably resulted in an increase in oligomerization of alpha-synuclein as it resulted in higher MW aggregates (lane 3) as compared to the control protein (lane 1). However, when incubated in the presence of 0.25% Fenfuro®, this enhanced oligomerization was arrested significantly as the higher 30 kD band seen in the case of the glycated sample was absent. Contrary to alpha-synuclein and lysozyme, clear results were not obtained for BSA (Figure 2C). However, many more bands were visible in the case of the glycated BSA adduct (lane 4) as compared to the control BSA (lane 1), and BSA glycated in the presence of Fenfuro® (lane 2), which was reflective of the protective action of Fenfuro® over glycation induced changes in structural makeup and oligomerization of the proteins.

Alpha-synuclein is a well-studied amyloidogenic protein implicated in several neurodegenerative disorders (Twohig and Nielsen, 2019; Calabresi et al. 2023), which oligomerizes under a host of small changes in physiological conditions. Glycation is known to potentiate alpha-synuclein oligomerization and fibrillation (Farzadfard et al. 2022). Similar results were also obtained for the alpha-synuclein MGO adduct in the present studies (Data not shown). BSA and lysozyme were carefully selected for the study owing to their self-aggregation properties and the resultant formation of amyloid-like entities (Swaminathan et al. 2011; Das et al. 2020). Therefore, the change in molecular weights as visible in SDS PAGE, was not solely attributable to the attachment of MGO residues but also to altered SDS-resistant aggregational behavior imparted by glycation. Thus, Fenfuro® served an effective role in preventing the glycation-induced structural and aggregational modulation and preserving the native structure of the protein.

There are only five lysine residues in human lysozyme, out of which three are solvent-accessible (Lins et al. 2003), 15 lysine residues in human alpha-synuclein out of which about 8 are accessible (Bell and Vendruscolo, 2021), and as many as 59 lysine residues in BSA about half which are available on the surface for MGO conjugation. Therefore, it is evident that the band retardations observed in SDS PAGE do not comply exactly with the molecular weights of the glycated proteins. Instead, the band patterns observed are reflective of the MGO-inflicted altered oligomerization of the proteins, which are grossly SDS-resistant in nature.

Prevention of amyloidogenic transition by Fenfuro®

After successfully demonstrating the arrest of glycation by Fenfuro®, its effect on subsequent glycation-induced structural alteration was also investigated in terms of amyloidogenic transition (Figure 3). A 0.25% solution of Fenfuro® arrested Thioflavin-T binding by about 35% for BSA (Figure 3A), 14% for lysozyme (Figure 3B) and 13% for alpha-synuclein (Figure 3C) respectively. No Fenfuro® associated Thioflavin-T binding was noted in either of the proteins. Thioflavin T binding is a hallmark for the formation of amyloid-like entities (Xue et al. 2017). The binding of this dye to amyloid fibril results in its immobilization,

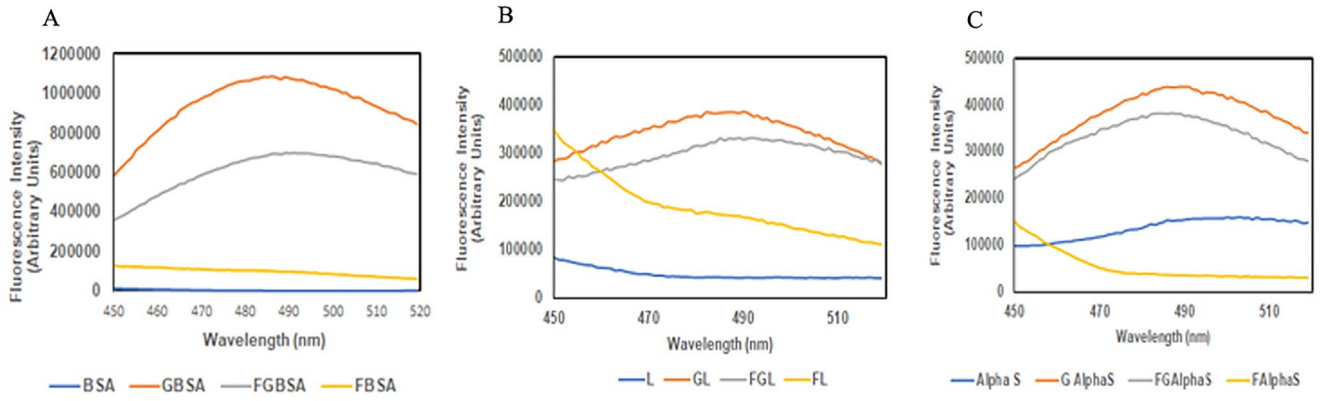

Figure 3. Analysis of amyloidogenic propensity by thioflavin T binding assay for A) BSA B) lysozyme and C) alpha-synuclein in presence and absence of Fenfuro®. the prefixes used before the three proteins stand respectively for F-in presence of Fenfuro®, G- glycated in presence of MGO and FG- glycated in presence of 0.25% (w/v) Fenfuro®. the same trend was obtained in all the three replicates of individual glycation reactions.

resulting in the emission of a characteristic strong fluorescence at around 480 nm. Although Thio-T binding is generally considered to be characteristic of cross beta fibril formation, it is also known to give positive signals for certain unique non-fibrillar entities undergoing transformation from alpha helix to beta-pleated sheet formation, which led to the terminology 'globular amyloid' (Chiti and Dobson, 2009) as has been observed for many globular proteins such as BSA (Nirwal et al. 2021), Lysozyme (Jafari and Mehrnejad, 2016), Insulin (Gancar et al. 2020) and RNAse (Sambashivan et al. 2005). The formation of these pseudo-amyloid entities has been reported to be enhanced by glycation (Wei et al. 2009; Das et al. 2020; Sirangelo and Iannuzzi, 2021; Banik, 2024). Glycation-associated amyloid fibril formation has been found to be most potent for alpha-synuclein, thus explaining the strong clinical correlation between chronic diabetes and susceptibility to cognitive disorders (Banik, 2024).

Prevention of glycation-induced aggregation by Fenfuro®

The attachment of sugars is known to impart a strong aggregation propensity to many proteins. A classic example of this phenomenon is constituted by the extensively glycosylated fungal extracellular enzymes, which result in strong concentration-driven, reversible self-aggregation (Banik et al. 2012; Das et al. 2019). Protein glycation, although mechanistically different in being a non-enzymatic process, has the same resultant effect on protein structure mediated by the attached sugar moieties. In order to investigate the glycation-induced protein aggregation and the preventive effect of Fenfuro®, the change in hydrodynamic radii of the protein samples was determined by Dynamic Light Scattering (Figure 4, Table 1). Methyl-glyoxal mediated adduct formation in alpha-synuclein resulted in the disappearance of the 9 nm species and a substantial increase in the size of the other two (Figure 4A and 4B of left panel), thus reflecting the glycation inflicted enhanced aggregation of alpha-synuclein. However, when glycation was carried out in the presence of 0.25% Fenfuro®, the smaller entity plausibly representing monomeric alpha-synuclein reappeared, which reaffirmed that MGO attachment was effectively prevented by Fenfuro® (Figure 4C of left panel). Similar observations were also noted for the other two proteins. In the case of lysozyme, the 3.85 nm species disappeared on glycation, which again reappeared in a significantly compacted form (5.7 nm) in the presence of Fenfuro® (Figure 4(A–C) of middle panel). Similar trends were also noted in the case of BSA (Figure 4 A–C of the right panel). The smallest 4.18 nm species disappeared in the case of the BSA-MGO adduct but was visible again when the glycation was carried out in the presence of Fenfuro®, albeit at a less compacted form of 11.07 nm. In all three proteins, the size of the highest oligomeric radii was also significantly enhanced by glycation. Fenfuro® was able to largely restore the original size distribution of the protein oligomers by arresting MGO attachment.

The changes in aggregation of the proteins were also investigated through native PAGE (Figure 5). In lysozyme (Figure 5A), enhanced aggregation was also noted in the case of the glycated sample (appearance of an additional band in lane 2) as compared to the control protein (lane 1). However, in the presence of Fenfuro®, the original PAGE pattern of the unmodified protein was restored (lane 3). Exactly similar inferences can also be drawn for alpha-synuclein as several extra bands were noted for the glycated sample (Figure 5B, lane 2), which were not present either for the control protein (lane 1) or for the protein glycated in the presence of Fenfuro® (lane 3). In the case of BSA, however, the native PAGE patterns were less conclusive (Figure 5C, lane 1 - BSA glycated in the presence of Fenfuro®, lane 2 - control BSA, and lane 3 - Glycated in BSA). However, it was apparent that the glycation-induced changes were successfully reversed by Fenfuro®.

Protein glycation is a potential agent for widespread cellular toxicity (Banik et al. 2020; Kuzan 2021). Moreover, glycation is mostly confined to the side chain lysine residues, which in turn are targets for ubiquitination-mediated cellular destruction (Swatek and Komander, 2016). Subsequently, glycated proteins become recalcitrant to ubiquitination and persist in the cellular environment for longer periods of time, thus augmenting the AGE-associated toxic effects. It is believed, therefore, that the AGE-RAGE axis has evolved as a protective mechanism to sequester the toxic protein aggregates from inducing a host of cellular malfunctions. There is also an endogenous secreted

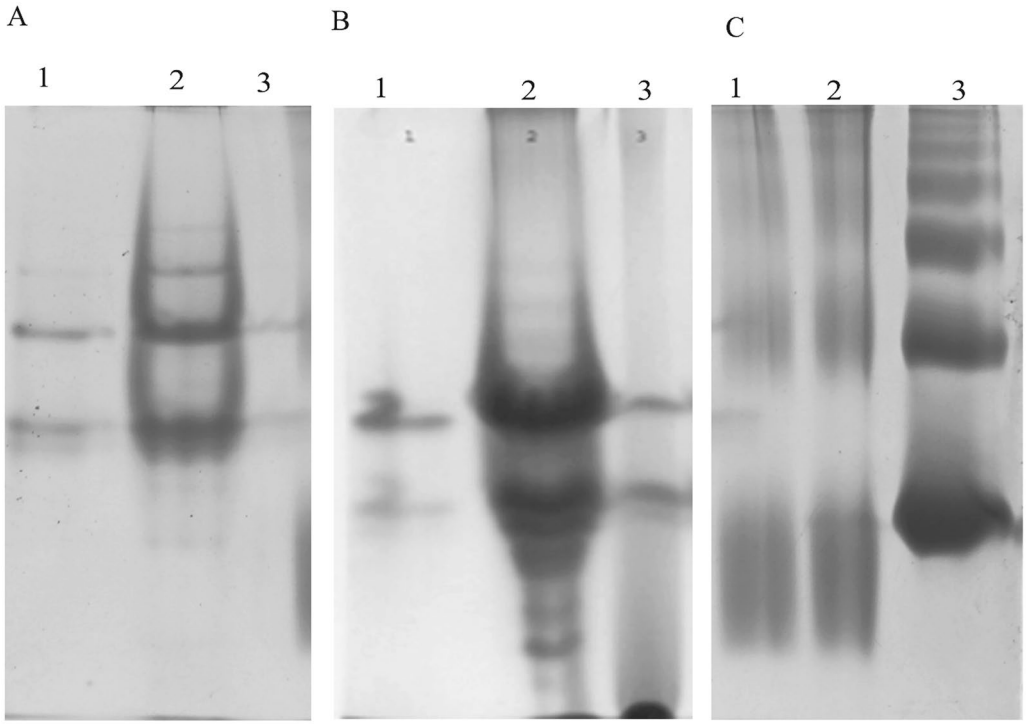

Figure 4. Native PAGE analysis in presence and absence of fenfuro® A) lysozyme lane 1- control protein, lane 2 – lysozyme + MGO, lane 3 – lysozyme + MGO + FG B) alpha-synuclein lane 1- control protein, lane 2 – alpha synuclein + MGO, lane 3 – alpha synuclein + MGO + FG C) BSA lane 1- control protein, lane 2 – BSA + MGO + FG, lane 3 – BSA + MGO. Similar migration profiles were obtained with samples prepared from three independent sets of glycation.

Table 1. DLS analysis of protein samples glycated in the presence and absence of Fenfuro®.

Sample	Hydrodynamic radius	Polydispersity index
Alpha -synuclein	421.3 ± 132.3, 80.03 ± 16.46, 9.28 ± 1.28	0.742
Glycated Alpha -synuclein	219.6 ± 114.8, 5032 ± 591.7	0.385
Alpha-synuclein + Methyl Glyoxal (MGO) + 0.25% (w/v) Fenfuro® (FG)	399.9 ± 122, 72.94 ± 19.52, 1.16 ± 0.2371	0.536
Lysozyme	944.2 ± 231.7, 158.3 ± 36.10, 3.851 ± 0.77	0.919
Glycated Lysozyme	1065 ± 178, 180.6 ± 24.77	0.785
Lysozyme + MGO + FG	787 ± 156, 5.7 ± 0.96, 157.1 ± 24.04	0.656
BSA	528.4 ± 242.8, 4.181 ± 0.94, 36.38 ± 10.35	0.656
Glycated BSA	17.05 ± 9.537, 138.7 ± 72.94, 3991 ± 1091	0.506
BSA + MGO + FG	274.2 ± 142.3, 11.07 ± 4.09, 47.55 ± 13.14	1

PS. The standard deviations of five replicate scans for each sample were calculated by the associated software of Malvern instruments.

form of RAGE (sRAGE or soluble RAGE), which protects different organs of the body from AGE-adduct-induced damage (Miyagawa et al. 2022). In addition to the many different manifestations of diabetes-associated ailments in the body, including obesity, cardiovascular diseases, and increased risk factors for some forms of cancer (Zhu and Qu, 2022), an emerging threat accompanying chronic hyperglycemia has been an onset of several different forms of cognitive impairments mostly owing to the accumulation of amyloid-like AGE adducts at several different tissues of the body.

The beneficial effects of phytotherapeutics such as furostanolic saponins from several different formulations of Fenugreek, such as Fenfuro®, have been unequivocally established over the last few years in long-term side effect-free management of chronic hyperglycemia. Many mechanistic interventions related to the improvement of insulin sensitivity, management of gluconeogenesis as well as optimized lipid metabolism have been proposed to explain the mode of action of the bioactives present in Fenugreek. The present studies represent one of the very limited numbers of systematic reports, if not the first one, to understand the direct intervention of Fenugreek extract in the prevention of protein glycation. One of the major advantages of this study stems from the fact that the product has already been clinically tested and undergone human trials to deliver the desired result without any side effects. At the same time, a current limitation is that at a concentration of Fenfuro® higher than 0.5% (w/v), no additional inhibition of glycation has been observed. Moreover, the secondary structure of the proteins was found to be affected at 0.5% (w/v) Fenfuro® concentration by Circular Dichroism spectroscopy, thus preventing its use at higher concentrations (data not shown). Therefore, further studies with more purified fractions of the active ingredients need to be carried out to achieve complete arrest of glycation. It will be worthwhile to investigate whether Fenfuro® can degrade or disassemble glycation-associated amyloid fibrils both *in vitro* and *in vivo* so that it can also be developed as a prospective therapeutic treatment for neurodegenerative disorders.

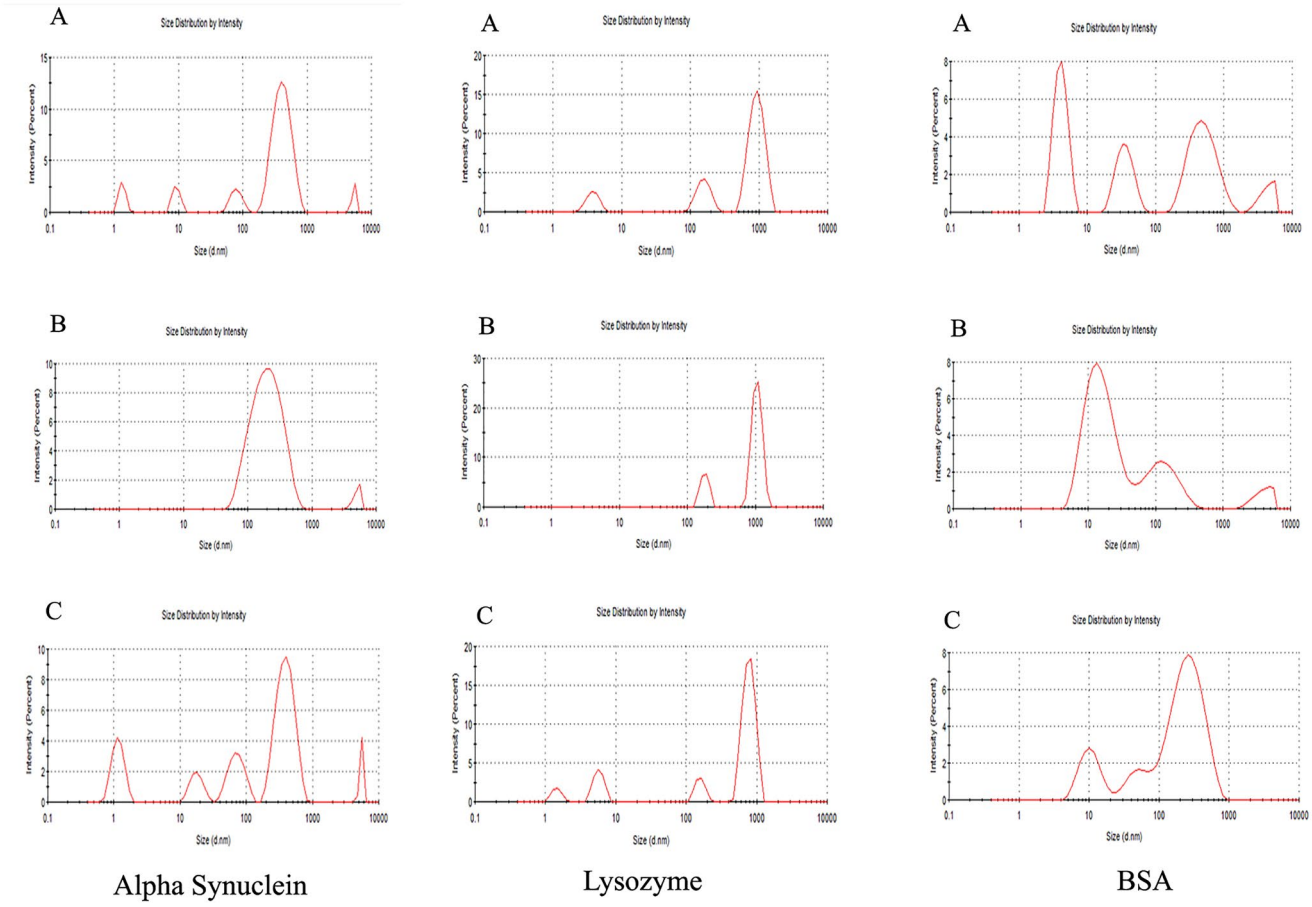

Figure 5. DLS analysis for estimation of hydrodynamic radii of proteins glycated in presence and absence of Fenfuro®. in each of the three panels corresponding to the three proteins, a represents control protein, B represents protein glycated in presence of MGO and C represents protein glycated in presence of MGO and 0.25% (w/v) Fenfuro®.

Conclusion

The studies established the direct intervention of active components present in Fenfuro® in arresting protein glycation and subsequent formation of amyloidogenic protein aggregates or the so called 'plaques'. This is a new mechanistic insight for explaining the anti-diabetic property of Fenugreek seed extract in addition to its proven role in improving insulin sensitivity. The studies are believed to provide significant insights toward the development of more optimized and targeted phytotherapeutics for the treatment of Type 2 diabetes.

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