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ASSESSING THE *IN VITRO* ANTI-GLYCATION EFFICACY OF VITAMINS A, C, D, E

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Accreted sugars in the blood react with protein's amino group via Schiff base to form Amadori compounds. Further, these compounds execute irreversible chemical modifications generating advanced glycation end products (AGEs). The current study investigated the effects of vitamins in a glycation-prone in vitro environment. Glycation model was reached by incubating BSA with 0.1 M glucose/fructose in 0.1 M phosphate-buffered saline. Intrinsic (tyrosine/tryptophan) and AGEs fluorescence was monitored with fluorescence spectrophotometer. Ellman's test depicted that native BSA contains more free thiol groups than glycated BSA. It was shown that BSA is more susceptible to glycation in the presence of fructose than glucose, and vitamin D followed by vitamin E and A can significantly rescue the BSA from glycation progression.

K e y w o r d s: advanced glycation end products, BSA, vitamins, anti-glycation, fluorescence, Ellman's test.

Lingered hyperglycemia provokes reducing sugars to non-enzymatically bind with protein leading to glycation, also known as the Maillard reaction. Where sugar's carbonyl group reacts with the free amino group of protein via Schiff base, this instigates a multifarious cascade of repeated condensations, rearrangements, and oxidative modifications, which anomaly protein's functional conformation and lead to the formation of irreversible advanced glycation end products (AGEs) [1-6]. Accretion of AGEs in body tissue is the foreground for the utmost happening health complications like age-related degeneracies, atherosclerosis, and diabetic difficulties such as retinopathy, nephropathy, and neuropathy [1, 3, 7-9]. An inclusive review of the current scientific works divulges that the inhibition of AGEs formation is one of the therapeutic approaches to avoiding the progressions of glycation due to hyperglycemia.

Albumin is the most abundant serum protein that aids in maintaining osmotic pressure, pH and transporting a wide variety of endogenous and exogenous compounds, including fatty acids, metals, amino acids, steroids, and drugs [1, 10]. Anomalies in albumin affect the loss of its biological functions. Bovine serum albumin (BSA) is a substitute for human serum albumin (HSA), having the selfsame structure and operations. It is a monomeric globular protein consisting of 583 amino acid residues with a molecular mass of 66,46 Da [11]. In the present study, BSA is subjected to illustrate protein glycation.

Vitamins are defined as a group of micronutrients that the human body cannot produce [12]. Vitamins play vital roles in human life, including regulating metabolic and cellular processes, preventing diseases, and promoting healthy reproduction and growth [13]. Individual vitamins have the capacity to preserve bodily homeostasis [14]. In the current study, we explored the antiglycation efficacy of 4 common vitamins, namely fat-soluble vitamins A, D, E, and water-soluble vitamin C, in a glycation-prone environment by glucose/fructose individually by implementing fluorometric and spectrometric properties.

Materials and Methods

5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman's reagent) was purchased from Sisco Research Laboratories Pvt Ltd Mumbai. BSA, sodium azide, D-glucose (G), D-fructose (F), vitamin A acetate (A), vitamin C (C), vitamin D_3 (D), vitamin E

(E), aminoguanidine (AMG) and di-sodium hydrogen phosphate were purchased from HiMedia Laboratories Pvt Ltd, Mumbai. In addition, all the other chemicals utilized were of analytical quality.

In vitro glycation and anti-glycation models. Glycation and anti-glycation of BSA were carried out with minor changes, as described by [15]. Briefly, the glycation model was reached by incubating BSA (5 mg/ml) with 0.1 M glucose/fructose in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. For the anti-glycation model, 0.1% individual standard vitamins A, D, E (in ethanol), and C (aqueous) were supplemented for the same recipe. Both the models were incubated at 37ºC for a stretch of 1, 2 & 3 weeks individually. Control was incubated accordingly without saccharide supplementation. BSA+AMG was treated as the positive control.

Fluorescence analysis. Fluorescence data was routed by the F-7000 fluorescence spectrophotometer (HITACHI, Japan), having a photomultiplier tube voltage (PMT voltage) of 500 V and excitation-emission slit of 5.0 nm. Fluorescence emission intensity was measured in terms of arbitrary units (AU).

Measurement of intrinsic fluorescence. Intrinsic fluorescence of glycated and anti-glycated models was determined as described by [16]. After three weeks, intrinsic tryptophan fluorescence of all the samples was obtained by excitation at 280 nm and recording the emission ranging from 300-400 nm at room temperature.

Fluorescence of AGEs. Fluorescence analysis for AGEs in two models was determined by excitation at 365 nm and emission at 440 nm. Quenching effects were partially decreased by diluting the aliquots with PBS (1:1 v/v) before recording the fluorescence spectra [17].

The % Inhibition nominated vitamins on glycation was calculated using the formula as manifested by [18].

The native BSA sample fluorescence was considered 100% inhibition for glucose and fructose treated models.

Estimation of free thiol groups. This method is based on spectrophotometric quantification of compounds containing free sulfhydryl groups (-SH). Ellman's reagent (DTNB) reacts with the free-SH group by forming a stable yellow-colored complex. The samples (1 ml) were taken along with 5 mM Ellman's reagent and incubated for 10 min at room temperature, the absorbance was measured at 412 nm (UV-1800, UV-spectrophotometer SHIMADZU), and concentration was extrapolated using the standard calibration curve [19].

Statistical analysis. All the experiments were done in triplicates, and the results expressed are the mean values. The error bar represents the standard deviation of the triplicates. The statistical significance of the results was evaluated by using a one-way analysis of variance (ANOVA). A $P < 0.05$ was considered statistically significant.

Results and Discussion.

Tryptophan intrinsic fluorescence. Intrinsic fluorescence was traced to discern the deviations in the native configuration of BSA by reducing sugar. Intrinsic fluorescence of native BSA showed the intensity of 6417 AU at 345 nm. In the anti-glycation model (Fig. 1, *a*), aminoguanidine (AMG), vitamin D, and vitamin E supplements showed slightly lesser and similar fluorescence to native BSA of 5331, 4897, 4780 AU, respectively. In anti-fructation, all the models exhibited more than 50% decreased fluorescence compared to native BSA (except AMG) (Fig. 1, *b*). AMG showed the nearest spectrum of all (3542 A.U), followed by vitamin D, E, and A having 2411, 2333, and 2248 AU, respectively. Vitamin C treated samples had the most negligible fluorescence (2368, 1685 AU), which was nearing and lesser than the fluorescence of both glucated and fructated samples with the intensity of 2986 and 2125 AU, respectively.

A decrease in the intensity of the spectra than native BSA was also observed by Ali Khan et al. [20]; Ahmed et al. [16], and many others, and it may be due to the formation of protein aggregates due to change in structural integrity. A wavelength of 280 nm was used as it excites both Try/Tyr as there are 2-tryptophan and 20 tyrosine residues in the native BSA [11].

Fluorescence of AGEs. The revealed fluorescence spectra of AGEs after 3 weeks of incubation were flat and broadened in the region of maximum fluorescence. Native BSA showed the least intensity (24.8 AU) and was considered 100% inhibition. In glycation models intensity of fructated BSA (222.2 AU) was more than twice that of the glucated BSA (87.2 AU) (Fig. 2, *a*, *b*). In the anti-glucation model, vitamin D showed the highest inhibition, 66.3%, followed by 49 and 48% by vitamin E and vitamin A, respectively (with not much difference). Similarly, in the anti-fructation model, vitamin D could inhibit the highest of 50%, followed by vita-

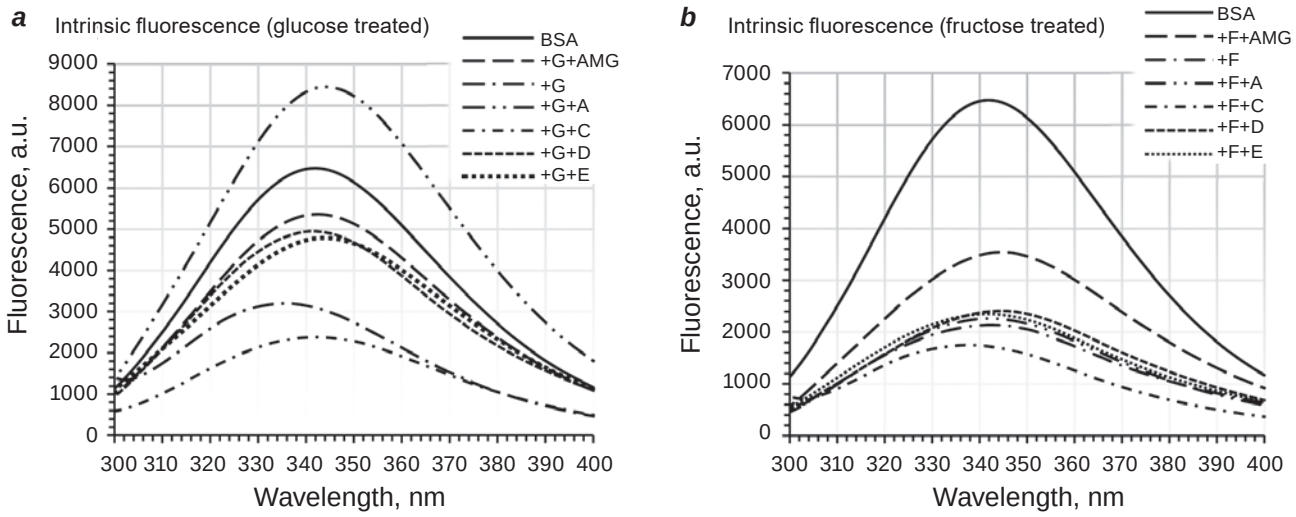

Fig. 1. Intrinsic tryptophan/tyrosine fluorescence spectrum excited at 280 nm and recorded at 300-400 nm: ***a****) glucose treated BSA model;* ***b****) fructose treated BSA model; (n = 3)*

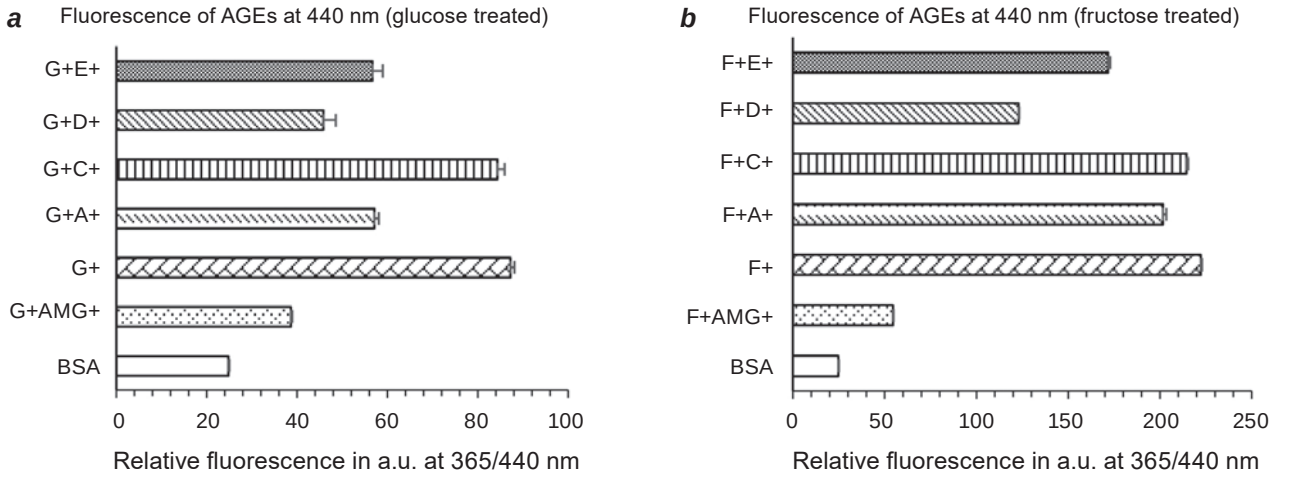

Fig. 2. Relative fluorescence of AGEs excited at 365 nm and emission recorded at 440 nm: ***a****) glucose treated model;* ***b****) fructose treated model; (n = 3)*

min E 25.6%. Vitamin A showed negligible inhibition of 10%. Whereas vitamin C showed no or very negligible inhibition (5 and 4%) while AMG inhibited the highest amount of 78 and 85% in glucose and fructose treated models, respectively.

The increase in fluorescence intensity compared to native BSA imply the formation of AGEs in all the incubated samples. Glycation inhibition majorly occurs by the chelation of sugars, as demonstrated in most inhibitors; the positive control AMG mechanizes similarly [21]. BSA treated with fructose was found more prone to form AGEs than the glucose treated ones, which agrees with Suárez et al., [22] and many others. Vitamin D halted the formation of AGEs remarkably among all the tested vitamins; Iqbal et al. [23] also found similar results.

Estimation of free thiol groups. Glycation-induced structural modifications unmask hidden sulfhydryl groups. Unmasking in BSA makes free cysteine residues available for oxidation by halting its overall antioxidant property. Ellman's protocol depicted that the free-SH group concentration decreased with an increasing incubation. Free-SH group concentration of native BSA (10.8 µg/ml) without incubation was treated as 100%, and upon 21 days of incubation, it slightly decreased to 83.3%. In glucation and fructation models, thiol group concentration dropped to 55.6% and 32.4%, respectively

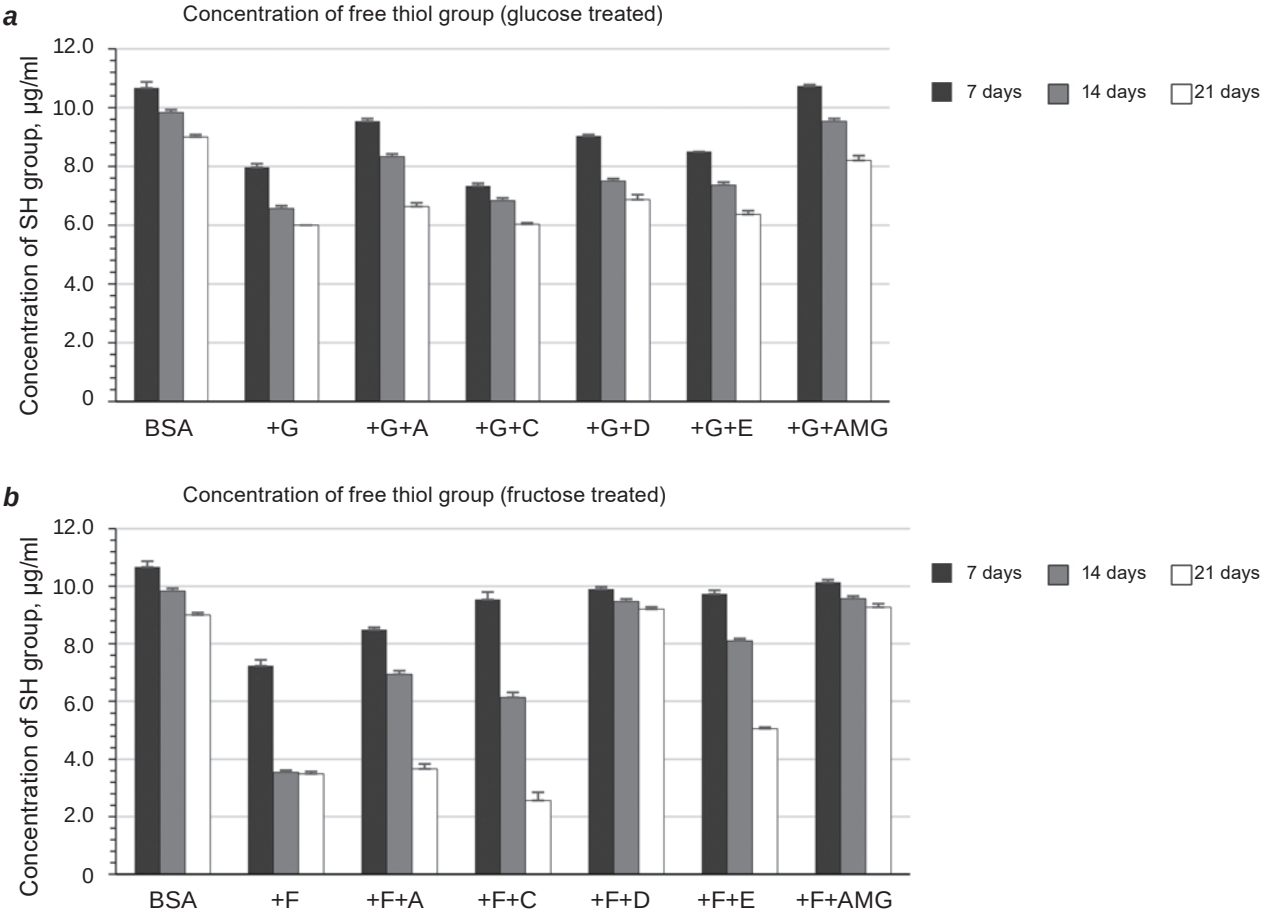

Fig. 3. Concentration of free thiol groups in glucated/fructated BSA in the presence of different vitamins: ***a)*** *glucose treated model;* ***b)*** *fructose treated model; (n = 3)*

(Fig. 3, *a*, *b*). Among the tested vitamins, vitamin D was most prominent in holding thiol group concentration and restrained to 63.9% in glucation and 85.2% in fructation. In comparison, the positive control restored the free thiol group to 76% and 84.3% in glucation and fructation models, respectively. Vitamin E and vitamin A moderately ceased the concentration to 59.3%, 47.2%, and 61.1%, 34.25% in glucose and fructose treated models, respectively. Vitamin C was least/not prominent in stopping the oxidation of the free thiol group; concentration ranged in line with glycated protein in both glucated and fructated models upon 21 days of sequential incubation.

Similar observations were reported showing the decrease in the thiol concentration after glycation [16, 21, 23, 24]. Single cysteine moiety has a free-SH group out of 35 total cysteine residues in BSA, where the remaining are arranged in 17 di-sulfhydryl bridges, contributing to the structural integrity of proteins [25].

Conclusions. Present *in vitro* studies demonstrated that BSA is more susceptible to glycation in the presence of fructose than glucose, and vitamin D followed by vitamin E and A can significantly rescue the BSA from glycation progressions. Hence, we deduce that supplementation of vitamins like vitamin D, vitamin E, and vitamin A aid in halting the complications of hyperglycemia. Further, research needs to be carried out to understand the mechanism of vitamin D action in anti-glycation.

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Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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Author contributions. Nagarjuna Prakash Dalbanjan, Arihant Jayawant Kadapure, Parvati Huded performed and analyzed the experiments. Nagarjuna Prakash Dalbanjan drafted the manuscript. Vishwanath B Chachadi; Sreenivasa Nayaka assisted in reviewing the manuscript and execution of the work. Praveen Kumar S.K designed and supervised the experiments.

Data availability. The data that supports the finding of this study is available upon request from the corresponding author.

ОЦІНКА АНТИГЛІКАЦІЙНОЇ ЕФЕКТИВНОСТІ ВІТАМІНІВ A, C, D, E *IN VITRO*

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Накопичений цукор у крові реагує з аміногрупою протеїну через основу Шиффа з утворенням сполук Амадорі. Ці ж сполуки здійснюють незворотні хімічні модифікації, утворюючи кінцеві продукти глікації (AGE). У роботі вивчали вплив вітамінів у схильному до глікації середовищі *in vitro*. Модель глікації була досягнута шляхом інкубації BSA з 0,1 М глюкозою/фруктозою в 0,1 М фосфатному буфері. Внутрішню флуоресценцію (тирозин/триптофан) і AGE контролювали за допомогою флуоресцентного спектрофотометра (HITACHI). Із застосуванням тесту Еллмана встановлено, що нативний BSA містить більше вільних тіолових груп, ніж глікований. Крім того, показано, що BSA більш сприйнятливий до глікації в присутності фруктози, ніж глюкози, і що вітамін D, а потім вітамін E та A можуть суттєво захистити BSA від глікації.

Ключові слова: кінцеві продукти гликозилування, BSA, вітаміни, антиглікація, флуоресценція, тест Еллмана.

References

1. Roohk HV, Zaidi AR, Patel D. Glycated albumin (GA) and inflammation: role of GA as a potential marker of inflammation. *Inflamm Res.* 2018; 67(1): 21-30.
2. Yousof Ali M, Zaib S, Mizanur Rahman M, Jannat S, Iqbal J, Kyu Park S, Seog Chang M. Poncirin, an orally active flavonoid exerts antidiabetic complications and improves glucose uptake activating PI3K/Akt signaling pathway in insulin resistant C2C12 cells with anti-glycation capacities. *Bioorg Chem.* 2020; 102: 104061.
3. Anis MA, Sreerama YN. Inhibition of protein glycoxidation and advanced glycation end-product formation by barnyard millet (*Echinochloa frumentacea*) phenolics. *Food Chem.* 2020; 315: 126265.
4. Taniguchi M, Kuda T, Shibayama J, Sasaki T, Michihata T, Takahashi H, Kimura B. *In vitro* antioxidant, anti-glycation and immunomodulation activities of fermented blue-green algae Aphanizomenon flos-aquae. *Mol Biol Rep.* 2019; 46(2): 1775-1786.
5. Khazaei MR, Bakhti M, Habibi-Rezaei M. Nicotine reduces the cytotoxic effect of glycated proteins on microglial cells. *Neurochem Res.* 2010; 35(4): 548-558.
6. Muñiz A, Garcia E, Gonzalez D, Zuñiga L. Antioxidant activity and *in vitro* antiglycation of the fruit of *Spondias purpurea*. *Evid Based Complement Alternat Med.* 2018; 2018: 5613704.
7. Booth AA, Khalifah RG, Todd P, Hudson BG. *In vitro* kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways. *J Biol Chem.* 1997; 272(9): 5430-5437.
8. Brownlee M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med.* 1995; 46: 223-234.
9. Rungratanawanich W, Qu Y, Wang X, Essa MM, Song BJ. Advanced glycation end products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury. *Exp Mol Med.* 2021; 53(2): 168-188.

10. Wani TA, Bakheit AH, Zargar S, Rizwana H, Al-Majed AA. Evaluation of competitive binding interaction of neratinib and tamoxifen to serum albumin in multidrug therapy. *Spectrochim Acta A Mol Biomol Spectrosc.* 2020; 227: 117691.
11. Carter DC, Ho JX. Structure of Serum Albumin. In: Anfinsen CB, Edsall JT, Richards FM, Eisenberg DS, eds. Advances in Protein Chemistry. Academic Press, 1994; 45: 153-203.
12. Ottaway PB. Stability of vitamins in food. In: Ottaway PB. (ed.). The Technology of Vitamins in Food. Springer, Boston, MA, 1993: 90-113.
13. Lešková E, Kubíková J, Kováčiková E, Košická M, Porubská J, Holčíková K. Vitamin losses: Retention during heat treatment and continual changes expressed by mathematical models. *J Food Composit Anal.* 2006; 19(4): 252-276.
14. Fletcher RH, Fairfield KM. Vitamins for chronic disease prevention in adults: clinical applications. AMA. 2002;287(23):3127-3129.
15. Adisakwattana S, Sompong W, Meeprom A, Ngamukote S, Yibchok-Anun S. Cinnamic acid and its derivatives inhibit fructose-mediated protein glycation. *Int J Mol Sci*. 2012; 13(2): 1778-1789.
16. Ahmed A, Shamsi A, Khan MS, Husain FM, Bano B. Methylglyoxal induced glycation and aggregation of human serum albumin: Biochemical and biophysical approach. *Int J Biol Macromol.* 2018; 113: 269-276.
17. Schmitt A, Gasic-Milenkovic J, Schmitt J. Characterization of advanced glycation end products: mass changes in correlation to side chain modifications. *Anal Biochem*. 2005; 346(1): 101-106.
18. Ramkissoon JS, Mahomoodally MF, Ahmed N, Subratty AH. Antioxidant and anti-glycation activities correlates with phenolic composition of tropical medicinal herbs. *Asian Pac J Trop Med*. 2013; 6(7): 561-569.
19. Riener CK, Kada G, Gruber HJ. Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4'-dithiodipyridine. *Anal Bioanal Chem*. 2002; 373(4-5): 266-276.
20. Khan MWA, Rasheed Z, Khan WA, Ali R. Biochemical, biophysical, and thermodynamic analysis of *in vitro* glycated human serum albumin. *Biochemistry (Mosc).* 2007; 72(2): 146-152.
21. Nagai R, Murray DB, Metz TO, Baynes JW. Chelation: a fundamental mechanism of action of AGE inhibitors, AGE breakers, and other inhibitors of diabetes complications. *Diabetes.* 2012; 61(3): 549-559.
22. Suárez G, Rajaram R, Oronsky AL, Gawinowicz MA. Nonenzymatic glycation of bovine serum albumin by fructose (fructation). Comparison with the Maillard reaction initiated by glucose. *J Biol Chem*. 1989; 264(7): 3674-3679.
23. Iqbal S, Alam MM, Naseem I. Vitamin D prevents glycation of proteins: an *in vitro* study. *FEBS Lett.* 2016; 590(16): 2725-2736.
24. Yousof Ali M, Jannat S, Mizanur Rahman M. Ginsenoside derivatives inhibit advanced glycation end-product formation and glucose-fructose mediated protein glycation *in vitro* via a specific structure-activity relationship. *Bioorg Chem*. 2021; 111: 104844.
25. Gülseren I, Güzey D, Bruce BD, Weiss J. Structural and functional changes in ultrasonicated bovine serum albumin solutions. *Ultrason Sonochem.* 2007; 14(2): 173-183.