CHAPTER 4: ANTIGLYCATION EFFECTS OF NUTRACEUTICALS

4.1 Introduction

The increased glucose level associated with diabetes results in the excessive non-enzymatic glycation and accumulation of advanced glycation end products (AGEs). AGEs activates a cascade of signaling pathways leading to the development of oxidative stress and severe pathological conditions such as retinopathy, neuropathy, nephropathy and micro-vascular complications [1, 2]. Non-enzymatic glycation also called as ‘Maillard reaction’ is a multi step process initiated due to the reaction of glucose and its intermediates with the free amino groups in proteins, lipids, and nucleic acids. The reaction of sugar with free amino groups in proteins gives rise to the formation of early glycation product also called Amadori product, which on oxidative modification leads to the formation of a highly heterogeneous class of compounds called as AGEs. AGEs are highly reactive and characterized by their specific properties such as florescence, cross-linking and non-cross-linking [3, 4].

Albumin is a transport protein in the blood, which serves as a carrier for many endogenous and exogenous ligands [5-8]. In addition to this, albumin is also the most abundant plasma protein and highly prone for non-enzymatic glycation. Glycation alters the structure and function of proteins leading to the conformational transition with complete loss of native helical structure towards the beta sheeted unordered conformation [9]. Glycation accelerates fibrillation and formation of amyloid like aggregation of proteins. Recently studies have shown that glycation increases the neuronal toxicity of amyloid peptide [10, 11].
Due to the critical role of glycation in the development of severe complications and pathological conditions, the recent research has been focused on small molecule inhibitors for prevention of non-enzymatic glycation. In spite of the strong antiglycation properties, the potent antiglycation compound such as aminoguanidine has been found to be associated with severe side effects which led to their withdrawal from the 3rd phase of the clinical trial [12]. Considering the side effects of synthetic compounds, the natural products are promising towards the development of a potent inhibitor of AGEs.

**Figure 4.1:** Structures of (a) silybin, (b) vanillin and (c) sinigrin.

Several natural and synthetic compounds, which have been proposed as AGE inhibitors are either limited to the *in vitro* studies or have been associated with undesirable
complications [13, 14]. Considering the side effects associated with the synthetic compounds, there is a need of an alternative approach to preventing non-enzymatic glycation. There is a need to adopt a healthy life style, which lowers AGEs intake in the form of diet, minimize exposure to smoke, oxidants, radiation, and obesity. With this motivation, we aimed to determine the inhibitory potential of edible greens, nutraceuticals, and essential amino acids against formation and accumulation of AGEs. In this work the antiglycation activity of three phytochemical (silybin, sinigrin and vanillin) and nine essential amino acids was evaluated considering their abundance in different food items. The structural features such as a) free hydroxyl groups b) amino groups c) sulphur containing compounds have been shown to be essential for the antiglycation effects. The structure of three different phytochemicals and nine essential amino acids are shown in Figure 4.1.

Silybin (SIB) is a flavonolignan and one of the major constituents of silymarin. SIB is a mixture of two diastereoisomers known as Silybin A and Silybin B in a ratio of 1:1 [15]. The antidiabetic, antioxidant, anticancer and hepatoprotective activities of silybin have been reported from both in vitro and in vivo examinations. Also, in vivo studies using mice have proven the clinical efficacy of silybin [16-18].

Sinigrin is a common ingredient present in cruciferous vegetables (such as cabbage, broccoli, cauliflower etc.), which are regularly used as food and in combination with other herbs for treatment of various diseases. Sinigrin has been found to significantly inhibit the proliferation of tumor cells and adipocyte differentiation [19]. Also, studies
have shown the potent antioxidant activity of sinigrin [20], and suppressive effects towards hypertriglyceridemia [21].

Vanillin is a major component of vanilla bean extract, which is extensively used as a natural food flavoring agent. The antioxidant, antidepressant, and anti-proliferative activities of vanillin have been reported based on both in vitro and in vivo studies [22]. Also, in vivo studies using mice have proven the clinical efficacy of vanillin [23].

Amino acids play a key role in human nutrition and maintenance of health. Essential amino acids cannot be synthesized by the body and thus need to be supplied in the form of diet [24]. There is a growing interest in biochemistry and physiology of amino acids in health and nutrition of humans [25]. Recently amino acids have been proposed to be classified into three classes namely; non-essential amino acids, which are biosynthesized by the body itself, essential amino acids; which body cannot synthesize and need to be supplied in diet, and third new class known as functional amino acids which play role in signaling pathways, growth and metabolic diseases [26]. Structures of nine essential amino acids used in this study are shown in the Figure 4.2.

The antiglycation potential of silybin, sinigrin and vanillin was determined by employing the in vitro glycation model of BSA. The inhibition activity for the early and advanced glycation end products (AGEs) was determine based on measurements of fructosamine (Amadori product) formation and fluorescent AGEs. Also, we determined the carbonyl content and protective effect of these compounds towards lysine modification due to glycation.
Figure 4.2: Structures of nine essential amino acids.

The inhibitory activity of these compounds towards glycation induced aggregation and fibrillation of albumin was determined using amyloid specific dyes i.e. Congo red (CR) and Thioflavin T (ThT). Confocal imaging was carried out to get better insights about the inhibition of aggregation using ThT.

4.2 Materials and Methods

4.2.1 Materials
Bovine serum albumin (BSA), silybin (SIB), sinigrin (SIN), vanillin (VAN), methylglyoxal (MG), aminoguanidine (AG), 2,4,6-trinitrobenzenesulfonic acid (TNBSA), Congo red (CR) and thioflavin T (ThT) were purchased from Sigma, USA. 2,4-dinitrophenyl hydrazine (DNPH), guanidine hydrochloride and nitro blue tetrazolium
(NBT) were obtained from Sisco Research Laboratory (SRL), India. All other chemicals of AR grade for the preparation of buffers were purchased from Merck, India.

4.2.2 In vitro Glycation of Albumin

Glycation of BSA was carried out using glucose and methylglyoxal under high sterile conditions. Briefly, 10 mg ml\(^{-1}\) of BSA was incubated in dark with glucose (25 mM) and methylglyoxal (10 mM) for the period of 30 days. A pre-incubation of 10 min was carried out with the test compound before the addition of glucose/methylglyoxal. The control samples include BSA alone, BSA+Glucose and BSA+Methylglyoxal. Aminoguanidine was used as a positive control. All the solutions were prepared using sodium phosphate buffer (50mM of pH-7.4).

4.2.3 Determination of Early Glycation Inhibition

Fructosamine assay was used to determine the level of early glycation product formed as described earlier [27] with slight modifications. Briefly, the glycated protein sample was incubated with NBT (150 µM) reagent prepared in sodium carbonate buffer for 30 min. The absorbance was recorded at 530 nm using Thermo Scientific Evolution-201 spectrophotometer. The positive control was setup using aminoguanidine (AG), which is known as a potent anti-glycation compound.

4.2.4 Determination of AGEs Inhibition Activity

The estimations of AGEs fluorescence were carried out for 10 mg ml\(^{-1}\) glycated protein using Jasco-FP8200 spectrofluorimeter. The excitation (\(\lambda_{\text{ex}}\)) and emission (\(\lambda_{\text{em}}\))
wavelengths for different AGEs have been given in Table 1. The results were expressed in the form of percent inhibition.

Table 4.1 The excitation ($\lambda_{exc}$) and emission ($\lambda_{em}$) wavelengths of different AGEs.

<table>
<thead>
<tr>
<th>Advance glycation end products (AGEs)</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AGEs</td>
<td>350</td>
<td>440</td>
<td>[28]</td>
</tr>
<tr>
<td>Argpyrimidine</td>
<td>320</td>
<td>385</td>
<td>[28]</td>
</tr>
<tr>
<td>Vesperlysine</td>
<td>350</td>
<td>405</td>
<td>[28]</td>
</tr>
<tr>
<td>Pentosidine</td>
<td>335</td>
<td>385</td>
<td>[28, 29]</td>
</tr>
<tr>
<td>Crossline</td>
<td>380</td>
<td>440</td>
<td>[30]</td>
</tr>
</tbody>
</table>

4.2.5 Determination of Lysine Modification

Quantification of lysine modification was carried out by using TNBSA assay [31]. Briefly, the 500 µl of glycated BSA (1 mg ml$^{-1}$) sample was incubated with 250 µl of 0.01% TNBSA for 2 hours at room temperature. After incubation 250 µl of 10% SDS and 1N HCl was added and absorbance was read at 335 nm using Thermo Scientific Evolution-201 spectrophotometer. TNBSA was prepared in 100 mM sodium bicarbonate buffer (pH 8.5) and native BSA was used as a control. Results were presented as the percent lysine modification.

4.2.6 Determination Carbonyl Content

Protein bound carbonyl groups in glycated protein sample was determined using DNPH [32]. The carbonyl content was expressed as nmol carbonyl mg$^{-1}$ protein based on the extinction coefficient for DNPH ($\epsilon = 22,000$ M$^{-1}$ cm$^{-1}$).
4.2.7 Determination of Protective Effects on Conformation of BSA

CD spectra of BSA (1 μM) was measured using Jasco J-715 Circular Dichroism Spectropolarimeter. A quartz cell (1 mm) was used for far-UV (195–260 nm) measurements with 1 nm bandwidth and the scan speed was set at 100 nm min\(^{-1}\). All spectra were derived from the average of three scans for each sample and analyzed using the DICHROWEB [33].

4.2.8 Inhibition of Aggregation and Fibrillation

4.2.8.1 Congo red (CR) Binding Assay

CR binding was performed based on the absorbance measurements for AGE modified BSA and native BSA separately, as well as for Congo red background based on the well established method described earlier [34]. Briefly, 800 μL of protein sample (100 μM) was incubated with 200 μL of CR (100 μM). Absorbance was recorded at 530 nm using Thermo Scientific Evolution-201 spectrophotometer. The results were expressed as the percent inhibition of amyloid formation.

4.2.8.2 Thioflavin T (ThT) Binding Assay

To determine the inhibitory activity for β aggregation, ThT binding assay was carried out. Glycated control and test samples (100 μL) were incubated with ThT (32 μM) for an hour at room temperature [35]. Fluorescence measurements were carried out at λ\(_{ex}\) and λ\(_{em}\) wavelengths of 435 and 485 nm (slit width, 10 nm) respectively.
4.2.8.3 Confocal Microscopy

Inhibition activity of all compounds towards aggregation and fibrillation due to glycation of BSA was analyzed using Carl Zeiss Confocal microscope (LSM-710). ThT (Excitation= 445 nm and Emission= 480 nm) was used to determine amyloid aggregation with protein samples (100 µM) glycated in the presence and absence of inhibitors.

4.2.9 Molecular Docking Studies

Molecular docking studies were carried out to determine the interactions of all the phytochemicals and essential amino with BSA. AutoDock Vina 4.0 [36] was used to carry out docking studies. The structure of protein and ligands and were retrieved from the Protein Data Bank [37] and PubChem, a chemical compounds database. Docked structures were compared for their binding affinity and interaction with BSA. Best poses were analyzed using Pymol [38] and lysine and arginine residues interacting with inhibitors were observed.

4.3 Results and Discussion

4.3.1 Antiglycation Activities of Silybin, Sinigrin, and Vanillin

In our approach towards the identifying, the antiglycation potential of food phytochemicals, the present study was carried out to evaluate the protective effects of silybin, sinigrin, and vanillin the major component of milk thistle seeds, cruciferous vegetables and vanilla beans respectively by employing an in vitro glycation model of albumin. Assays were carried out for the determination of fructosamine formation (early glycation adduct), advanced glycation end products (fluorescent AGEs), carbonyl content, lysine modification, anti-fibrillation (based on CR and ThT binding),
conformational changes using circular dichroism, and molecular interaction pattern with BSA.

4.3.1.1 Effect on Early Glycation (Amadori Modification) of Albumin

Fructosamine is a highly useful marker in the diagnosis of hyperglycemia and determination of early stage glycation level in diabetic patients [39]. Fructosamine assay was carried out in order to investigate the inhibitory potential of these phytochemicals at the early stage of glycation reaction.

![Figure 4.3: Effect of silybin, sinigrin, and vanillin on early glycation (Amadori modification) of albumin.](image)

The results are presented in the Figure 4.3, which showed that the presence of SIB, SIN and VAN was able to inhibit Amadori modification, as the intensity of Amadori modified BSA decreased in presence of these compounds. Based on the results it was evident that the extent of glycation was higher in the case of BSA glycated in the absence of inhibitors, followed by the BSA treated with aminoguanidine and phytochemicals. The
inhibition activity of VAN and SIB was found to be even higher when compared to aminoguanidine. Sinigrin also showed potent inhibitory activity for the early glycation of albumin. Fructosamine is a strong predictor of microvascular disease [40] and accumulation of Amadori albumin in conditions of hyperglycemia could participate in the development of secondary complications [41]. The reduced level of early glycation product as seen in the presence of VAN, SIB and SIN suggests their preservative effect towards the non-enzymatic glycation mediated modification of albumin.

4.3.1.2 Effect on Advanced Glycation End Products (AGEs)

AGEs exhibits high reactivity and are fundamental in the development of severe complications in diabetes such as retinopathy, nephropathy, and neurological disorders. Assays were carried out to determine the inhibition activity of SIB, SIN and VAN against the formation of fluorescent AGEs and results are presented in Figure 4.4. In addition to the estimation of total fluorescent AGEs inhibition activity of SIB, SIN and VAN, we furthermore determined the fluorescence intensity of four specific AGEs whose excitation and emission spectra are well characterized [28, 42, 43]. The excitation and emission wavelength used for the determination of fluorescent AGEs is given in the Table 4.1. It was evident from the results that presence of phytochemicals showed comparatively reduced fluorescence intensity and the AGEs inhibition activity was found to be up to 80% in the presence of VAN. It was observed that VAN showed highest AGEs inhibitory activity when compared to SIB and SIN. Also, SIB and VAN were found to exhibit better antiglycation activity than AG. The reason for specifically
analyzing these four AGEs was their role in the development of complications in body and their well characterized fluorescence properties.

**Figure 4.4:** Effect of silybin, sinigrin, and vanillin on advanced glycation end products.

The level of inhibitory potential of SIB for different fluorescent AGEs studied was found to be vesperlysine > argpyrimidine > pentosidine > crossline. The SIB showed comparatively better inhibitory activity for AGEs when compared to the aminoguanidine.

**4.3.1.3 Effect on Carbonyl Content**

Protein bound carbonyls have been proposed to serve as a marker for the oxidative stress and also as an intermediate for the formation of AGEs [44, 45]. Investigations were carried out to determine the carbonyl content using 2,4-DNPH assay and results are presented in the Figure 4.5. The results demonstrated the carbonyl trapping ability of SIB, SIN and VAN, which was evident from the reduced carbonyl content observed in the
The presence of these phytochemicals. Carbonyl scavenging activity has been proposed to be a key mechanism for the antiglycation activity of several phytochemicals. The reduction in the carbonyl content as observed in the presence of SIB, SIN and VAN could be helpful in order to prevent the formation and accumulation of AGEs.

![Figure 4.5](image)

**Figure 4.5:** Effect of silybin, sinigrin, and vanillin on carbonyl content.

### 4.3.1.4 Effect on Lysine Modification Due to Glycation

Lysine residues have been identified as a major site of glycation in the case of many proteins and albumin contains 59 lysine residues out of which 34 have been reported to be glycated in various studies [46]. Glycation of the free amino group in the lysine side chain gives rise to the formation of a range of advanced glycation end products such as Carboxymethyllysine (CML) and Carboxyethyllysine (CEL), Vesperlysine (VESP) etc. TNBSA assay was carried out to determine the protective effects of SIB, SIN and VAN.
towards the modification of lysine residues in albumin and results are presented in Figure 4.6.

![Figure 4.6](image)

**Figure 4.6:** Effect of silybin, sinigrin and vanillin on lysine modification in albumin.

Glycated BSA showed up to 80% modification of lysine residues, which was reduced in the presence of SIB, SIN and VAN. The extent of lysine modification was found to be reduced in the presence of SIB, SIN and VAN with VAN exhibiting the maximum protective effect. These results suggest the protective effect of these compounds towards lysine modification due to glycation.

4.3.1.5 **Effect on Conformational of Albumin**

Circular dichroism spectropolarimetry was used to determine the effect of non-enzymatic glycation on albumin conformation and protective effects of inhibitors. CD spectra of native BSA were recorded at the wavelength range of 195 to 260 nm and results are
shown in Figure 4.7. Secondary structural changes in BSA were determined based on the ellipticity measurements. The shift in the CD signals in case of glycated BSA suggests the loss of helicity and increase in beta sheets content. It was confirmed based on the CD results that presence of SIB, SIN and VAN imparted preservative effect on albumin and prevented the glycation mediated conformational changes in BSA.

![Figure 4.7: Effect of silybin, sinigrin and vanillin on the conformation of BSA.](image)

Based on the secondary structure analysis it was seen that native BSA showed 87% helix with very low (0.5%) beta sheet content whereas glycated BSA showed loss of helicity with 10% helix and increased beta sheet (41%) content. At 500 µM, SIB and VAN exhibited maximum protective effects for the structure of BSA, which showed similar to native like CD spectra with 67% helix and only 2% beta sheet. The tendency of SIB and VAN to exhibit the protective effect for the native helical conformation suggests its possible chemical chaperone like activity. Results from the CD analysis were suggestive
of the possible mechanism for the potent antiglycation activity of SIB, SIN and VAN by protecting the native helical conformation of albumin.

4.3.1.6 Congo Red (CR) and Thioflavin T (ThT) Binding

Amyloid aggregation and fibrillation of proteins are known to be fundamental in the development of several incurable disorders such as Parkinson’s disease, Prion disease, Alzheimer’s disease etc [47]. It has been shown that non-enzymatic glycation stimulates amyloid aggregation of albumin [9]. CR binding assay has been widely used to characterize \textit{in vitro} amyloid fibrillation [48, 49] based on the absorbance at 530 nm. Results for the CR binding assay are presented in the Figure 4.8a, which suggests a decrease in the CR intensity in the presence of SIB, SIN and VAN. The overall decrease in the CR intensity (at 500 µM) was found to be up to 63%, which signifies the protective effect of these phytochemicals towards glycation induced fibrillation of BSA.

Thioflavin T (ThT) exhibits characteristic fluorescence intensity at around 482 nm on binding to beta sheets [49]. Glycation is known to induce conformational transition in albumin from the native helical state to a beta-sheeted form. ThT binding showed very high intensity for glycated BSA (Figure 4.8b), which indicates amyloid aggregation. The presence of SIB, SIN and VAN showed up to 69% decrease in the ThT fluorescence intensity, which reaffirm the anti-fibrillation activity of these phytochemicals. Moreover, ThT binding has been used frequently for the characterization of albumin fibrillation in earlier studies [50].
4.3.1.7 Confocal Microscopic Imaging

Confocal microscopic examinations were carried out using ThT to determine the morphology of aggregates and effect of SIB, SIN, and VAN. ThT gives green blue fluorescence on binding to beta sheeted amyloid fibrils. Figure 4.9 displays the results of the confocal imaging for the ThT binding for native and BSA glycated in the presence and absence of SIB, SIN, and VAN. Glycated BSA showed fibrillar aggregation with significantly high green blue fluorescence when compared to the native BSA, which does not show such aggregation.

The presence of SIB, SIN and VAN prevented post-glycation aggregation of BSA, which was evident from the confocal microscopic images with very less ThT positive aggregates. These results suggested the potent activity of these food phytochemicals in the prevention glycation induced aggregation of albumin.
Figure 4.9: Confocal imaging of albumin aggregates in the presence and absence of nutraceuticals.

4.3.1.7 Molecular Interaction of Silybin, Sinigrin, and Vanillin with BSA

Antiglycation compounds can work through different the mechanisms such as, by masking the free amino groups, and thus preventing glycation reaction by sugars or scavenging the carbonyl compounds formed from reducing sugars, thereby leading to the reduced formation of Amadori products and AGEs. Molecular docking studies were performed in order to understand the binding pattern of SIB, SIN and VAN with BSA and find the residues involved in the interaction. The scoring function for AutoDock Vina includes evaluations of the hydrogen bonding, dispersion/repulsion, electrostatics interactions and desolvation energy. SIB was found to have a very high affinity with BSA i.e. -8.6 kcal/mol. The interaction pattern of SIB with BSA and residues involved in the
hydrogen bonding are shown in Figure 4.10. SIB binds with BSA by the forming two hydrogen bonds with Arg194 and His247 along with the hydrophobic forces.

![Figure 4.10: Molecular interaction of silybin with albumin.](image)

The distance of two hydrogen bonds was found 2.6Å and 1.9Å respectively with Arg194 and His247. The high affinity of SIB for BSA further provides insights about its inhibitory activity in the glycation mediated aggregation and fibrillation of BSA.

SIN was found to bind with BSA in the Sudlow’s site I that has been characterized to have high affinity for the anionic heterocyclic ligands with extended conformation. Based on the results of the binding affinity it was evident that sinigrin binds with the high affinity (−6.2 kcal mol⁻¹) with BSA. The representative structure of the complex was
derived from the binding pose with minimal binding energy $-6.2$ kcal mol$^{-1}$ is presented in Figure 4.11.

Figure 4.11: Molecular interactions of sinigrin with albumin.

There were totally 4 hydrogen bonds (h-bond) which stabilize the sinigrin BSA complex. The major interacting residues were found to be Glu-152, Arg-217, Ala-290, and Glu-291. The distance of four h-bonds stabilizing SIN-BSA complex were as follows; Glu-152 (2.1 Å), Arg-217 (3.5 Å), Ala-290 (2.1 Å), and Glu-29 (3.3 Å). Also, these results suggested that the affinity of sinigrin to BSA is predominantly determined due to the hydrogen bonding and hydrophobic interaction.
The binding affinity of VAN was found to be -5.5 Kcal mol⁻¹. Figure 4.12 displays the best binding pose of VAN with BSA showing the hydrogen bonds (H-bond) in the form of the yellow dashed line. It can be seen that VAN closely interacts with three arginine residues i.e. Arg-194, Arg-198 and Arg-217 while forming H-bond with Arg-198. Masking of lysine and arginine residues, which are most susceptible for the non-enzymatic glycation has been proposed to be one of the mechanisms for the inhibition of glycation [51].
Figure 4.13: Schematic representation showing the antiglycation effect and chemical chaperon like function of vanillin by preventing conformational transition from α-helix to beta sheeted structure and aggregation of albumin.

In this regard, the interaction of SIB, SIN, and VAN with lysine and arginine residues could prevent their glycation modification and eventually lower the early and advanced glycation end products (AGEs). Figure 4.13 shows the schematic representation for the antiglycation effect and chemical chaperon like function of vanillin by preventing the conformational transition from α-helix to beta sheeted structure and aggregation of albumin. Based on the outcome from different assays carried out we could hypothesize
that the antiglycation and anti-fibrillation activity of silybin, sinigrin, and vanillin was mainly due to their protective effects towards the native conformation of albumin.

4.3.2 Antiglycation Effects of Essential Amino Acids

Small molecules with amino groups such as aminoguanidine, metformin etc. have been already found to exhibit potent antiglycation activities [12]. The antiglycation activities of only three amino acids i.e. D-lysine [52] L-arginine [53] and recently L-cysteine [54] have been reported and the role of essential amino acids which are the key part of nutrition and human health was not reported in the literature.

4.3.2.1 Effect on Early Glycation (Amadori Inhibition) of Albumin

Fructosamine is also a marker for the glycemic level in the diagnosis of diabetes patients. NBT assay was carried out to determine the inhibitory potential of EAA against at the early stage of glycation and results are presents in Figure 4.14. NBT reacts with the fructosamine and gives rise to the blue color product, which exhibits absorbance at 530 nm. It was observed that EAA reduces early glycation and formation of Amadori product, which was evident from the reduced absorbance at 530 nm. From results, it was evident that EAA exhibited differential inhibitory activity towards early glycation of BSA. Tryptophan showed highest inhibitory activity followed by PHE, LYS and HIS and other amino acids. Inhibition of Maillard reaction at the early stage of glycation could be helpful in avoiding the formation of more reactive, and toxic advanced glycation end products.
Figure 4.14: Effect of essential amino acids on early glycation product (Fructosamine). Results are means ± standard deviations of three different assays.

4.3.2.2 Effect on Advanced Glycation End Product (AGEs)

AGEs are highly reactive and fundamental in the development of various complications in diabetes such as nephrological disorders, Alzheimer’s disease, and skin aging. AGEs are characterized by their specific properties such as fluorescence, cross-linking and activation of signaling cascades for inflammatory pathways [55]. Fluorescence spectroscopy was used to determine the inhibitory activity of EAA against the formation of fluorescent AGEs. Results are presented in the Figure 4.15 in the form of percent inhibition activity of different EAA. In addition, to the total AGEs, the fluorescence intensity estimations were also carried out for the four specific AGEs (Argpyrimidine, pentosidine, vesperlysine and crossline). The main reason for considering these four specific AGEs was their role in the development of severe complications and their well defined fluorescence properties.
It was seen that when compared to the negative control (glycated BSA), the presence of EAA showed comparatively reduced fluorescence intensity. TRP showed the least fluorescence intensity and thus highest inhibitory activity when compared to the other EAA. The maximum AGES inhibition achieved was found to be up to 80%. Also the AGES inhibitory activity of PHE, TRP and LYS was found to be greater or similar to that of aminoguanidinedine, which was used a positive control.

4.3.2.3 Effect on the Carbonyl Content and Lysine Modification

Both synthetic and natural compound with high scavenging potential for carbonyl compounds have been found to be the potent inhibitor of glycation [56]. DNPH assay was carried out in order to determine the level of protein bound carbonyl groups and results are presented in the Figure 4.16. From results, it was evident that there was a reduction in the level of carbonyl content in the presence of EAA. The carbonyl
scavenging potential of TRP and PHE was found to be similar to that of aminoguanidine. These observations suggest the effective role of EAA as a potent scavenger of carbonyl compounds, which serve as an intermediate of AGEs.

![Figure 4.16: Effect of essential amino acids on protein bound carbonyl content. Results are means ± standard deviations of three different assays.](image)

Lysine residues in proteins have been found to be most susceptible for the non-enzymatic glycation modification, giving rise to the formation of a range of AGEs such as Carboxymethyllysine (CML), Carboxyethyllysine (CEL) and Vesperlysine (VESP) etc. Mass spectroscopic studies in the recent have shown that out of the 59 lysine residues in BSA 34 serve as a site of glycation [46]. Thus, determining the level of lysine modification could provide the information about the protective effects of essential amino acids towards non-enzymatic glycation of BSA. TNBSA assay was used for the quantification of lysine modification and results for are presented in the Figure 4.17. The
extent of lysine modification was found to be as high as 80% in case glycated BSA control. As shown in the figure the presence of EAA showed a comparatively low level of lysine modification. In case of TRP, lysine modification was reduced to be as low as 23%, which was comparatively better than aminoguanidine (25%). Other than TRP, PHE, LYS and HIS also showed a significant decrease in the lysine modification.

![Figure 4.17: Effect of essential amino acids on lysine modification. Results are means ± standard deviations of three different assays.](image)

Valine showed almost no effect with least reduction in lysine modification due to glycation. These observations were indicative of the protective effect of EAA towards non-enzymatic glycation of lysine.
4.3.2.4 Congo Red (CR) and Thioflavin T (ThT) Binding

Glycation modulates structure of albumin and induces amyloid like fibrillation [9]. Protein fibrillation and accumulation of amyloid like aggregates is a key step in the development of several neurological disorders such as Parkinson’s disease, Prion disease etc [57]. In order to determine the inhibition of glycation induced aggregation and fibrillation of BSA, ThT, and CR binding assay was carried out and results are presented in the Figure 4.18a & b. ThT specifically binds to the amyloid fibrils and shows enhanced fluorescence intensity at 485 nm. From the results, it was evident that amino acids restrained glycation induced aggregation and fibrillation of BSA.

Figure 4.18: Effect on essential amino acids on glycation induced fibrillation of albumin, a) CR binding b) Thioflavin T binding. Results are means ± standard deviations of three different assays.

Out of all essential amino acids TRP, MET, LYS, and PHE showed the maximum reduction in the fluorescence intensity when compared to the glycated BSA control. VAL
and THR showed the least reduction in the ThT fluorescence when compared to the other essential amino acids. Inhibitors of protein aggregation and fibrillation could play a preventive role in the development of severe neurological disorders such as Alzheimer’s disease. These results were indicative of the anti-fibrillation potential of essential amino acids.

4.3.2.5 Confocal Imaging

Confocal microscopic examinations were carried out using ThT to determine the morphology of aggregates and effect of different essential amino acids. Figure 4.19 shows the results of the confocal imaging for the ThT binding for native and BSA glycated in the presence and absence of EAA. As expected glycated BSA showed fibrillar aggregation with significantly high green blue fluorescence when compared to the native BSA, which does not show such aggregation. The presence of EAA showed differential effects on post-glycation aggregation of BSA, which was evident from the confocal microscopic images. It was Tryptophan, phenylalanine, and lysine, which showed maximum inhibition of glycation induced aggregation of albumin when compared to other amino acids. Vanillin showed no inhibition towards aggregation of albumin.

4.3.2.6 Effect of EAA on Conformation of albumin

Non-enzymatic glycation induces the conformational transition in albumin leading to the secondary structural changes from helical to a beta-sheeted structure. CD analysis can provide useful information about glycation mediated conformational changes in proteins. The protective effect of essential amino acids for the structure of BSA was determined based on circular dichroism (CD) analysis and CD spectra are presented in Figure 4.20.
Glycated BSA showed beta-sheeted conformation when compared to the native helical BSA. The protective effect of EAA for glycation induced conformational changes in BSA was evident from the CD spectra of BSA glycated in the presence of amino acids. Compared to the other EAA, TRP showed the highest activity and CD spectra similar to native BSA. Even earlier studies have showed amino acids such as L-arginine and L-
lysine exhibits chemical chaperon like function and stabilize proteins against aggregation [58, 59].

![Figure 2.20: Effect of essential amino acids on the conformation of albumin.](image)

Such protective effect of EAA towards the native conformation of BSA could also helpful in the prevention of glycation induced amyloid. Moreover, this protective effect of EAA for native conformation of BSA could be attributed to their chemical chaperon like activity.

4.3.2.7 Molecular Interaction of EAA with BSA
Docking studies were used in order to get insights about the molecular interaction pattern of all nine essential amino acids with BSA. Masking of free amino groups in proteins has been proposed to be one of the mechanisms for the antiglycation effects of small
molecule inhibitors of glycation [51]. The binding energies (kcal mol\(^{-1}\)) as determined from the molecular docking studies of all EAA and BSA complex are given in Figure 4.21. It can be seen that PHE (-4.9), TRP (-4.8) and THR (-4.4) showed the highest affinity for BSA when compared to the other amino acids.

![Figure 4.21: Binding energy of all nine essential amino acids as determined from molecular docking approach.](image)

The affinity of EAA with BSA was mainly determined by hydrogen bonding interaction. Furthermore, observations for the molecular interaction of EAA-BSA complexes were carried out and the best poses obtained from the molecule docking simulations were presented in the Figure 4.22.
Figure 4.22: The molecular interaction pattern of nine essential amino acids with albumin as determined from molecular modeling studies. Hydrogen bonds are represented with yellow color dashed lines.

The residues interacting with the EAA are shown in figure and hydrogen bonds (H-bond) are represented as the yellow dashed line. The distance of H-bonds formed by EAA is given in angstrom (Å). From the observations of docked structure, it was evident that EAA bind with BSA by mainly interacting with both lysine and arginine residues.
Interestingly, there was no H-bonding interaction seen in case of valine, which also showed least antiglycation activity. The results from the molecular interaction studies of EAA were in good agreement with the other results from the assays to determine the antiglycation activity. Based on these observations this can be hypothesized that the masking of lysine and arginine residues is possibly one of the mechanisms responsible for the potent antiglycation activity of EAA.

4.4 Conclusion

In summary, this is the first study to demonstrate the antiglycation potential of SIB, SIN and VAN, the three abundant nutraceuticals present in different vegetables and food products, based on the combination of in vitro and in silico approach. The results demonstrated that SIB, SIN and VAN exhibit a) inhibition of both early and advanced glycation end products b) carbonyl quenching and protective effects towards lysine modification c) inhibition of glycation induced fibrillation of BSA as determined by CR and ThT binding assay d) protective effects towards glycation induced conformational change in BSA and e) inhibition for lens protein glycation. Out the nine essential amino acids used to determine the antiglycation activity tryptophan, phenylalanine and lysine showed highest antiglycation activity while valine showed least activity. These findings suggest that the antiglycation activity of SIB, SIN and VAN was largely due to their carbonyl scavenging potential and interaction with albumin by chaperone like function. Moreover, these phytochemicals and amino acids are a regular part of the diet in the form of various vegetables and food products and thus, it could be of great benefits in preventing the non-enzymatic glycation in vivo.
4.5 References


