THE INHIBITORY EFFECT OF *SILYBUM MARIANUM* (MILK THISTLE) SEEDS EXTRACT ON SERUM ALBUMIN GLYCATION BY GLUCOSE, FRUCTOSE, AND GALACTOSE


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Abstract. Protein glycation consists of the non-enzymatic attachment of monosaccharides to proteins. This leads to the formation of advanced glycation end products (AGEs) that are held responsible for diabetes complications. There are some drugs that inhibit AGEs, but their usage is limited by side effects. Plant-based therapeutic strategies could be useful in overcoming this limitation. *Silybum marianum* (Milk thistle), a plant used to treat liver problems, was also proved useful in the treatment of type 2 diabetes. Here we investigated the ability of *S. marianum* extract to inhibit the *in vitro* glycation of bovine serum albumin (BSA) by three physiologically relevant monosaccharides, namely glucose, fructose and galactose. BSA was glycated in the absence and in the presence of 0.1 %, 1 % and 5 % *S. marianum* seeds hydroalcoholic extract. Measurements on fructosamine, AGEs and amyloid cross-β structures formation showed that the plant extract inhibited these processes in the case of the three monosaccharides, especially in the case of glucose and galactose. The inhibition was dose-dependent and time-dependent. Our results demonstrate the ability of the plant extract to inhibit the *in vitro* glycation of BSA.

Key words: Diabetes, protein glycation inhibition, *Silybum marianum* extract, glucose, fructose, galactose.

INTRODUCTION

Protein glycation is a spontaneous reaction in which reducing sugars attach to free amino groups without the aid of an enzyme [17, 31, 45]. The process, also known as the Maillard reaction, involves several stages, starting with the formation of an unstable Schiff’s base that rearranges to Amadori products. Both types of products are considered early glycation products. In the later stage of glycation, Amadori products go through rearrangement and dehydration reactions to give
advanced glycation end products (AGEs) [17, 31, 45]. Proteins with AGE modifications can form protease resistant cross-links that further promote protein aggregation [14, 21].

The glycation process is significant in aging [17] and in diabetes, when the glycation level is correlated with the glycemic control of patients [16]. In the human body, the main monosaccharides that are assimilated from dietary sources are glucose, fructose and galactose [30]. The liver removes fructose and galactose from the circulation, which makes glucose the major circulating monosaccharide [30] and the major glycat ing agent in diabetes [37]. Glycation by fructose is relevant in organs that synthesize fructose trough the polyol pathway like ocular lenses, nerves and kidneys [20]. Galactose, that accumulates in galactosemia and aging, is also an important glycation agent, being more reactive than glucose [43].

Human health is influenced by both exogenous and endogenous glycation products. Exogenous AGEs from food can be absorbed into the systemic circulation or cause inflammatory reactions at the colon level, as reviewed in [16]. The early glycation products from food were shown to promote prostate tumor growth more than AGEs [7]. Regardless of source (Maillard reaction or exogenous), AGEs may cause a direct destruction of the extracellular matrix or can trigger a receptor-mediated signaling cascade that results in the production of free radicals and pro-inflammatory molecules [28]. All these processes contribute to the development of diabetic complications [28].

Given the inflammatory effect of AGEs, there is an interest in developing agents that can limit their effect. One strategy is to target the receptor of AGEs (RAGE) and prevent their interaction [4], with some inhibitors being currently under clinical trials [13, 33]. A different strategy involves inhibiting AGEs formation by compounds that have antioxidant activity, scavenge free radicals, trap intermediate products from Maillard reaction or break the cross-linked products [33, 41]. Several synthetic compounds were developed to inhibit AGEs, like aminoguanidine, a candidate that did not pass clinical trials due to its secondary effects [5]. Natural products with diverse chemical structures (polyphenols, polysaccharides, terpenoids, vitamins, alkaloids, peptides) appear as potent and safer AGEs inhibitors [33, 41]. These are extensively reviewed in [41].

Silybum marianum (milk thistle) is known for its benefits in liver and gallbladder diseases. It was also proven to have hypoglycemic and hypolipidemic effects, appearing as an insulin sensitizer and a useful plant in type 2 diabetes [15, 25]. Previous studies have shown the inhibitory effect of S. marianum in vivo, in living human skin explants model and in vitro [35, 40, 44]. In vitro studies investigated the effect of bovine serum albumin (BSA) glycation by glucose [35, 44]. BSA is a model protein often used in biophysical studies due to its structural similarity with human serum albumin, its low cost and high availability [12].

Here we addressed the anti-glycation effect of a hydroalcoholic extract of S. marianum seeds on in vitro BSA glycation by glucose, fructose and galactose. Considering the different reactivities of the three monosaccharides in the Maillard
reaction – fructose and galactose being more reactive than glucose [11, 19], and their physiological relevance in diabetes [9, 11], we investigated whether the plant extract is effective in inhibiting the glycation by all considered sugars.

MATERIALS AND METHODS

MATERIALS

Bovine serum albumin (fraction V, > 98% purity) was purchased from Carl Roth (Karlsruhe, Germany). *S. marianum* seeds extract (1:3.75) in hydroalcoholic solution (35/65 ethanol/water) was purchased from Dacia Plant (Strada Hărmanului, Bod, 507015). D-(-)-galactose was purchased from Merck KGaA (Darmstadt, Germany), D-(-)-glucose and D-(+)-fructose were purchased from Carl Roth (Karlsruhe, Germany). The monosaccharides, as well as all reagents used were of analytical grade.

TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) in plant extract was assessed with Folin and Ciocalteu reagents, using a slightly modified protocol from that of Singleton and Rossi [38]. Briefly, a calibration curve was performed using increasing concentrations of gallic acid (0 – 0.5 mg/mL). Gallic acid or plant extract aliquots (0.1 mL) were mixed with 0.2 mL of 10% Folin-Ciocalteu reagent and 0.8 mL of sodium carbonate solution (700 mM). Samples were incubated for 30 minutes. During the incubation, a blue color develops, whose intensity is given by the total phenolic content [3, 6, 38]. The optical density of samples was measured at 765 nm. The total phenolic content of plant extract is then calculated in gallic acid equivalents (mg GAE/g) using the determined calibration curve ($y = 6.533 x + 0.277, R^2 = 0.990$) and the equation:

$$C = c \cdot \frac{v}{m}$$  \hspace{1cm} (1)

where $C$ is the TPC expressed in mg GAE/g, $c$ is the gallic acid concentration determined using the calibration curve (measured in mg/mL), $v$ is the volume of plant extract used in the assay (measured in mL) and $m$ is the weight of the plant extract used in the assay (measured in g).

IN VITRO GLYCATION OF BSA

BSA glycation by glucose, fructose and galactose was performed using the fast thermal glycation protocol of Bhatwadekar and Ghole [2]. This involved
incubating 50 mg/mL BSA with 0.5 M monosaccharides in 0.1 M phosphate buffered saline (PBS), pH 7.4 for 4 days at 50 °C without plant extract (positive control) or with 0.1 %, 1 % and 5 % S. marianum extract (samples). The negative control was considered a 50 mg/mL BSA solution without monosaccharides that was thermally prepared as the positive control and the samples. In order to observe the dynamics of the glycation process in the absence and presence of plant extract, we analyzed aliquots of the glycation mixtures after 2, 3 and 4 days of incubation by applying the methods that will be presented in the following sections.

FRUCTOSAMINE DETERMINATION

Fructosamine concentration was determined using the nitroblue tetrazolium (NBT) assay, as presented in [42], with some modifications. For the assay, we mixed 40 µL of glycated BSA samples with 80 µL of 0.5 mM NTB solution in 0.1 M carbonate buffer (pH 10.35). The mixtures were incubated for 30 minutes at 37 °C. Afterwards, their absorption was read at 530 nm. The inhibition of fructosamine formation due to the treatment with S. marianum was evaluated as the percentage of inhibition using Eq (2) [8], where $A_c$ is the absorbance value of the control samples after 2, 3 or 4 incubation days and $A_s$ is the absorbance of BSA samples glycated in the presence of plant extract at the corresponding incubation day. The measurement was performed using a Jasco V-560 UV/VIS Spectrophotometer.

\[
\% \text{ inhibition} = \left(1 - \frac{A_s}{A_c}\right) \cdot 100 \%
\]  

DETERMINATION OF AGES FORMATION

AGEs formation was determined by measuring the fluorescence of samples at 460 nm when using an excitation wavelength of 335 nm [27, 32]. Measurements were performed using a Jasco FP750 spectrofluorometer. The inhibitory effect of the three concentrations of plant extract on AGE formation in the case of BSA glycated by glucose, fructose and galactose was estimated based on the measured fluorescence of the glycation solutions without ($F_c$) and with plant extract ($F_s$), as given by equation (3) [24].

\[
\% \text{ inhibition} = \left(1 - \frac{F_s}{F_c}\right) \cdot 100 \%
\]  

DETERMINATION OF PROTEIN AGGREGATION

Congo Red (CR) binding assay was used to address the aggregation of BSA upon glycation in the absence and in the presence of S. marianum extracts. The protocol was derived from [18], involving mixing of 0.5 mL of sample (BSA
incubated with monosaccharides, without or with plant extract) with 0.5 mL of CR solution (100 µM CR freshly prepared in PBS with 10 % v/v ethanol). The mixtures were incubated for 30 minutes at room temperature, followed by reading their optical densities at 530 nm. The percentage of inhibition on amyloid cross-β structures formation was calculated using Eq. (2).

STATISTICAL ANALYSIS

The results are reported as mean and standard deviation calculated over four measurements. The statistical significance of results was determined by applying one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSIONS

In the present study we investigated the activity of S. marianum extract in inhibiting the in vitro glycation of BSA by three monosaccharides with different reactivities, namely glucose, fructose and galactose. Initially we determined the plant extract TPC and then we investigated its inhibitory effect at three levels – early and late albumin glycation, albumin oxidation and amyloid aggregation.

TOTAL PHENOLIC CONTENT OF S. MARIANUM EXTRACT

TPC content of the hydroalcoholic S. marianum seeds extract that we analyzed was 0.332 ± 0.008 mg GAE/g. The concentration of phenolic compounds extracted from S. marianum varies depending on plant part used in the study [34, 35], on the quality of seeds and extraction method [23]. The concentration obtained when analyzing the present seed extract is about half of the concentration obtained by Serçe et al. from defatted seeds using ethanol [34] and significantly lower than that obtained in other studies using different solvents like ethanol, n-hexane, and petroleum using conventional extraction or supercritical fluid extraction [23]. Nevertheless, water and ethanol are green solvents, and the plant extracts prepared using water-ethanol mixtures are promising antioxidants to be used in food [29] or the pharmaceutical industry [26].

THE EFFECT OF S. MARIANUM EXTRACT ON FRUCTOSAMINE FORMATION

In the initial stages of glycation, acyclic monosaccharides attach to free amino groups of arginine or lysine residues to form unstable Schiff base products. These may undergo Amadori rearrangements to form fructosamine derivatives through ketoamine linkages [1]. Fructosamine levels are indicative on the glycemic control of diabetic patients over shorter periods than glycated hemoglobin [36].
Here we measured fructosamine levels in BSA samples incubated with glucose, fructose and galactose, in the absence and in the presence of 0.1 %, 1 % and 5 % S. marianum extract. Results are presented in Figure 1. In the positive control condition, we observe fructosamine formation in BSA samples incubated with glucose, fructose and galactose, the process being enhanced with incubation time. By comparing the positive controls, we observe that fructosamine levels are higher when BSA is incubated with fructose (1.19 mmol/L after 4 days of incubation) or galactose (1.09 mmol/L after 4 days of incubation) relative to glucose (0.90 mmol/L after 4 days of incubation), which is consistent with the increased reactivity of fructose and galactose [11, 19].

As shown in Figure 1, the treatment with S. marianum extract decreases fructosamine formation in a dose dependent manner, namely the treatment with 5 % plant extract is the most efficient. The percentages of fructosamine formation inhibition due to the treatment with S. marianum extract are presented in Table 1. These percentages are calculated relative to the fructosamine concentration in the positive control at the corresponding day of incubation. When the samples were treated with 0.1 % plant extract, the inhibition percentages increase with incubation time, the highest percentages being seen in the case of BSA samples incubated with galactose. The same dependency was determined in the case of BSA incubated with glucose or fructose treated with 1 % plant extract and in the case of BSA with fructose treated with 5 % plant extract. In the case of BSA with galactose treated with 1 % plant extract and BSA with glucose or galactose treated with 5 % plant extract, we observe that the inhibition is stronger after 2 days and decreases after 3 and 4 days of incubation. In the lowest concentration (0.1 %), the extract is more effective in the case of glycation by glucose and galactose, but higher concentrations (1 % and 5 %) are effective even in the case of fructose. These results prove that S. marianum seeds extract inhibits the formation of fructosamine in the samples incubated with glucose, but also with the more reactive fructose and galactose.

Table 1
Inhibition percentage of fructosamine formation due to the treatment with S. marianum seeds extract. The samples are BSA incubated with glucose (BSA+Glc), BSA incubated with fructose (BSA+Fru) and BSA incubated with galactose (BSA+Gal).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day</th>
<th>Concentration of S. marianum extract in sample</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1 %</td>
</tr>
<tr>
<td>BSA+Glc</td>
<td>2</td>
<td>8.3 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34.39 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.71 %</td>
</tr>
<tr>
<td>BSA+Fru</td>
<td>2</td>
<td>10.99 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18.63 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25.10 %</td>
</tr>
<tr>
<td>BSA+Gal</td>
<td>2</td>
<td>32.72 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34.19 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>39.75 %</td>
</tr>
</tbody>
</table>
Fig. 1. Fructosamine levels in thermally prepared BSA, BSA incubated with monosaccharides and BSA incubated with monosaccharides in the presence of S. marianum extract (SIB) for 2, 3 and 4 days. Results on samples with glucose (Glu) are shown in (a), with fructose (Fru) are shown in (b) and with galactose (Gal) are shown in (c). The results are expressed as mean ± SEM (n = 4). The statistical significance (One way ANOVA test considering BSA incubated with the corresponding monosaccharide as positive control) is represented as c for p < 0.001.
THE EFFECT OF S. MARIANUM EXTRACT ON AGES FORMATION

As presented in the Introduction section, AGEs are glycation products resulted through the Maillard reaction. Their accumulation from dietary and endogenous sources is in strong relationship with human diseases like cardiovascular, insulin resistance, etc. [22]. Not all AGEs are fluorescent, but the levels of fluorescent AGEs are indicators of diabetes complications [10, 22].

Here we addressed the ability of S. marianum extract to inhibit fluorescent AGEs formation in the samples of BSA incubated with glucose, fructose and galactose. The fluorescence emission intensities of AGEs formed in the samples \((\lambda_{ex} = 335 \text{ nm}, \lambda_{em} = 460 \text{ nm})\) are plotted in Figure 2. The fluorescence of samples is correlated with the concentration of AGEs produced in each sample. The results in the positive controls show that AGEs are formed in the samples in a time-dependent and reducing monosaccharide-dependent manner. The largest levels of AGEs are seen after 4 days of incubation. The incubation with galactose produces more AGESs, with 43.66 % more than in the case of fructose and with 73.85 % more than in the case of glucose (the percentages were calculated considering fluorescence intensities after 4 days of incubation).

The calculated inhibition percentages of AGEs formation in the samples incubated with S. marianum extract are shown in Table 2. The inhibition depends on extract concentration, with the 5 % concentration being the most effective. In all cases, the inhibition is more effective after 2 days of incubation and less effective after 4 days of incubation. When looking at the impact of reducing monosaccharide, we observe that the plant extract is the most effective in the case of samples glycated by glucose, followed by the samples glycated by galactose and last are the samples glycated by fructose. As seen in Figure 2, in the case of BSA with glucose samples incubated with 5 % plant extract, the intensity of AGEs fluorescence is similar to the fluorescence measured for thermally prepared BSA in the absence of monosaccharides (control BSA). This suggests that the extract almost fully inhibits AGEs formation. In the case of the other sugars, AGEs fluorescence significantly exceeds the results of control BSA, even if the extract has a certain inhibitory effect on AGEs formation. Moreover, we calculated larger inhibition percentages of S. marianum extract on AGEs formation in the samples with galactose, but the AGEs fluorescence emission intensity in these samples still exceeds that of samples incubated with fructose. This shows that in the case of glycation by fructose or galactose, the plant extract slows down the formation of AGEs, but does not stop it.
** BS A glycation inhibition by *S. marianum* extract **

**Table 2**

Inhibition percentage of AGEs formation due to the treatment with *S. marianum* seeds extract. The samples are BSA incubated with glucose (BSA+Glc), BSA incubated with fructose (BSA+Fru) and BSA incubated with galactose (BSA+Gal).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day</th>
<th>Concentration of <em>S. marianum</em> extract in sample</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1 %</td>
</tr>
<tr>
<td>BSA+Glc</td>
<td>2</td>
<td>55.08 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34.97 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11.65 %</td>
</tr>
<tr>
<td>BSA+Fru</td>
<td>2</td>
<td>33.43 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27.82 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.42 %</td>
</tr>
<tr>
<td>BSA+Gal</td>
<td>2</td>
<td>39.87 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>33.26 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.94 %</td>
</tr>
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</table>

**Table 3**

Inhibition percentage of amyloid cross-β structures formation due to the treatment with *S. marianum* seeds extract. The samples are BSA incubated with glucose (BSA+Glc), BSA incubated with fructose (BSA+Fru) and BSA incubated with galactose (BSA+Gal).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Day</th>
<th>Concentration of <em>S. marianum</em> extract in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1 %</td>
</tr>
<tr>
<td>BSA+Glc</td>
<td>2</td>
<td>1.69 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.81 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.08 %</td>
</tr>
<tr>
<td>BSA+Fru</td>
<td>2</td>
<td>2.74 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.86 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.52 %</td>
</tr>
<tr>
<td>BSA+Gal</td>
<td>2</td>
<td>1.80 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.09 %</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.02 %</td>
</tr>
</tbody>
</table>
Fig. 2. Fluorescence intensity (λ_{ex} = 335 nm, λ_{em} = 460 nm) measured on thermally prepared BSA. BSA incubated with monosaccharides and BSA incubated with monosaccharides in the presence of S. marianum extract (SIB) for 2, 3 and 4 days. Results on samples with glucose (Glu) are shown in (a), with fructose (Fru) are shown in (b) and with galactose (Gala) are shown in (c). The results are expressed as mean ± SEM (n = 4). The statistical significance values (One way ANOVA test considering BSA incubated with the corresponding monosaccharide as positive control) are represented as a for p < 0.05, b for p < 0.01, c for p < 0.001 and ns for non-significant.
S. MARIANUM EFFECT ON PROTEIN AGGREGATION

Fig. 3. UV absorption at 350 nm measured on thermally prepared BSA. BSA incubated with monosaccharides and BSA incubated with monosaccharides in the presence of S. marianum extract (SIB) for 2, 3 and 4 days. Results on samples with glucose (Glu) are shown in (a), with fructose (Fru) are shown in (b) and with galactose (Gala) are shown in (c). The results are expressed as mean ± SEM (n = 4). The statistical significance values (One way ANOVA test considering BSA incubated with the corresponding monosaccharide as positive control) are represented as a for p < 0.05, b for p < 0.01, c for p < 0.001 and ns for non-significant.
The glycation of serum albumins has a negative impact on their conformation and stability, which promotes the formation of cross-β structures that trigger their amyloid aggregation [39]. We analyzed the formation of amyloid cross-β structures in our samples of BSA incubated with reducing monosaccharides in the absence and in the presence of *S. marianum* seeds extract. For this purpose, we used the Congo red binding assay as Congo red is a amyloid-specific dye [18]. In Figure 3 we report the absorbance of samples measured at 530 nm. The increase in absorbance shows an increase in amyloid structures in the sample.

In BSA incubated with the monosaccharides without plant extract, we observe an increase in absorbance relative to the control, showing that amyloid structures were formed. The process increases with incubation time. As in the case of AGEs formation, the absorbance of samples with galactose is the highest, increased with 3.18 % relative to the samples with fructose after 4 days of incubation and with 7.33 % relative to the samples with glucose after 4 days of incubation.

The determined inhibition percentages of *S. marianum* seeds extract on amyloid structures formation in BSA samples incubated with glucose, fructose or galactose are given in Table 4. The 5 % plant extract has an enhanced inhibitory effect, especially after 2 days of incubation. Its efficiency decreases with incubation time in the case of glucose and galactose, while it increases in the case of fructose. The 5 % plant extract is more efficient in the case of samples glycated by galactose, followed by fructose. In the case of glucose, it is initially efficient (after 2 days of incubation), but its efficiency decreases significantly with incubation time.

**CONCLUSIONS**

Here we investigated the inhibitory effect of a hydroalcoholic extract of *S. marianum* seeds on the glycation of BSA by three monosaccharides with different reactivities in the glycation reaction. We performed a rapid thermal glycation protocol without plant extract and with 0.1 %, 1 % and 5 % plant extract. Samples were analyzed after 2, 3 and 4 days of incubation by measuring the fructosamine levels, AGEs and amyloid cross-β structures formation.

The analysis of positive control samples showed that galactose triggers the formation of the largest levels of fructosamine, AGEs and amyloid structures, fructose leads to intermediate values and glucose induces the lowest levels of glycation products.

We have shown that the effect of *S. marianum* extract in inhibiting BSA glycation depends on concentration, reaction time and monosaccharide type. The 5 % plant extract proved to be more efficient than the lower concentrations of plant extract. In what concerns the time, there are clear cases when the inhibitory effect is the strongest after 2 days of incubation and then decreases in time, like in the case
of AGEs formation, regardless of reducing monosaccharide or plant extract concentration. In other cases, like fructosamine or amyloid structures formation, we see a decrease or an increase of inhibitory effect in time, depending on plant extract concentration or on the reducing sugar. The strongest inhibitory effect of the plant extract was seen in the case of glucose and galactose. The extract also inhibits fructosamine, AGEs and amyloid structures formation in the case of samples glycated by fructose, but to a lesser degree than in the case of other monosaccharides.

Our conclusion is that the hydroalcoholic extract of S. marianum seeds can inhibit protein glycation, being effective in the case of protein glycation by glucose or by more reactive monosaccharides like galactose and fructose.

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