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RESEARCH ARTICLE
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ANTIGLYCATION AND ANTI-AGES ACTIVITY OF SULFOSALICYLIC ACID (IN VITRO STUDY)

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ABSTRACT
Diabetes mellitus is one of the most common diseases that is present nearly all over the world and it is characterized by hyperglycemia. Hyperglycemia results from protein glycation and the continuing increase of AGEs. These AGEs form on biomolecules and possess complex structures that produce protein fluorescence and cross-linking that leads to tissue damage. Salicylic acid is known as a potent inhibitor of glycation and AGEs. A number of its derivatives i.e. salicylate, 5-aminosalicylic acid, acetylsalicylic acid (aspirin), and para-amino salicylic acid are known to have the potential to reduce glycation levels and AGEs. Our study investigated glycation and AGEs inhibitory activity of sulfosalicylic acid, a synthetic compound using glucose-BSA *invitro* conditions. The study was performed by making sixteen combinations using different concentrations of glucose, inhibitors with BSA and these were incubated at 37^{o}C and 50^{o}C simultaneously for five weeks. Glycation level was assessed by TBA and periodate assays. ELISA measured AGEs. The formation of glycated BSA was quantitated by measuring browning intensity. The results showed that browning was increased from 1st to 5th week of incubation due to an increase in glycation. Our results showed that a 10 *mM* concentration of sulfosalicylic acid showed a good response to minimize the glycation and AGE production as compared to its lower concentrations. The results of this study demonstrated that sulfosalicylic acid inhibited the glycation of BSA by glucose in a dose-dependent manner. Periodate borohydride assay showed that it is a more suitable glycation method when compared to the TBA method.

Key Words: Hyperglycemia, advanced glycation end products (AGEs), Sulfosalicylic acid, Periodate borohydride, TBA, bovine serum albumin (BSA).

Introduction
Diabetes mellitus is one of the most prevalent chronic diseases worldwide, and increasing in figures with every passing day. The most common reasons behind its prevalence are a changed lifestyle, reduced physical activity, and obesity [1]. Among all types of diabetes type II diabetes is escalating

at an alarming rate and it is one of the major threats to human health in the contemporary era, in both developed and developing nations [2]. DM affects almost 1–2% of the population around the globe [3]. World Health Organization (WHO) survey said that developing countries will have to tolerate the impact of disease soon. Presently, in low- and middle-revenue countries more than 70% of the population suffers from diabetes. Approximately 285 million people which is equivalent to 6.4% of the total world's adult people were diagnosed with diabetes in 2010. This figure will be augmented to 438 million by 2030, which is equivalent to 7.8% of the total world's adult people [4]. World Health Organization (WHO) predicted that in Pakistan frequency of Type 2 diabetes was 5.2 million in 2000 and it would be raised to 13.8 million by 2030 [5] Diabetes mellitus is a disease that is caused by hyperglycemia and hyperglycemia results from the complete absence of insulin or lack of insulin and insulin resistance [3].

Conventionally, diabetes has been divided into micro and macrovascular complications. Among both complications, macrovascular complications are well researched and studied but microvascular complications are unique to diabetes. Hyperglycemia is a key contributor to the progress of microvascular complications. So, most diabetic complications are caused by hyperglycemia [6].

Non-enzymatic glycation or Maillard reaction is a process in which an aldehydic group of reducing sugars like glucose [7] reacts spontaneously with free amino groups of biological molecules such as proteins, lipids, and nucleic acids [8]. These reactions take place through various phases that start from glucosylamines (Schiff bases), fructosamines (Amadori compounds), and aminoaldoses (Heyns compounds) and finally convert into irreversible end products, which comprise crosslinks, aromatic heterocycles, and oxidized compounds, that are described together as advanced glycation end products (AGEs). AGEs are a heterogeneous group of molecules [9]. The concentrations of AGEs are raised in diabetes, and it has been verified from intense research work that the production of AGEs and non-enzymatic glycation may lead to diabetic complications. Pathology of diabetic complications shows that the main cause of developing diabetic complications is nonenzymatic glycation. [9]. This process was first time recognized in 1912 and is described as the Maillard or “browning” reaction because this reaction results in the production of yellow-brown color change [10] as described by Louis Camille Maillard [11]. Protein glycation by free glucose and their further changes, jointly named as Maillard reaction, produce a heterogeneous array of advanced glycation end products, characterized by alkylated amino acids, fluorescence residues, and a variety of intra- and intermolecular cross-linkages [12]. It is a spontaneous process and depends on the degree and duration of hyperglycemia, the half-life of the protein, and the permeability of the tissue to free glucose [3].

AGEs are principally significant, they are formed both intra- and extracellularly and can be harmful, independent of hyperglycemia. They are associated with the development of macrovascular disease, nephropathy, neuropathy, retinopathy [10], and impaired wound healing [3]. Numerous compounds, e.g., *N*-carboxymethyl-lysine, pentosidine, or methylglyoxal derivatives, serve as examples of well-characterized and broadly studied AGEs. A series of subsequent reactions, including successions of dehydrations, oxidation-reduction reactions, and other arrangements lead to the production of AGEs. An important characteristic of certain reactive/precursor AGEs is their ability for covalent crosslink formation among proteins, which varies their structure and function, as in cellular matrix, basement membranes, and vessel-wall components. AGEs buildup in collagen, a long-lived structural protein in the extracellular matrix region of the kidney, is supposed to affect alterations in elasticity, ionic charge, thickness, and turnover of basement membrane components [13]. Other main features of AGEs that narrate their interaction with a variety of cell-surface AGE-binding receptors, leading either to their endocytosis and degradation or to cellular activation and pro-oxidant, pro-inflammatory events. A large number of indications suggest that AGEs are important pathogenetic mediators of almost all diabetes complications that are grouped into micro- or macroangiopathies. AGEs also form in a normal body at a constant but slow rate, their formation starts in early embryonic development and accumulates with time. However, their development is significantly enhanced in diabetes because of the increased availability of glucose [6]. Nature has planned several humoral and cellular defense mechanisms to guard tissues from the harmful effects of carbonyl stress and the accumulation of AGE.

These comprise the glyoxylase system (I and II) and aldose reductase that catalyze the deglycation of methylglyoxal (MG), the most common reactive intermediates of AGE to D-lactate. Moreover, a novel class of enzymes found in *Aspergillus* named Amadoriases catalyze the deglycation of Amadori products. Most recently, human fructosamine-3-kinase (FN3K) has been recognized which phosphorylates fructose-lysine (F1) residues on glycated proteins to F1-3-phosphate and leading to its spontaneous breakdown, thereby reversing the non-enzymatic glycation process at an early stage [13].

Several natural and synthetic/chemical compounds have been discovered and suggested, and are now presently being used as AGE inhibitors. Some antioxidant compounds like vitamin C, and vitamin E decrease the protein glycation both in vitro and in vivo while some other antioxidant compounds, including 5-aminosalicylic acid (5-ASA), N-acetylcysteine, lipoic acid, lipoic acid amide, taurine, para-aminobenzoic acid (PABA), para-amino salicylic acid, aspirin, benzoic acid, salicylic acid, inositol, and probucol, are also known as the inhibitor of AGEs. Salicylic acid, PABA, and benzoic acid were found to have moderate AGE-inhibitory effects while inositol and probucol were strong AGE-inhibitors. Historically, high-dose salicylate treatment was found to reduce glycosuria in diabetic patients. The first compound that has been comprehensively studied in vitro and in vivo to be a powerful inhibitor of AGE formation is aminoguanidine. Thiamine pyrophosphate and pyridoxamine were shown to be effective inhibitors of AGE formation of the post-Amadori type. Other compounds such as acetylsalicylic acid, ibuprofen, indomethacin, and Benfotiamine were also reported to be inhibitors of glycation [13]. In the present study, the inhibitory effect of chemical/synthetic inhibitor 5-sulfosalicylic Acid against glycation level and glycation end products was studied *in vitro* conditions.

Experimental Method

Non-enzymatic glycation and advanced glycation end products (AGEs) inhibition was studied by chemical/synthetic inhibitor Sulfosalicylic acid (SSA).

Selection of conditions and concentrations:

For this study, Bovine Serum Albumin (BSA) was used as a protein. The concentration of BSA protein was 20mg/ml used in this experimental study. Four concentrations of glucose (G_1=500 mM, G_2=250 mM, G_3=50 mM, and G_4=5.5 mM) and three different concentrations of inhibitor Sulfosalicylic acid (I_1= 10 mM, I_2= 5 mM, and I_3 = 1 mM) were also used is in this study.

Selection of Combinations:

To study the inhibitory effects of Sulfosalicylic acid (SSA) on glycation of bovine serum albumin protein with glucose (*invitro*) sixteen combinations (Table 1) were made and all were placed at 37^{o}C and 50^{o}C simultaneously for five weeks. Samples were drawn after the 1st, 3^{rd,} and 5th week of incubation to perform the experiments for glycation, glycation inhibition, and advanced glycation end products (AGEs) estimation. Combinations were stored at -20^{o}C until used.

Table: 1 Different combination for glycation inhibition

S. No	Combinations
1.	P_{20} G_1 B
2.	P_{20} I_1 G_1
3.	P_{20} I_2 G_1
4.	P_{20} I_3 G_1
5.	P_{20} G_2 B
6.	P_{20} I_1 G_2
7.	P_{20} I_2 G_2
8.	P_{20} I_3 G_2
9.	P_{20} G_3 B

10.	P_{20} I_1 G_3
11.	P_{20} I_2 G_3
12.	P_{20} I_3 G_3
13.	P_{20} G_4 B
14.	P_{20} I_1 G_4
15.	P_{20} I_2 G_4
16.	P_{20} I_3 G_4

In-vitro Glycation of BSA (Preparation of BSA- AGEs)

BSA was incubated with all glucose and inhibitor concentrations in a buffer at 37^{o}C and 50 for 1-5 weeks at the same time for the detection of glycation and AGEs inhibition. Phosphate buffer saline (containing 1**%** Sodium Azide: 0.075M, pH: 7.4) was used in this study.

Protein samples were dialyzed to remove free glucose that was present in samples after incubation because free glucose is the main interruption in the determination of the level of glycation and advanced glycation end products. Glycated BSA was dialyzed against distilled water for twenty-four hours continuously at room temperature with constant stirring. After dialysis, samples were again placed at -20^{o}C until these were used. Glucose was estimated before and after dialysis by commercially available kit method to confirm whether conc. of glucose is reduced or not. Total proteins were estimated by the Biuret method before and after dialysis before carrying out glycation assays/experiments [14].

Two methods Thiobarbituric Acid (Colometric Technique) and Periodate Borohydride were used for measurement of glycation level. Glycation level was determined in mole/mole of glucose/protein. All samples in these experiments were taken in triplicates.

Thiobarbituric Acid (TBA) Colorimetric Technique

TBA method was used for the estimation of both enzymatic and non-enzymatic glycation [15]. The thiobarbituric acid assay is based on the reaction between fructose, amino acids, and weak acid that produces 5- a hydroxymethyl furfural (HMF) compound. Non-enzymatic glycation was measured as given below.

Non-Enzymatic Glycation = (Total Glycation + Enzymatic Glycation) – Enzymatic Glycation

Periodate Borohydride Assay

This method is based on the production of formaldehyde by the reaction that is periodate oxidation of cis-diol, aminol, ketol or ketoamine structures [16,17]. One molecule of hexose sugar produces two moles of formaldehyde. The quantity of formaldehyde formed was measured as the fluorescent adduct that is produced by the condensation of formaldehyde with ammonia and acetylacetone. In this method, fructose was used as standard, because fructose is the analogue of the ketoamine form of the glycogroups.

Advanced Glycation End Products Detection

Advanced glycation end products were measured by estimation of browning production at 370 nm through a spectrophotometer. Blank samples were used to make the reading zero.

Enzyme Linked Immunosorbent Assay (ELISA)

Advanced glycation end products (AGEs) were determined by using an enzyme-linked immunosorbent assay. ELISA was performed by using enzyme alkaline phosphatase and para nitrophenyl phosphate was used as a substrate. Anti-AGEs immunoglobulin was commercially purchased from Sigma. Bovine serum albumin was used for the preparation of AGE-BSA [18] with minor modifications in the original method according to our laboratory situations. Absorbance was taken by using a microtiter ELISA plate reader at 405 nm.

Results and Discussion

Estimation of glycation level

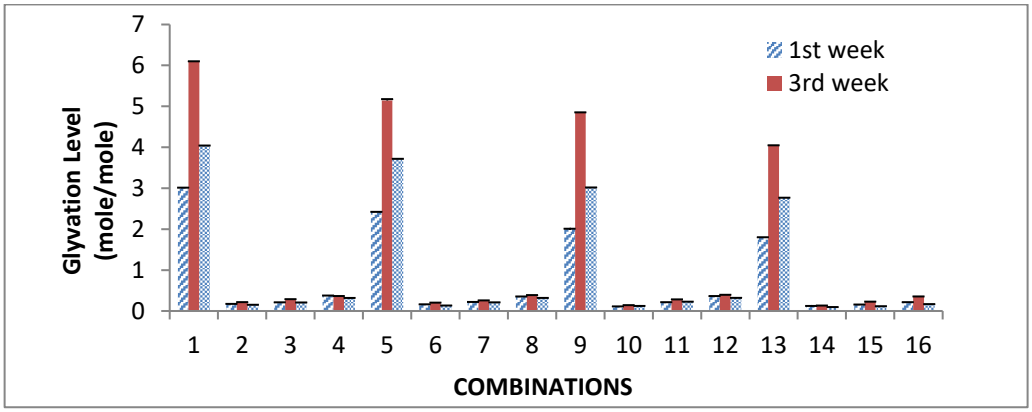

Figure 1: Effect of sulfosalicylic acid on glycation level with TBA at 37 ºC

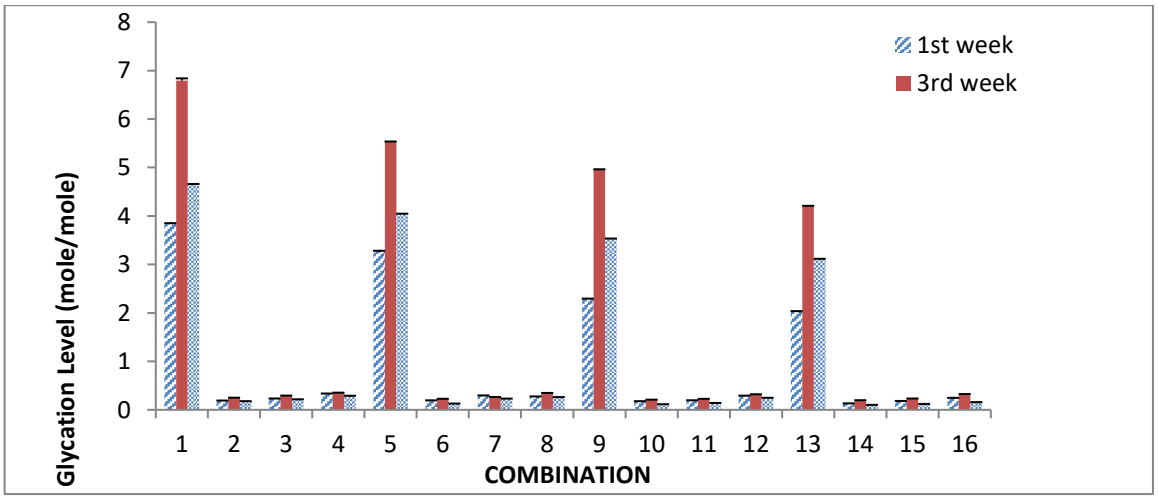

Figure 2: Effect of sulfosalicylic acid on glycation level with TBA at 50 ºC

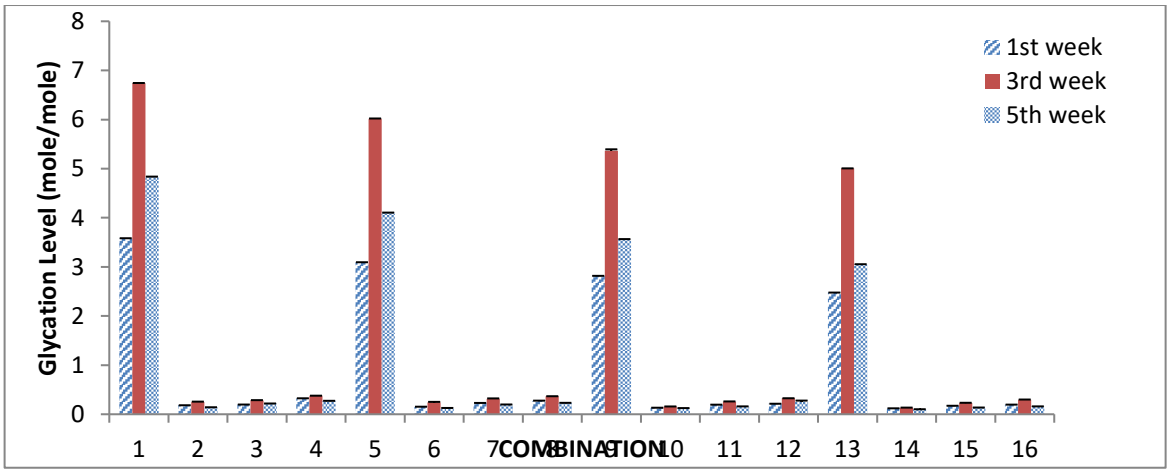

Figure 3: Effect of sulfosalicylic acid on glycation level with Periodate borohydride at 37 ºC

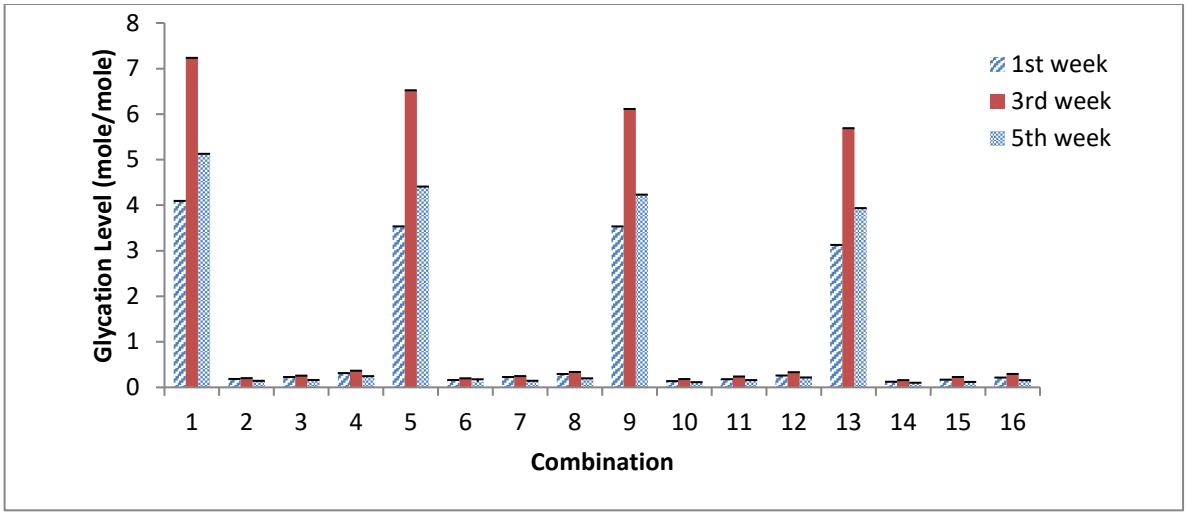

Figure 4: Effect of sulfosalicylic acid on glycation level with Periodate borohydride at 50 ºC

Effect of sulfosalicylic acid on glycation level with TBA

Results accomplished by using sulfosalicylic acid as glycation inhibitor by TBA revealed that 500 (G_1) concentration of glucose exhibited maximum glycation level with BSA in all weeks at both temperatures 37ºC and 50ºC (Fig.1 and Fig. 2). 5.5mM (G_4) concentration of glucose indicated least glycation level with BSA in all weeks at both temperature 37ºC and 50ºC (Fig.1 and Fig. 2). Our finding suggested that glycation level increases from 1st week and it became maximum in 3rd week of incubation after 3rd week of incubation glycation level decreases because after 3rd-week glycation reaction products converted into AGEs. Thus, results revealed that all glucose concentrations demonstrated maximum glycation level with BSA in 3rd week as compared to 1st and 5th week at both temperatures 37ºC and 50 (Fig.1 and Fig. 2). By TBA method maximum glycation level (6.098 mole/mole) was measured by 500mM (G_1) concentration of glucose after 3rd week of incubation that is reduced to (1.801mole/mole) by 5.5mM (G_4) concentration of glucose after 1st week of incubation at 37ºC (Fig. 1). When TBA method was used to measured glycation level at 50ºC, maximum glycation level (6.796 mole/mole) was observed by 500mM (G_1) concentration of glucose after 3rd week of incubation that is decreased to (2.034 mole/mole) by 5.5mM (G_4) concentration of glucose after 1st week of incubation. All glucose concentrations with BSA showed a dominant glycation level at 50ºC as compared to 37ºC (Fig.1 and Fig. 2) as determined by the TBA method. Maximum glycation inhibition was seen by I_1 (10mM) concentration of sulfosalicylic acid at both temperatures 37ºC and 50ºC (Fig.1 and Fig. 2). Above results demonstrated that I_1 (10mM) concentration of sulfosalicylic acid showed a dominant response against glycation level as compared to other two inhibitor concentration.

Effect of sulfosalicylic acid on glycation level with Periodate borohydride

Results obtained by using periodate borohydride assay showed that 500mM (G_1) concentration of glucose displayed maximum glycation level with BSA in all weeks at both temperature 37ºC and 50ºC (Fig.3 and Fig.4) while 5.5mM (G_4) concentration of glucose showed minimum glycation level with BSA in all weeks at both temperature 37ºC and 50ºC (Fig.3 and Fig. 4). Our results also revealed that all glucose concentration demonstrated maximum glycation level with BSA at 3rd week as compared to 1st and 5th week of incubation at both temperatures 37ºC and 50ºC (Fig.3 and Fig.4). By periodate borohydride method maximum glycation level (6.741 mole/mole) was measured by 500mM (G_1) concentration of glucose after 3rd week of incubation while lowest glycation level (2.475mole/mole) was monitored by 5.5mM (G_4) concentration of glucose after 1st week of incubation at 37ºC. While at 50ºC, the maximum glycation level (7.233mole/mole) was observed by 500mM (G_1) concentration of glucose after 3rd week of incubation (Fig. 4). The Minimum glycation level (3.126mole/mole) was observed at 5.5mM (G_4) concentration of glucose after 1st week of incubation (Fig. 4). As measured by periodate borohydride assay, all glucose concentrations with BSA showed highest glycation level

at 50ºC as compared to 37ºC (Fig.3 and Fig.4). Maximum glycation inhibition was given by I_1 (10mM) concentration of sulfosalicylic acid at both temperature 37ºC and 50ºC (Fig.3 and Fig.4). Above results revealed that I_1 (10mM) concentration of sulfosalicylic acid showed more activity against glycation level as compared to I_1 and I_2 concentration of inhibitor.

Our results indicated that both the glycation assays, TBA and periodate borohydride showed a similar trend of glycation measurement. However, periodate borohydride proved to be a more suitable, reliable, and effective method for the measurement of glycation level as compared to TBA. The above results also showed that a 10mM (I_1) concentration of sulfosalicylic acid showed that it was most active for inhibition of glycation at both temperatures as compared to I_1 and I_2 concentration of inhibitor. Our results are supported by Huby and Harding [19] who discovered that aspirin inhibits the reaction with galactose in a dose-related manner. Pre-incubation of crystallins with aspirin, before incubation with galactose in the absence of aspirin, showed that aspirin modified the crystallins permanently. Yue *et al.* [20] also observed the same type of inhibition by aspirin while Rao *et al.* [21] proposed that aspirin acted by acetylating the glycation sites. Our results are supported by Duraisamy, *et al*. [22] who investigated that aminosalicylic acid and aminoguanidine reduce the antiproliferative effect of hyperglycemia (high glucose) and advanced glycation endproducts(BSA-AGE). They used different glucose and inhibitor concentrations. In their study aminosalicylic acid at a concentration of 200µmole/L proved to be more effective than equimolar concentrations of aminoguanidine in protecting endothelial cells against the antiproliferative effects of both high (30 mmol/L glucose) and (50 mmol/L) BSA-AGE. As described by Rahbar and Figarola, [23] nearly a century ago, high-dose salicylate treatment was found to reduce glycosuria in diabetic patients. They also described the AGE inhibitory activities of antioxidant compounds, including 5-aminosalicylic acid (5-ASA) para-aminosalicylic acid, aspirin, and salicylic acid which was confirmed by several in vitro assay methods. Ahmad, [3] in review also described that aspirin has been shown to reduce glycation in vitro, and in animal experiments, probably by acetylation of amino groups. So, it may protect against glycation. However, other analgesics such as paracetamol, and ibuprofen also protect against glycation but cannot acetylate proteins.

Our results accomplished by using sulfosalicylic acid as glycation inhibitor revealed that 500mM (G_1) concentration of glucose exhibited maximum glycation level with BSA in all weeks at both temperatures 37ºC and 50ºC (Fig.1 and Fig. 2). 5.5mM (G_4) concentration of glucose indicated least glycation level with BSA in all weeks at both temperature 37ºC and 50ºC (Fig.1 and Fig. 2). According to Hatton *et al*. [24], the non-enzymatic glycation occurs when protein is present in the solution of sugar. The product of the reaction is a covalently linked glycated protein. As the above description shows the glycation level was elevated in 1st week and it was becoming maximum in 3rd week but the glycation level decreased in 5th week because after 3rd week of incubation glycation or Maillard reaction products (early and intermediate products) were converted into Advance glycation end-products (AGEs). It was clear from the above results that all glucose concentrations demonstrated maximum glycation levels with BSA in 3rd week as compared to 1st and 5th week at both temperatures 37ºC and 50 (Fig.1 and Fig. 2). All glucose concentrations with BSA showed the highest glycation level at 50ºC as compared to 37ºC (Fig.3 and Fig.4). Different variable's effect on glycation i.e. temperature, time, pH, glucose and protein concentration, etc was also studied. We also observed that the glycation level was increased with the increase in glucose concentrations. Similar results are also observed by Winocour *et al*. [25]. Our results also follow Eble *et al.* [26] and Brownlee *et al.* [27] who found that glucose concentration and incubation time are the most clinically relevant variables affecting the extent of glycation. Our results also relate with the results of Vinson and Howard, [28] who stated that glucose and other reducing sugars with protein reversibly produce Amadori products and over a long period irreversible advanced glycation end products by using bovine albumin as the model protein. They used a mixture of 25 mM glucose/fructose as the glycating agent. The Amadori product was quantitated by thiobarbituric acid colorimetry after hydrolysis. They measured advanced glycation end products by their intrinsic fluorescence. They studied the effect of several inhibitors on glycation.

Estimation of Advance Glycation End Products

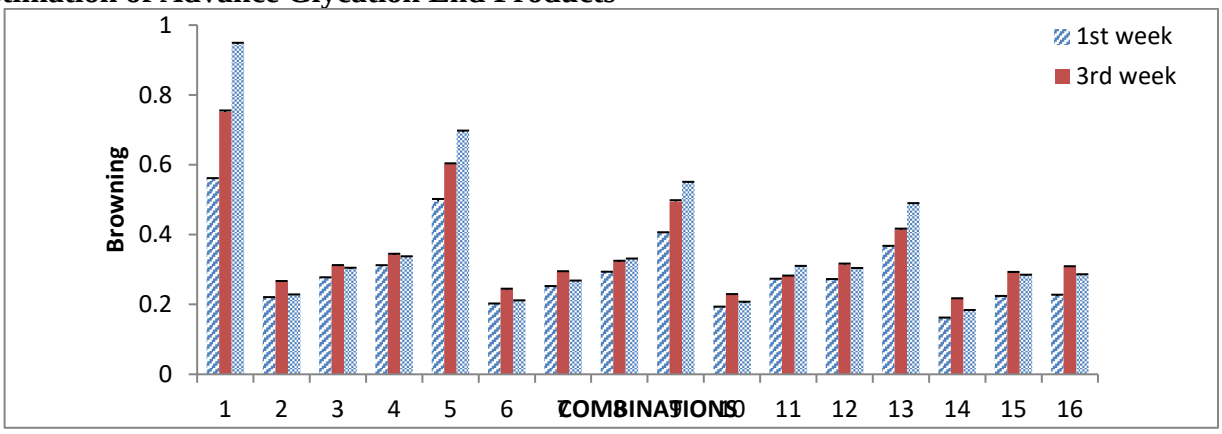

Figure 5: Effect of sulfosalicylic acid on browning/Fluorescence at 37 ºC

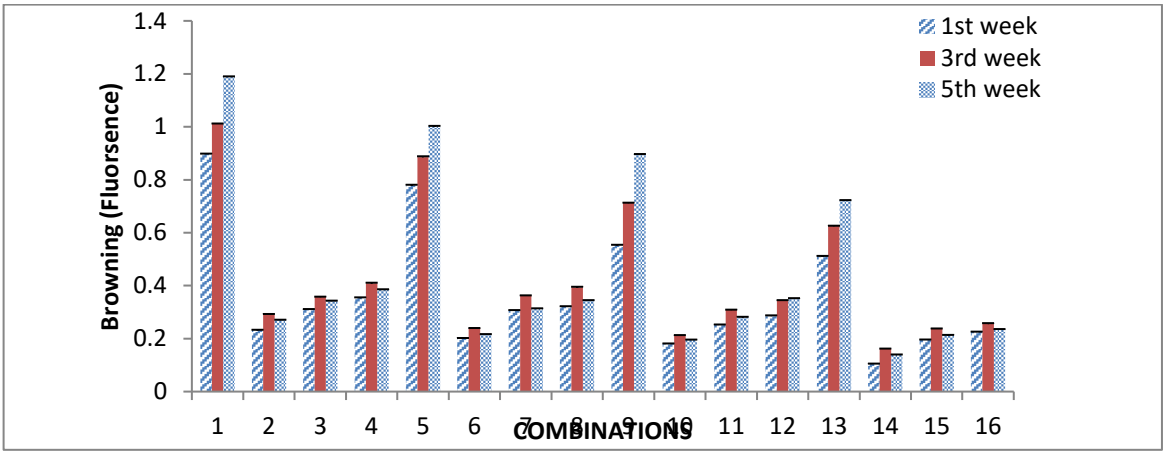

Figure 6: Effect of sulfosalicylic acid on Browning/Fluorescence at 50º

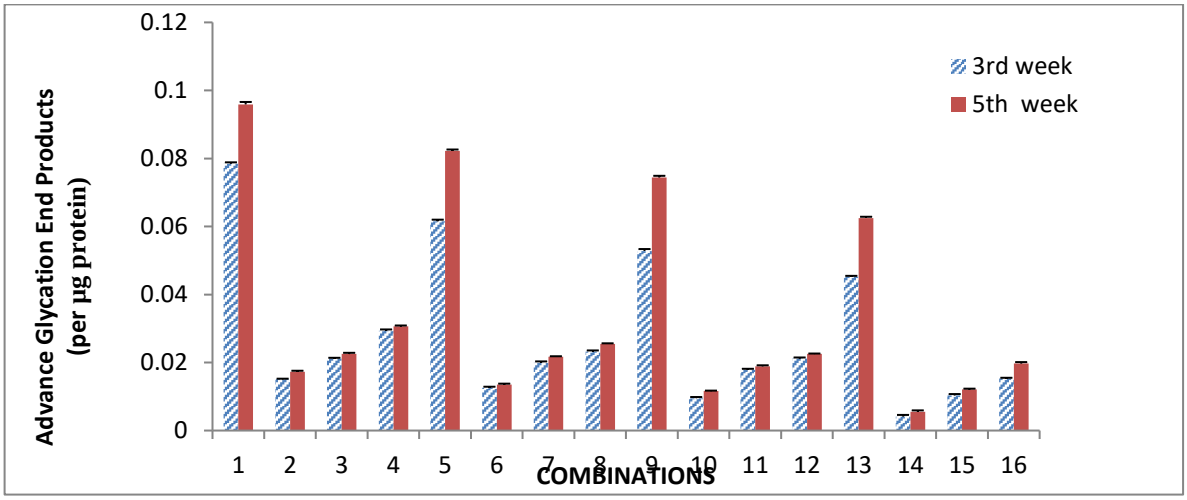

Figure 7: Effect of sulfosalicylic acid on AGEs level at 37 ºC measured by ELISA

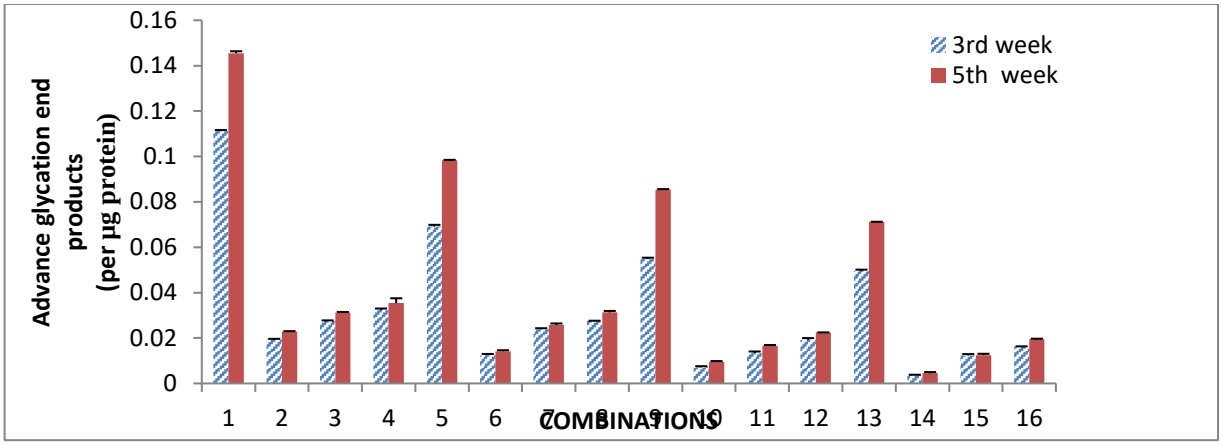

Figure 8: Effect of sulfosalicylic acid on AGEs level at different weeks at 50 ºC measured by ELISA

Effect of sulfosalicylic acid on Browning/Fluorescence

AGEs are complex, heterogeneous molecules that cause protein cross-linking, exhibit browning and generate fluorescence. AGEs are diverse molecules either fluorescent or non-fluorescent compounds. Browning shows the cross-linking of glucose and protein. In disease conditions e.g. diabetes crosslinking of glucose increases due to persistent hyperglycemia, which in turn increases browning /fluorescence. Results showed that browning/fluorescence was significantly increased from 1st to 5th week of incubation. Browning/fluorescence becomes maximum after the 5th week of incubation. 500mM (G_1) concentration of glucose showed maximum browning/fluorescence with BSA in all weeks at both temperatures 37^{o}C and 50^{o}C (Fig.5 and Fig.6) While 5.5mM (G_4) concentration of glucose showed minimum browning/fluorescence with BSA in all weeks at both temperature 37^{o}C and 50^{o}C (Fig.5 and Fig.6) as compared to other glucose concentrations. 250mM (G_2) concentration of glucose showed variable response and 50mM (G_3) concentration of glucose showed lesser browning/fluorescence as compared to G_1 and G_2 at both temperatures 37^{o}C and 50 (Fig.5 and Fig.6). All concentrations of sulfosalicylic acid produced good response against AGEs inhibition but 10mM (I_1) inhibitor concentration gives maximum inhibition of AGEs or minimum browning/fluorescence with all glucose concentration at both temperature 37^{o}C and 50^{o}C (Fig.5 and Fig.6) as compared to 5mM (1_2) and 1mM (I_3) concentration of sulfosalicylic acid. Our results indicated that 10mM (I_1) concentration sulfosalicylic acid has more ability to inhibit AGEs production at both temperatures. 5mM (1_2) and 1mM (I_3) concentrations of sulfosalicylic acid were determined less effective on AGEs inhibition as compared to 10mM concentration of sulfosalicylic acid. Our results also indicated that the temperature affects browning/fluorescence production. Browning/fluorescence increases with the increase in temperature. Higher temperatures produce changes in protein structure that facilitate the advanced glycation end-product formation. According to our results, all glucose concentrations with bovine serum albumin showed more browning/fluorescence at 50^{o}C as compared to 37^{o}C. The above results showed that browning more increased at 50^{o}C than at 37^{o}C because browning increased more at high temperatures.

Effect of sulfosalicylic acid on AGEs level measured by ELISA

Glycation level was less in 1st week and it was going on increasing from 3rd to 5th week. The glycation level becomes maximum in 3rd week and it decreases in 5th week because after 3rd week early and intermediate glycation products are converted into advanced glycation end products (AGEs). Thus, it indicates that AGEs formation started after 1st week and it went on increasing from 3rd week and became maximum in 5th week when Maillard reaction products converted into AGEs. In the present study, ELISA method was used to measure AGEs in the 3rd and 5th week of incubation. It is a sensitive and reliable method for the efficient quantification of advanced glycation end products (AGEs). According to our finding, it was indicated that 500mM (G_1) concentration of glucose showed maximum AGEs production after 3rd and 5th week of incubation with values 0.0785 and 0.0959/µg of

BSA respectively While 5.5mM (G_4) concentration of glucose showed minimum AGEs production after 3rd and 5th week of incubation with values 0.0452 and 0.0625/µg of BSA respectively at 37°C (Fig7). At 50°C, 500mM (G_1) concentration of glucose showed maximum AGEs production after 3rd and 5th week of incubation with values 0.1112/µg and 0.1455 of BSA respectively while 5.5mM (G_4) concentration of glucose showed minimum AGEs production after 3rd and 5th week of incubation with values 0.0495 and 0.0711/µg of BSA respectively (Fig.8). When AGEs inhibition was monitored by sulfosalicylic acid at 37°C and 50°C, maximum AGEs inhibition was given by 10mM (I_1) concentration of inhibitor with all glucose concentration at both temperature 37°C and 50°C (Fig.7 and Fig.8) as compared to 5mM (1_2) and 1mM (I_3) concentration of sulfosalicylic acid. Thus, 10mM (I_1) concentration of sulfosalicylic acid was more effective than 5mM (1_2) and 1mM (I_3) concentration of sulfosalicylic acid (Fig.7 and Fig.8).

Elisa is a more reliable method for the measurement of AGEs as compared to browning estimation because in ELISA specific antibodies are used for the measurement of AGEs. The above description showed that the Glycation level was decreased by inhibitor action. Therefore, sulfosalicylic acid inhibits the fluorescence in *in-vitro* conditions. The same type of inhibition by aspirin with serum/plasma proteins and collagen was observed by Kennedy *et al.* [29]. Rendell *et al.* [30] stated that Aspirin (acetylsalicylic acid or ASA) is known to inhibit the glycosylation (glycation) of albumin *in vitro*. They conclude that ASA inhibits glycation by a very rapid acetylation process, but the dose requirement was considerably higher and Swamy and Abraham [31] also reported the decreased glycation and high molecular weight aggregate formation (HMWA) by aspirin *invitro* and in-vivo glycation of lens crystalline. Similarly, when AGE inhibition was studied by Rahbar and Figarola, [23], they found that 0.1, 1, and 10mM concentrations of aminosalicylic acid can cleave AGE-BSA collagen cross-links but 10mM concentration is better than others. Their finding is comparable to our results. Our results are also supported by Stoynev *et al.* [32]. Their results indicate that glycation can be inhibited by acetylsalicylic acid (aspirin), thiamine, and pyridoxine. Zhang *et al*. [33] also studied and compared the protective effects of three different anti-glycation compounds, aspirin, D-penicillamine, and vitamin E, against high glucose and advanced glycation end product (AGE) mediated toxicity in cultured bovine aortic endothelial cells. Their proliferation was assessed in culture in different concentrations of glucose (5.5-100 mmol/l) with and without these inhibitors. All three compounds protect against the anti-proliferative effects of high glucose, with vitamin E being the most effective. Our finding also suggests that at higher temperatures AGEs production increases (Fig.7 and Fig.8). According to our results all glucose concentrations with bovine serum albumin showed more advanced glycation end products (AGEs) production at 50°C as compared to 37°C (Fig.7 and Fig.8). According to our browning intensity was significantly higher at 5th week of incubation. Similarly, an increasing trend in the level of glycation with increasing protein concentration was observed by Gillery [34] that non-enzymatic glycation, i.e. binding of monosaccharide to amino groups of proteins, gives rise to complex components called "advance glycation end-products" (AGEs), which alter protein structure and function. Similar results also were determined by Day *et al.* [35,36] but they used different conditions. These were determined as the site of glycation of albumin; they observed that the addition of aspirin to the incubation mixture (serum + glucose) resulted in a 50% inhibition of the rate of incorporation of labeled glucose, although the concentration of aspirin was one-tenth of glucose.

Conclusion

According to our results, it was concluded that sulfosalicylic acid can inhibit glycation and AGEs production. Hence, a higher concentration (10mM) of sulfosalicylic acid is a more effective inhibitor against glycation as compared to its lower concentrations. Thus, sulfosalicyclic acid inhibited glycation (BSA-AGEs) in a dose-dependent manner. 500 mM concentration of glucose produces maximum glycation at both temperatures compared to its lower concentrations. All glucose concentrations with BSA showed a dominant glycation level at 50°C compared to 37°C, indicating that glycation and AGEs formation increase with the temperature increase. Periodate borohydride

proved to be a more suitable and effective method for the measurement of glycation as compared to TBA. Elisa is a profound technique for measuring AGEs.

Conflict of Interest; Authors have no conflict of interest.

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