ANNUAL REPORT OF WORK DONE (2018-2019) ON THE PROJECT

1.	Project Report No.	RSSDI/HQ/GRANTS/2017/42
2.	Period of Report	June 2018 to June 2019 (First Year)
3.	Title of the Research Project	“Study on the glycation of protein by sweeteners"
4.	Name of the Principal Investigator	Dr. Ahmad Ali
	Departmental Address	Department of Life Sciences University of Mumbai Vidyanagari, Santacruz (E) Mumbai – 400 098.
5.	Name of the Co-Investigator	Dr. (Mrs.) S. Sivakami
	Departmental Address	Department of Biophysics University of Mumbai Vidyanagari, Santacruz (E) Mumbai – 400 098.
6.	Report of the work done	Enclosed

Study on the glycation of protein by sweeteners

(Report of work done during the first year)

INTRODUCTION

Diabetes and related disorders are imparting huge socio-economic health burden worldwide. All types of diabetes have common features like hyperglycemia and glucotoxicity. In hyperglycaemia, glucose interferes with the functions of proteins by forming adducts through the non-enzymatic process called glycation. The amino group of proteins, DNA and lipids interacts with carbonyl group of reducing sugars which forms Schiff's base, Amadori products in early stage of glycation. It is an important mechanism in physiological aging and the pathogenesis of diabetic complications and neurodegenerative disorders. It is often regarded by scientists as one of the unavoidable and deleterious modifications. Glycation leads to generation of early and advanced end products. These products have been implicated in altering the structure of biological macromolecules. The toxicity of glycation is due to its ability to inhibit specific functions of proteins through cross linkage, aggregation and precipitation as well as to produce reactive radicals, leading to damage to nucleic acids (Ali and Sharma, 2015).

The patients suffering from diabetes are advised to take sugar free diet as a part of diabetes management. For this and the other health benefits sweeteners are being preferred in the food and pharmaceutical industries. Some of the very commonly used sweeteners (natural and artificial) are aspartame (Asp), acesulfame potassium (Ace-K) saccharin (Sac), neotame (Neo), stevia, sucralose (Sucl), etc. which are approved by Food and Drug Administration (FDA). Glycation research till date has been carried out with sugars, sugar phosphates and other dicarbonyl compounds. However, there are a very few reports on the involvement of sweeteners in the process of glycation. The current study is based on the fact that artificial sweeteners mentioned above contain carbonyl groups which can react with amino groups of macromolecules inducing damage by glycation and glycoxidation. In the present study, artificial sweeteners were used to assess their glycating properties by using established methods like browning, fructosamine and carbonyl assays.

METHODOLOGY

MATERIALS

The following chemicals were purchased as indicated: Glucose from MERCK chemicals. BSA from Sigma chemicals; Sodium azide, Nitrobluetetrazolium chloride (NBT), 2,4-dinitrophenylhydrazine (DNPH), Guanidine hydrochloride and Trichloroacetic acid (TCA)

from Himedia chemicals; Acesulfame-K, Saccharine and Sucralose from NeelChem Mumbai. All other chemicals were used of analytical high grade.

METHODS

In vitro glycation of artificial sweeteners

Bovine serum albumin (BSA) (10 mg/mL) was modified *in vitro* by glucose (100 mg/mL) and acesulfame-K/saccharine/sucralose (100 mg/mL) at 37 °C for 28 days. All the incubations were carried out in 0.1 M phosphate buffer (pH 7.4) and 3 mM sodium azide to prevent bacterial contamination.

Measurement of browning

Browning was measured at 420 nm by using Shimadzu UV-Vis 1800 Spectrophotometer (Rondeau *et al*., 2007).

Measurement of Fructosamine

The concentration of Fructosamine, an Amadori product, was measured by Nitro blue tetrazolium (NBT) assay (Ali *et al*., 2013) with slight modifications. 10.0 µL of the glycated sample was incubated with 100.0 µL of 0.5 mmol/L NBT in 0.1 mol/L carbonate buffer (pH 10.4) at 37 °C for 15 minutes. The absorbance was measured at 530 nm using a Shimadzu UV 1800 spectrophotometer.

Determination of carbonyl content

The conventional 2, 4-dinitrophenylhydrazine (DNPH) method for carbonyl group determination in glycated sample (Meeprom *et al*., 2013). In this method, 400.0 µL of DNPH (10 mM) was added to 100.0 µL of glycated samples and incubated in dark for an hour. Then, 500.0 µL of 20% (w/v) Trichloroacetic acid (TCA) was added and kept on ice for 5 min for proteins precipitation. Centrifugation at 10,000 rpm for 10 min at 4°C was carried out and followed with pellet washing with 500.0 µL of ethanol/ethyl acetate (1:1) mixture three times. Lastly, the pellet was resuspended in 100.0 µL of 6 M guanidine hydrochloride and volume made sufficient with D/W for Spectroscopic measurement at 370 nm.

Determination of Aggregation Index

For aggregation index, the absorbance of incubated samples was taken at 280 nm and 340 nm by using a Shimadzu UV 1800 spectrophotometer. The mathematical formula was applied for determining the aggregation index.

Aggregation index = $(A_{340}/ (A_{280} – A_{340})) * 100$

Determination of amyloid β-structures by Congo red Assay

The quantitative analysis of amyloid β-structures was done by Congored method with slight modifications. 50.0 µL of the glycated sample was incubated with 50.0 µL of 100 µM Congo

red dye at 25 °C for 20 minutes. The volume of the reaction mixture was made up to 1.0 mL with distilled water (D/W) and optical density was measured at 530 nm using a Shimadzu UV 1800 spectrophotometer.

RESULTS

Browning

The browning was observed and it was found that Ace-K, Sac and Sucl quantitatively reduced the browning of BSA by 58.43%, 71.91% and 60.83% respectively after 28 days incubation at 37˚C. Ace-K has indicated results on same reduction pattern in BSA-MG glycation system. In both MG concentrations (1mM and 5 mM), Ace-K significantly reduced the browning.

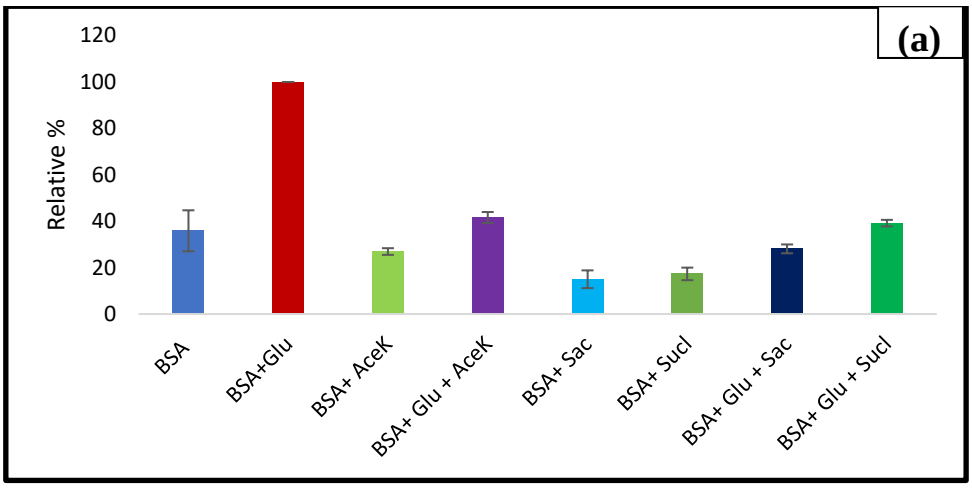

Figure 1a: Browning of BSA incubated with glucose and artificial sweetener. (BSA- bovine serum albumin, Glu- glucose, Ace-K- acesulfame-K, Sac- saccharine and Sucl- sucralose). Results are expressed as means ± SE (*n* =4) and p-value < 0.05.

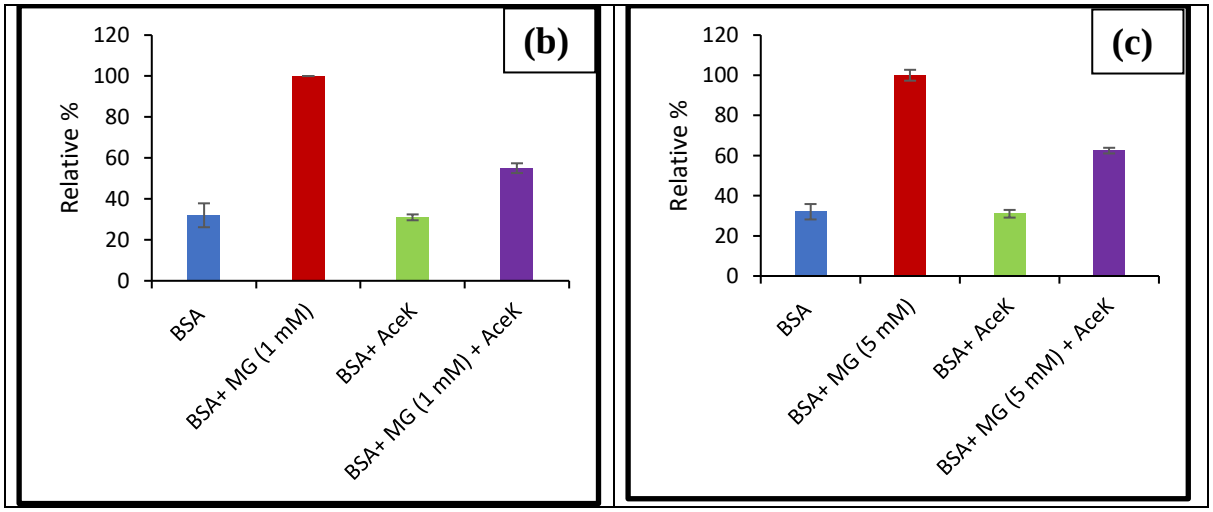

Figure 1(b & c): Browning of BSA incubated with MG and Ace-K. (BSA- bovine serum albumin, MG- methyl glyoxal, Ace-K- acesulfame-K). Results are expressed as means ± SE (*n* =2) and p-value < 0.05.

Early Glycation Products Measurement

After 28 days it was found that Ace-K, Sac and Sucl suppressed the formation of fructosamine as well as Amadori Product to 19.31, 24.16 and 27.13 µmol/mg protein in comparison of glycated BSA (28.71 µmol/mg). In MG glycation system, Ace-K reduced the formation of early glycation products to 2.25 and 13.37 µmol/mg protein as compared to 3.32 and 15.86 µmol/mg protein in MG glycated BSA at both concentration (1 mM and 5 mM) respectively. It showed that Ace-K has ability to hinder the glycation process markedly.

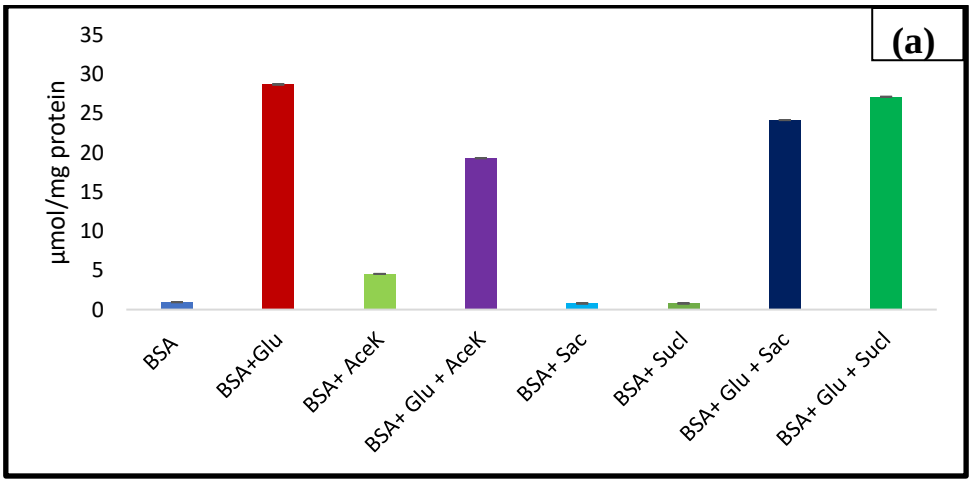

Figure 2a: Fructosamine Content measurement. (BSA- bovine serum albumin, Glu- glucose, Ace-K- acesulfame-K, Sac- saccharine and Sucl- sucralose). Results are expressed as means ± SE (*n* =4) and p-value < 0.05.

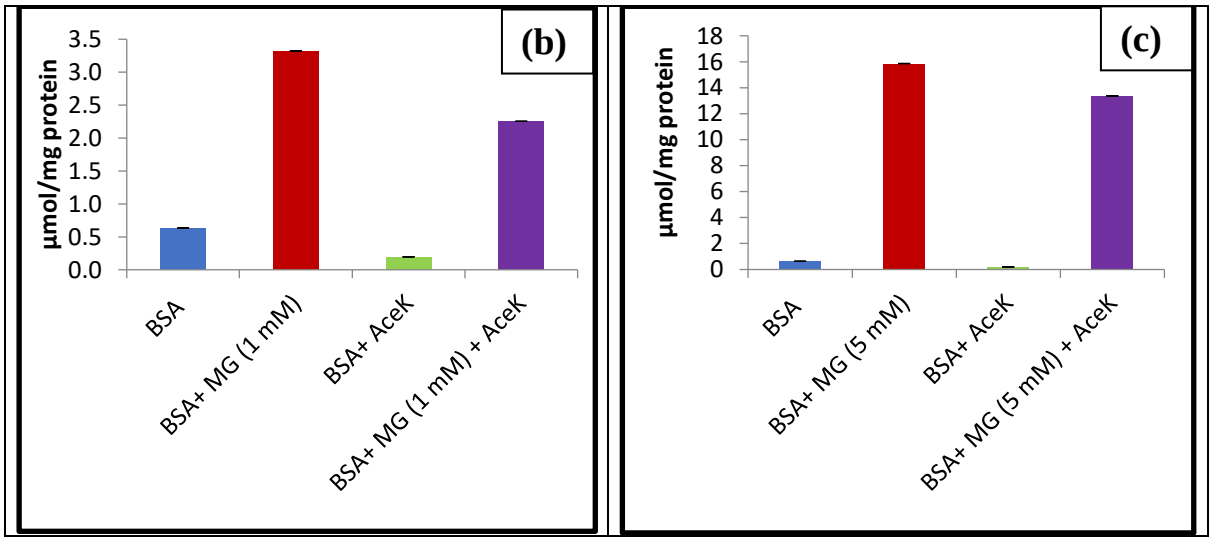

Figure 2(b & c): Fructosamine Content measurement (MG and Ace-K). (BSA- bovine serum albumin, MG- methyl glyoxal, Ace-K- acesulfame-K). Results are expressed as means ± SE (*n* =2) and p-value < 0.05.

Carbonyl Content

The carbonyl content was found to be reduced due to effect of Ace-K, Sac and Sucl compared to glycated BSA. Quantitatively, it was reduced maximum to 6.63, 5.09 and 7.06 µmol/mg by Ace-K, Sac and Sucl. Ace-K has also supressed the carbonyl content to 19.27 and 107.09 µmol/mg protein as compared to 26.43 and 121.77 µmol/mg protein in MG glycated BSA at both concentration (1 mM and 5 mM) respectively. It indicated formation of Amadori products and AGEs were prevented in the presence of sweeteners.

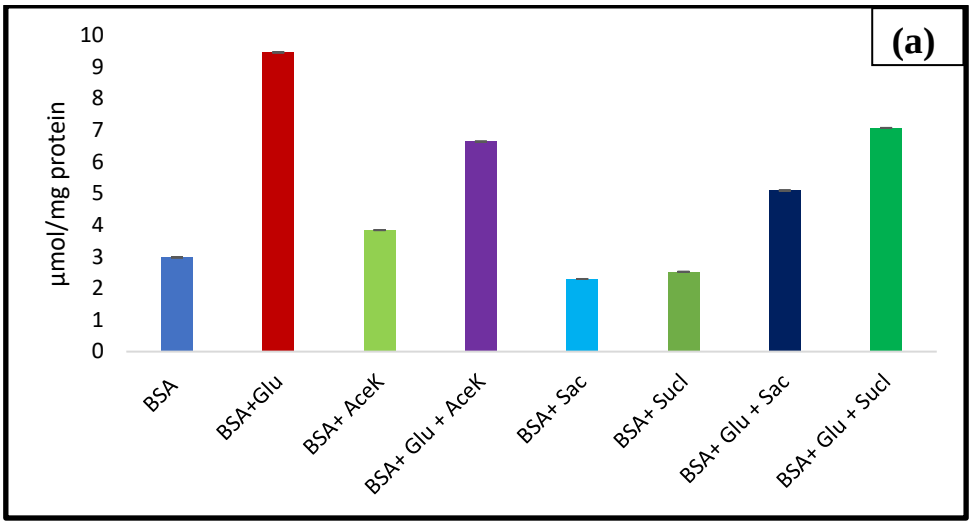

Figure 3a: Carbonyl Content measurement. (BSA- bovine serum albumin, Glu- glucose, Ace-K- acesulfame-K, Sac- saccharine and Sucl- sucralose). Results are expressed as means ± SE (*n* =4) and p-value < 0.05.

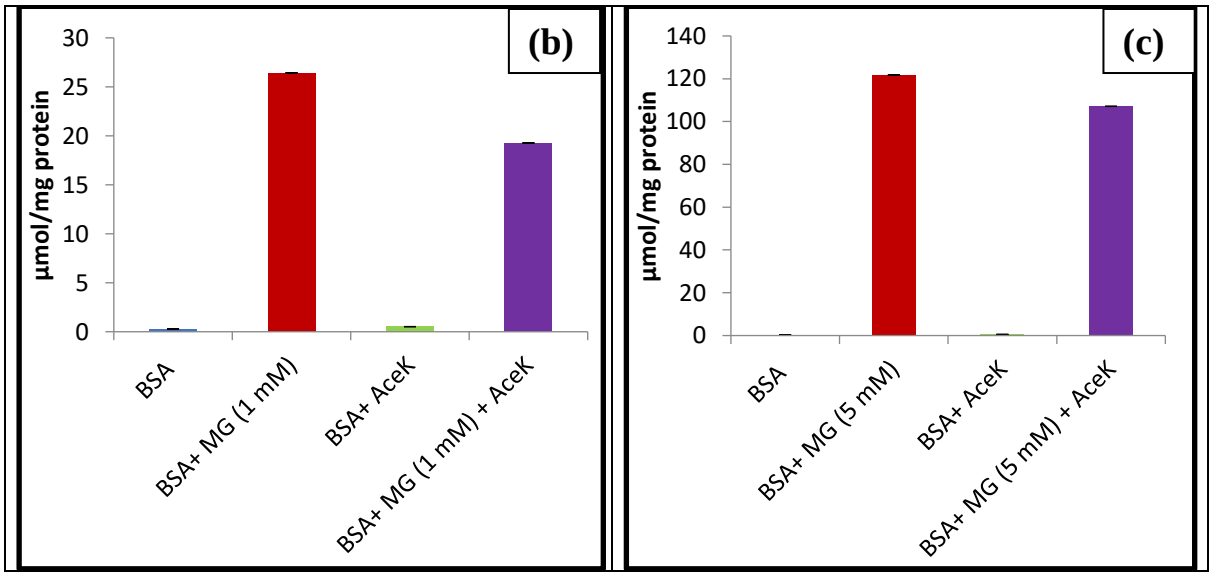

Figure 3(b & c): Carbonyl Content measurement (MG and Ace-K). (BSA- bovine serum albumin, MG- methyl glyoxal, Ace-K- acesulfame-K). Results are expressed as means ± SE (*n* =2) and p-value < 0.05.

Aggregation Index

Protein aggregation index (%) was calculated from spectroscopic absorbance at 280 nm and 340 nm. It indicates no aggregate (0-2%), moderate (2-5%), and aggregated (>5%) protein aggregation. Protein aggregation index of amyloid β-structure in the glycated BSA showed 3.15%, 6.61% and 12.15% in the presence of Ace-K, Sac and Sucl respectively as compared to BSA along with glucose 17.53%. Aggregation has also reduced in 1 mM MG glycation system (from 23.48% to 15.73%) and 5 mM MG glycation system in presence of Ace-K.

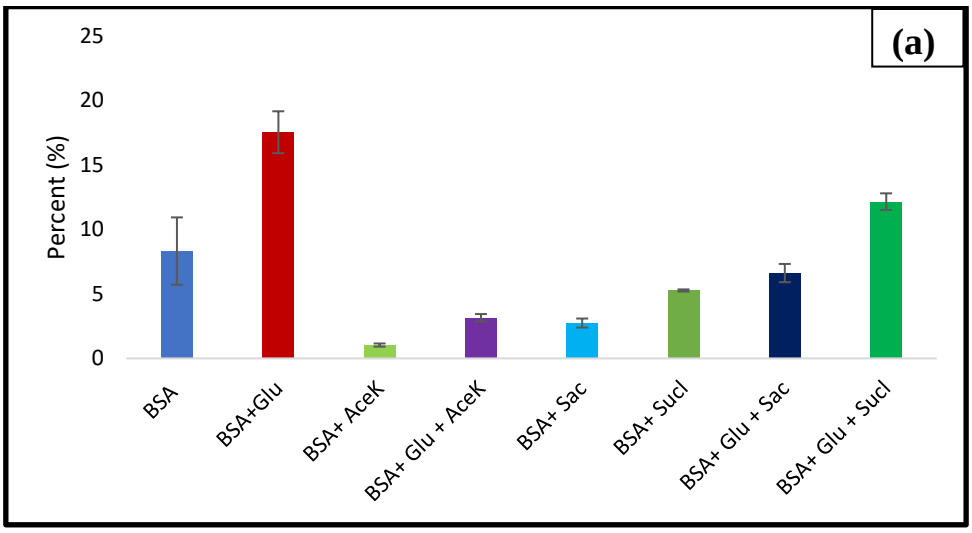

Figure 4a: Protein aggregation Index. (BSA- bovine serum albumin, Glu- glucose, Ace-K- acesulfame-K, Sac- saccharine and Sucl- sucralose). Results are expressed as means ± SE (*n* =2) and p-value < 0.05.

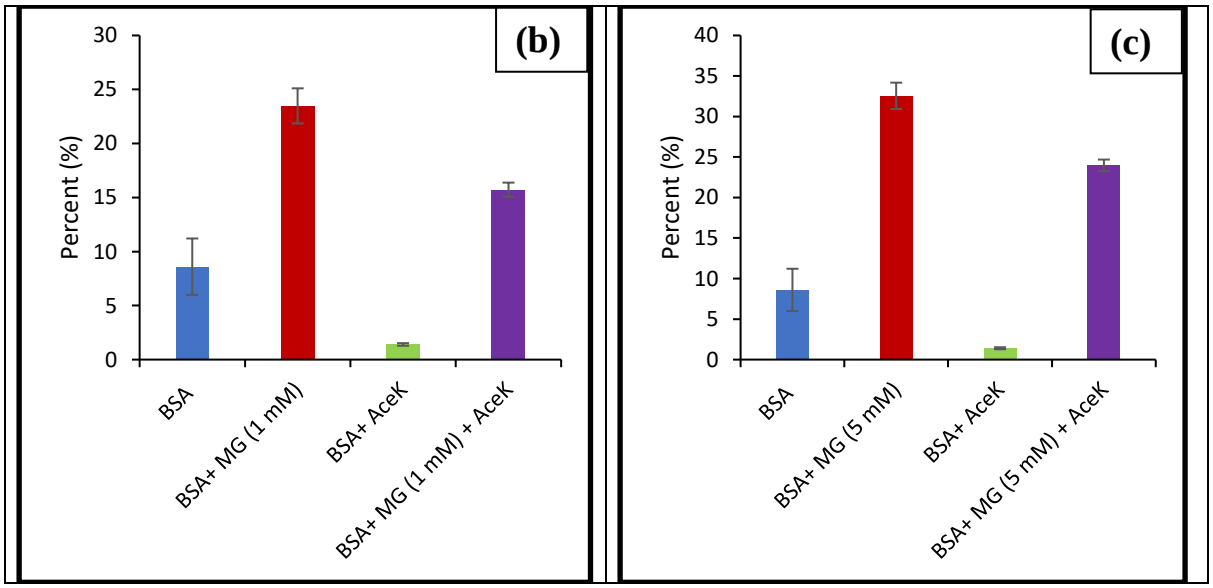

Figure 4(b & c): Protein aggregation Index (MG and Ace-K). (BSA- bovine serum albumin, MG- methyl glyoxal, Ace-K- acesulfame-K). Results are expressed as means ± SE (*n* =2) and p-value < 0.05.

Determination of amyloid β-structures by Congo red

We found that Ace-K, Sac and Sucl suppressed formation of aggregation of beta-amyloid by 59.00 %, 61.25% and 43.75% respectively at day 28. Ace-K also showed the reduction in MG mediated glycation at two different concentration of MG (1 mM and 5 mM). It means glycation and aggregation prevented in the presence of sweeteners significantly.

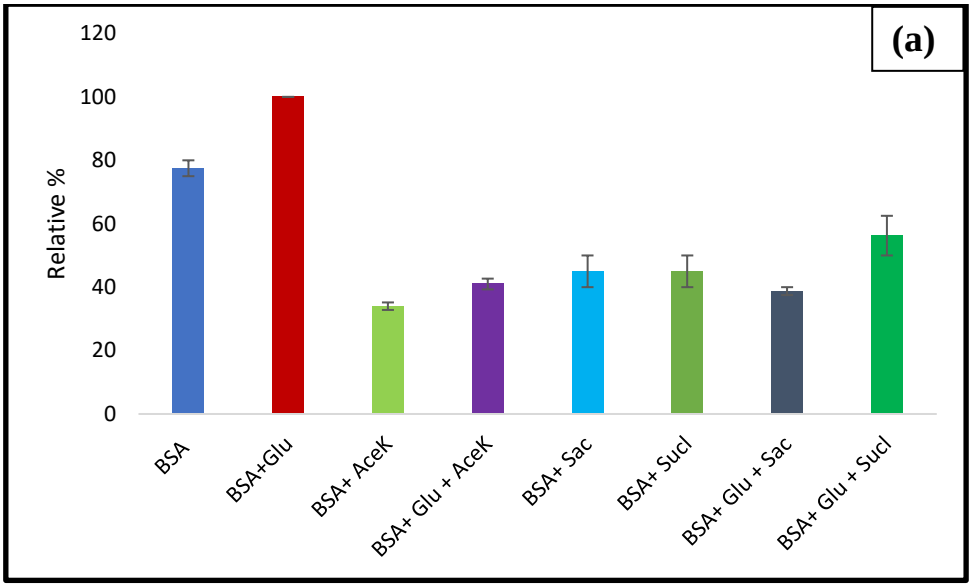

Figure 5a: Determination of amyloid β-structures by Congo red assay (BSA- bovine serum albumin, Glu- glucose, Ace-K- acesulfame-K, Sac- saccharine and Sucl- sucralose). Results are expressed as means ± SE (*n* =4) and p-value < 0.05.

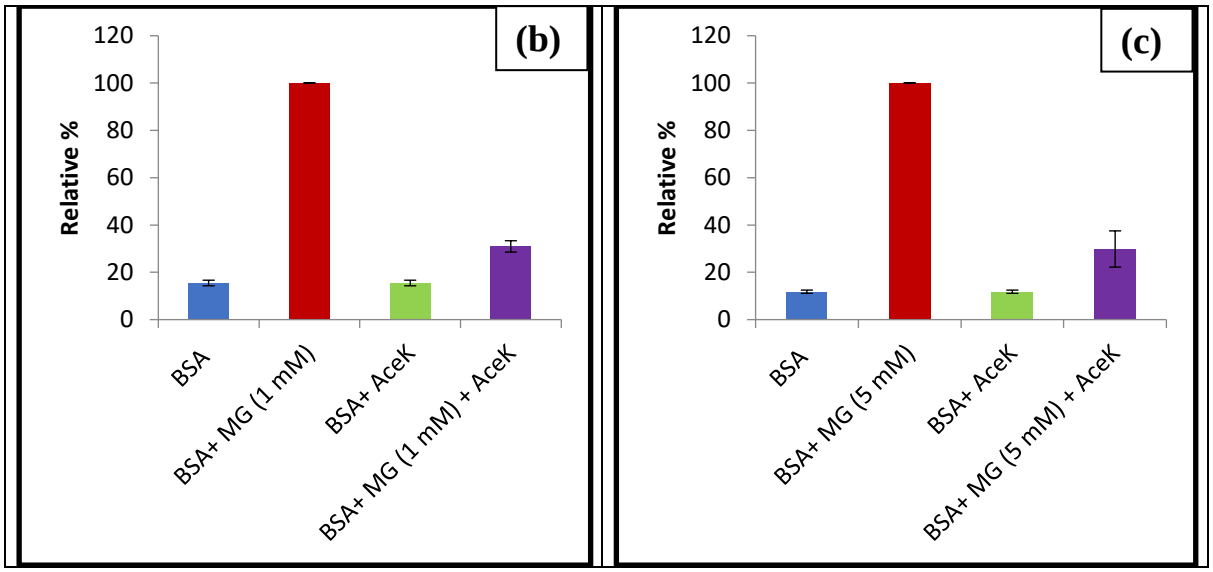

Figure 5(b & c): Determination of amyloid β-structures by Congo red assay (MG and Ace-K). (BSA- bovine serum albumin, MG- methyl glyoxal, Ace-K- acesulfame-K). Results are expressed as means ± SE (*n* =2) and p-value < 0.05.

MAJOR FINDINGS

- The present study has focused on the role of artificial sweeteners on protein glycation. The initial indicator of glycation (browning) were observed the sweeteners have able to clearly reduced the coloration of monocarbonyl (Glu) and dicarbonyl (MG) mediated glycated BSA.
- The early stage glycation products (fructosamine) formation were interfered by sweeteners (Ace-K, Sac and Sucl). Ace-K has showed reduction in both monocarbonyl (Glu) and dicarbonyl (MG) mediated glycation system.
- The carbonyl content has supressed by sweeteners (Ace-K, Sac and Sucl) in glucose mediated system. Ace-K has also reduced carbonyl content in MG mediated glycation system. The early and late stage glycation products (fructosamine and AGEs) formation has supressed by sweeteners.
- The aggregation of beta-amyloid structures has been prevented in glycation process by artificial sweeteners. The aggregation index and Congo red assay have significantly showed reduction in presence of Ace-K, Sac and Sucl.
- Acesulfame potassium was found to be most potent anti-glycating agent among artificial sweeteners. Hence it has been selected for further characterisation of inhibitory roles of artificial sweeteners in the process of glycation.

FUTURE PLANS

- AGEs will be identified on the basis of their fluorescent and nonfluorescent properties spectrofluorometrically.
- The structural alteration of glycated proteins in presence of Ace-K with the help of electrophoretic and blotting techniques.
- Electron microscopy will be used for identification of anti-aggregation and anti-glycation in presence of Ace-K.

OUTPUT (As Research Publication)

- Jha, P., Momin, A. R., Kumar, D., **Ali, A.** (2018). Reversal of glycoxidative damage of DNA and protein by antioxidants, Annals of Phytomedicine. 7: 101-105.
- Ahire, K., Kumar, D., **Ali, A.** (2018). Differential glycation of arginine and lysine by glucose and inhibition by acesulfame potassium, Journal of Bioscience and Biotechenology. 7: 11-15.
- Kumar, D., **Ali, A.** (2019). Antiglycation and antiaggregation potential of thymoquinone, Natural Volatiles & Essential Oils. 6 (2019) 25-33.
- Paramanya, A., Jain, Y., **Ali, A**. 2020. Obesity: its complications and medications. Journal of Health Sciences of Kocaeli University. 6(1): 1-9.

Conference Presentations

- Kumar, D., **Ali, A.** 2019. (Oral). Glycation induced structural alteration of Biomolecules and their reversal by some artificial and natural compounds. 5th International Mediterranean Symposium on Medicinal and Aromatic Plants (MESMAP5) and The 5th International Symposium on Pharmaceutical and Biomedical Sciences JOINT MEETING. April 24-28, 2019, Cappadocia – TURKEY
- Kumar, D., **Ali, A.** 2018. (Oral). Artificial Sweeteners and their Preventive Role in the Process of Glycation. International Health Sciences Conference (IHSC 2018). Dicle University, Diyarbakir, Turkey. November 14-17, 2018.
- Kumar, D., **Ali, A.** 2018. (Invited speaker). Artificial sweeteners prevent the accumulation of advanced glycation end products (AGEs). 2nd International Conference, Advances in Agricultural, Biological and Applied Sciences for Sustainable Future (ABAS 2018), Subharti Univrsity, Meerut, U.P., India. Oct. 20-22, 2018. **(Received second prize).**