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In Vitro Antioxidant and Antiglycation properties of extracts from *Zingiber officinale, Allium sativum, Caralluma fimbriata* and *Momordica charantia* by using Nanofluorometry

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ABSTRACT

Non-enzymatic glycation is a major source of irreversible AGEs production with oxidative stress and can cause major diabetic complications such as neuropathy, nephropathy, and cataract by changing the structure and function of proteins. Common medicinal food having antiglycation and antioxidant activity can be a good therapeutic agent for the treatment of diabetes. The present study was undertaken to investigate the antiglycation and antioxidant activity and their correlation among extracts of four household condiments namely *Allium sativum*, *Zingiber officinale*, *Caralluma fimbriata* and *Momordica charantia*. *In vitro* Bovine Serum Albumin assay (BSA) was used for measurement of antiglycation activity with four different solvent extracts (acetone, methanol, ethanol and distilled water). 1-diphenyl-2-picrylhydrazyl (DPPH) assay was used for detection of antioxidant activity. Methanolic and acetone extract of all condiments showed significant antiglycation and antioxidant activity and strong correlation (r=0.98). *M. charantia* showed the highest antiglycation activity with acetone extract as well as free radical scavenging activity 89.80%±1.37 and 93.43%±0.44 at 2mg/ml concentrations on the 7th day of incubation, respectively. All the other plants have shown their maximum percentage inhibition of glycation and antioxidation in methanol and acetone extract. Hence, it is suggested that these plants have a synergetic effect in many complications of diabetes.

Keywords: Amino guanidine, Rutin, Advanced glycation end products, Diabetes mellitus, Carbonyl stress, Microvascular disease, Glycation, Antiglycation, Antioxidation, Polyphenols, Free radicals.

INTRODUCTION

Hyperglycemia is an indication of diabetes mellitus consisting of severe pathologies and long-term complications. It is increasing day by day at a distressing rate. By 2010, approximately 221 million people may be found to suffer from diabetes and it is anticipated that this number will be doubled by 2030 (Uribarri et al., 2010, West et al., 2014). Long-term complications of diabetes mellitus include cataract, retinopathy, atherosclerosis, nephropathy, neuropathy, impaired wound healing and some cardiovascular diseases. The molecular basis of the major complication of diabetes is glycation, which is a non-enzymatic reaction between the carbonyl group of reducing sugars and the amino group of proteins. This reaction yield fluorescent and insoluble AGEs, which is notorious as advanced glycation end products and accumulates in the body with storage proteins that, can change their function and structure (Odjakova et al., 2012). The formation of AGEs is a complex cascade of different reaction such as condensation, rearrangement, fragmentation and oxidative modification which result in complex heterogeneous molecules forming a complex network of crosslink's (Bucala & Cerami, 1992). Although this is a significant progress in elucidating, and this mechanism of glycation is not completely understood yet.

Advanced glycation end products (AGEs)

Author's Contribution: **A.A. & A.S.**, Conducted experimental work, data analysis and wrote up primary draft; **N.M.A. & R.R.**, Reviewed, did analysis and editing; **S.M.**, Co-supervisor, did study design, review, analysis and editing; **Q.S.B.**, analysis and editing; **N.Z.**, Supervised, study design, manuscript review and editing

formation reaction is divided into three stages viz early stage, intermediate stage, and late stage. The unstable Schiff bases formation occur in early stage and by acid-base catalysis, it converts into stable Amadori products. In the intermediate stage, many complex reactions such as condensation, reduction, oxidation, dehydration, take place to form more reactive carbonyl compounds. These carbonyl compounds act as propagator by reacting with other free amino groups in a chemical reaction. In the last stage, the formation of AGEs occurs that can change the structure and function of storage proteins in the body. The first two stages are reversible and proceed fast while the last one is irreversible and slow proceeding stage, where the AGEs formation usually takes place (Lapolla et al., 2006).

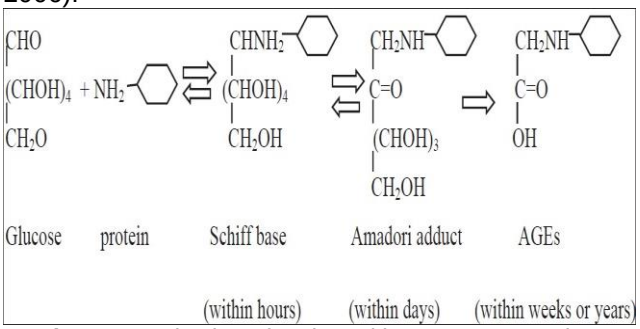

Fig.1.1: Production of early and late non enzymatic glycation products (Neelofar & Ahmad, 2015)

Glucose, fructose, reducing sugars and carbohydrates derivatives such as ascorbic acid are inheritably highly reactive towards nucleophilic nitrogenous bases of proteins, which are involved in fast preceding of the glycation reaction (Bunn et al., 1978). These possibly react with the α-amino group of lysine residue of protein to form a Schiff base (Paulsen & Pflughaupt, 1980). The arrangement of Schiff base into Amadori product occurs through open-chain enol form, which is intermediate. Schiff base formation is a relatively fast and highly reversible reaction, while the Amadori formation is comparatively a slow reaction; hence, the AGEs tend to accumulate on the stored proteins.

Glycated proteins produce free radicals, which are responsible for the protein fragmentation and oxidation of lipids and nucleic acid (Xi et al., 2008, Baynes, 1991). Several scientists reported that the formation of AGEs was accelerated by the oxidation reaction. During the formation of AGEs, auto-oxidation chemical reaction forming radicals and some other bioactive intermediates are formed along with the reactive oxygen species (Bonnefont-Rousselot, 2002)

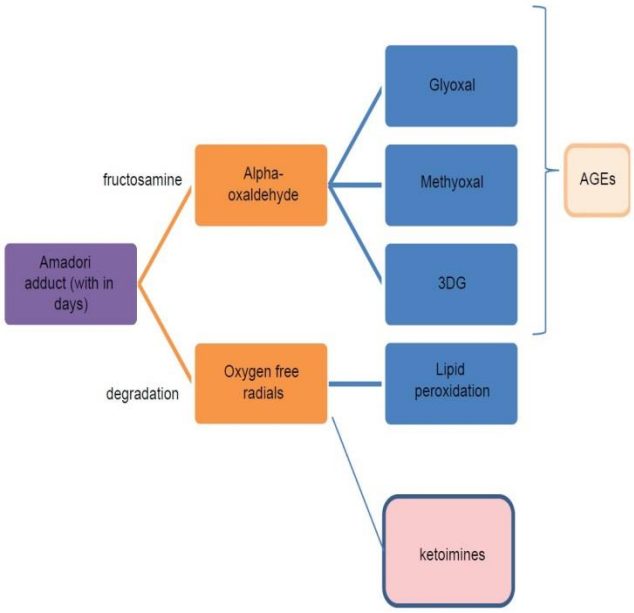

Fig. 1.2: Amadori adduct outcomes

Type 2 diabetes associated with reduced antioxidant activity may be a primary factor in diabetes-related vascular complications (Ceriello & Testa, 2009). Free radicals, which are produced during glycation are known as glycation-derived free radicals. These free radicals may cause several complications associated with diabetes. Antioxidants are the best therapeutic agents (Manzoor et al., 2016) that can prevent cells from free radicals harm. Therefore, the synergetic effects of plant extracts and compounds that have antiglycation and antioxidant activity can treat diabetes very efficiently (Kousar et al., 2008). It is a known fact that plants which possess pharmacological properties with antioxidant activity must have polyphenolic compounds particularly, flavonoids and phenolic acids (Ignat et al., 2011). These are secondary metabolites of plant cells (Ghaffari & Mojab, 2007). In plants polyphenols, based on aglycones, are divided into three subgroups: 1- phenolic acids, which are present in almost every fruits and vegetable (Tsao, 2010). 2- flavonoid, which are present in various types of teas, vegetables, and red wine. 3- terpenes, carotenoids, and tannins, which are present in many herbal plants (Shahidi et al., 1992, Chung et al., 1998). Their primary structure having three benzene rings and hydroxyl groups make them very efficient antioxidant and they play a key role in

trapping free radicals (Fraga, 2007). At present, many plants identified for polyphenolic contents, which are considered antioxidants *in vitro*.

The food ingredients exhibiting antioxidant activity are getting more importance in diabetes cure. Hence, the mechanism of AGEs formation may be blocked by antioxidant agents because they can prevent further oxidation of Amadori product and glucose oxidation catalyzed by metal ions. For this purpose, many synthetic compounds use polyphenolic compounds such as aminoguanidine, rutin, (Broadhurst et al., 2000). Synthetic drugs display many side effects along with benefits. Thus, many natural compounds that are well known for antioxidant activity (such as turmeric, curcumin, spices, etc.), have been shown to inhibit the glycation *in vivo* and *in vitro* (Patra & Saxena, 2010). Food consisting of plant sources can maintain the overall health of the diabetic patient. Fruits and vegetables are a good source of antioxidant activity and can inhibit glycation (Kelble, 2005). The present study has been conducted for the study of four plant species for antiglycation and antioxidant activity; *Allium sativum* (Garlic), bud, *Zingiber officinale* (Ginger), root (Kazeem et al., 2012), *Momordica charantia* (Bitter gourd), fruit (Kusirisin et al., 2009) and *Caralluma Fimbriata* (Choung), whole plant (Bellamakondi et al., 2014). These plants containing polyphenolic contents can serve as therapeutic agents for diabetes complications. *Allium sativum* belonging to the family of Liliaceae (common name is garlic) is a well-known food ingredient that is used in everyday diet. Allicin the compound of garlic induces the hypoglycemia (Gulati et al., 1973). S-allyl-Cysteine sulfoxide acts to the same extent as insulin, besides this, it can produce insulin in the beta cell of the pancreas *in vitro*, hence can be a good inhibitor for diabetic complications (Augusti & Sheela, 1996).

Ginger is also reported to be quite effective in reducing the complications of 5-hydroxytryptamine-induced type 1 diabetes by diminishing fasting blood glucose (FBG), blood serum lipids pressure and increasing glucose tolerance (Akhani et al., 2004). Ginger has polyphenolic compounds and can decrease blood glucose level in hyperglycemic condition (Eidi et al., 2006). Gingerol is the main bioactive compound reported in ginger for antiglycation activity and having properties like insulin (Matsuzaka et al., 2004). At present scientists claimed that the Caralluma species have anti-diabetic properties. However, just a few scientists are able to prove these findings (Habibuddin et al., 2008). The bioactive compound of this species includes Glycosides, like Carumbilloside-I to Carumilloside-V, flavones, etc. The phytochemical studies showed that this species has polyphenolic compounds, It is suggested that it has antiglycation activity as well as antioxidant activity. *Momordica charantia* is widely used in pharmaceuticals and it can treat several diseases such as stomachache, liver diseases, hypertension, diabetes, and cancer (Rajesh & Latha, 2004). It has bioactive compounds momordenol, momordicins, momordicinin, erythrodiol, galacturonic acids, and gentisic acid, etc. can inhibit glycation (Grover & Yadav, 2004).

MATERIALS AND METHODS

Plant material

Four plant species *Alliums sativum* (Garlic a bud), *Zingiber officinal* (Ginger a root), *Momordica charantia* (Bitter Gourd a fruit) and *Caralluma fimbriata* (Choung a whole plant) were purchased from the local market of Gujranwala, Pakistan and identified by Department of Botany, University of Gujarat.

Chemicals

BSA (bovine serum albumin), DPPH (1-diphenyl, 2 picrylhydrazyl), D- Glucose, TCA (Trichloroacetic acid), sodium azide, potassium phosphate, potassium dihydrogen phosphate, sodium phosphate, sodium dihydrogen phosphate, quercetin, ethanol, acetone, methanol, and distilled water all chemicals were obtained from the Department of Biochemistry, University of Gujrat.

Preparation of crude plant extracts

The plants were dried in dark at room temperature for 30 days. They were grounded into a fine powder and were stored in plastic jars, kept in refrigerators at 4°C. For extraction, 50g of each sample were dissolved into 400ml of solvents (distilled water, 80% Acetone, 80% Methanol and 80% ethanol) separately and kept on shaking incubator for 24 hours at room temperature. After that, the extracts were filtered by using a filtration assembly. The filtrate was concentrated under reduced pressure on a rotary evaporator at 45°C temperature. The temperature was optimized for deionized water, methanol, and acetone during rotary evaporation. Concentrated extracts were stored at 4°C in the refrigerator for further experiments (Ramkissoon et al., 2013, Shabbir et al., 2009)

Antiglycation assay

Bovine serum albumin assay (BSA) was used for the determination of antiglycation activity

using following procedure (Al-Musayeib et al., 2011). The sample extract was weighed and adjusted for two concentrations (2mg/ml, 1mg/ml). The final reaction volume was 1ml for all experiments, taken in 1.5ml Eppendorf tubes. For positive control, 500µl bovine serum albumin (2mg/ml concentration adjusted) was incubated with 400µl glucose (500mM concentration) and 100µl potassium phosphate buffer saline (0.2 M, pH adjusted as 7.4) was used.

For negative control, 500µl BSA (2mg/ml concentration adjusted) was incubated with 500µl potassium phosphate buffer saline (0.2 M, pH: 7.4) under the same experimental conditions. For treated sample 500µl bovine serum albumin (2mg/ml concentration adjusted) was incubated with 400µl glucose (500mM concentration), 100µl potassium phosphate buffer saline and 100µl of each plant extract separately. For standard reference, 3mM rutin (2mg/ml concentration) in methanol was used. The reaction was allowed to proceed for 14 days at room temperature. After 7 days, the reaction was stopped by adding 10µl of 100% (w/v) trichloroacetic acid (TCA). Then the mixture was kept for 10 minutes thereafter and centrifuged at 13000g for 4 minutes. The supernatant was discarded and the pellet was redissolved in potassium phosphate buffer saline and was quantified for the relative amount of glycated BSA based on fluorescence intensity by a Nanodrop 3300 (Fluorospectrometer). The excitation wavelength was set at 370nm and the emission wavelength was adjusted at 440nm. Each sample extract was assayed in two concentrations and was repeated in triplicate. Percentage of inhibition was calculated by the formula given below:

$$\% \text{ Inhibition} = \frac{1 - \text{Fluorescence of Specimen}}{\text{Fluorescence of BSA/Glucose}} \times 100$$

Antioxidant activity

Free radical scavenging activity was measured by the 1-diphenyl-2-picrylhydrazyl (DPPH) with some modification (Perez Gutierrez et al., 2012). The sample was prepared with the concentrations ~2mg/ml and 1mg/ml by using methanol to check activity at minimum concentration. 3ml of each sample with different concentrations and 1ml of DPPH (0.1mM in methanol) were taken in a test tube, followed good mixing and was incubated for 30 minutes in dark at room temperature. The absorbance was measured by the spectrophotometer (Think HS 3300). The wavelength was adjusted at 517nm. The percentage of radical scavenging activity was measured by comparing the samples reading with the blank. methanol was set as blank and standard is quercetin solution (2mg/ml). Percentage activity was measured by the formula given below.

AA-AB / AA x 100

All reactions were carried out in triplicate.

Statistical Analysis

All the results were presented as mean±SD. Correlation between variables were quantified by the correlation factor "r" and linear regression analyses were performed using Microsoft Excel 2016. In each analyses r-value near to 1 and $p<0.05$ were also considered statistically significant.

RESULTS

Inhibitory effect of plants extracts on the formation of advanced glycation products (AGEs)

Inhibition of glycation reactions with four plants (*Alliums sativum*, *Zingiber officinal*, *Momordica charantia and Caralluma fimbriata*) in four different solvents (water, acetone, methanol and ethanol) was studied to find the percentage inhibition of glycation between the bovine serum albumin protein used and glucose *in vitro*. The standard reference control rutin inhibited the glycation reaction ($p<0.05$) by 93.45%±0.91 and also the treated sample values were significantly different ($P<0.05$) from negative control. The extracts of all plants showed inhibition of glycation at 7th day and 14th day of incubation period with almost the same pattern (Table I and Table II). The *Allium sativum* showed the highest antiglycation of ~80.54%±1.30 and ~65.59%±1.09 with 2mg/ml, 1mg/ml concentration in aqueous extract respectively. The Momordica charantia showed the highest antiglycation activity of ~89.80%±1.37 and ~84.22%±1.62 with 2mg/ml, 1mg/ml concentration in acetone extract respectively. While the *Caralluma fimbriata* showed the highest antiglycation activity of ~74.05%±1.76 and ~67.17%±1.96, and Zingiber officinal showed the highest antiglycation activity of ~75.83%±1.79 and ~61.20%±1.51 with 2mg/ml, 1mg/ml concentration, respectively in methanolic extracts on 7th day of incubation (Table I). The same pattern bwere also seen in percentage glycation inhibition of four plants in four different solvents (Table II).

DPPH-radical-scavenging activity of four plants extracts using four different solvents (water, acetone, methanol and ethanol)

All plants extracts were found to be antioxidant. The antioxidant activity of four plants (*Alliums sativum*, *Zingiber officinal*, *Momordica charantia* and *Caralluma fimbriata*) in four different solvents (water, acetone, methanol, and ethanol) was studied. They all showed significant activity with interesting varying pattern in different solvents at two different concentrations 1mg/ml and 2mg/ml when compared with the standard reference control Quercetin, which showed the antioxidation activity of 95.12%±0.53 ($p<0.05$).

A.sativum exhibited maximum antioxidant activity with methanol extract (~88.97%) at the concentration of 2mg/ml and exhibited minimum activity (~71.61%) in ethanol extract. The *C. fimbriata* also exhibited highest activity with methanol extract (~90.61%) at 2mg/ml concentration and minimum activity with distilled water (~82.55%) at same concentration. The *Z. officinale* exhibited maximum activity (~85.51%) at the concentration of 2mg/ml in acetone and exhibited minimum activity (~75.64%) at same concentration in ethanol. The *M. charantia* exhibited maximum activity with acetone extract (~93.43%) at 2mg/ml concentration and minimum activity with ethanol extract (~77.95%) at the same concentration. The free radical scavenging activity with all extracts and percent antioxidant activity is presented as mean ± SD (Table III).

Table I: Inhibition of glycation by four plants extracts (water, acetone, methanol and ethanol at 7th day incubation.

Plants	% Inhibition							
	Water		Acetone		Methanol		Ethanol	
	1mg/ml	2mg/ml	1mg/ml	2mg/ml	1mg/ml	2mg/ml	1mg/ml	2mg/ml
Allium. sativum	65.59 ±1.09	80.54 ±1.30	57.34 ±1.23	76.12 ±1.33	66.34 ±1.34	78.26 ±1.75	62.23 ±0.76	75.54 ±1.32
Zingiber. officinate	63.63 ±1.07	69.68 ±1.20	64.48 ±1.86	71.85 ±0.37	61.20 ±1.51	75.83 ±1.79	59.75 ±0.91	65.15 ±1.10
Momordica charantia	74.06 ±1.84	83.73 ±1.18	84.22 ±1.62	89.80 ±1.37	82.99 ±1.38	86.84 ±1.52	77.75 ±1.76	80.15 ±0.68
Caralluma fimbriata	61.46 ±1.0	67.10 ±1.2	54.50 ±1.02	60.43 ±1.13	67.17 ±1.96	74.05 ±1.76	67.78 ±1.88	70.33 ±1.15

Table II: Inhibition of glycation by four plants extracts (water, acetone, methanol and ethanol at 14th day incubation.

Plants	% Inhibition							
	Water		Acetone		Methanol		Ethanol	
	1mg/ml	2mg/ml	1mg/ml	2mg/ml	1mg/ml	2mg/ml	1mg/ml	2mg/ml
Allium. sativum	75.68 ±1.48	88.11 ±0.37	71.83 ±1.7	81.99 ±0.96	78.98 ±1.33	85.71 ±1.11	67.65 ±0.95	79.15 ±1.25
Zingiber. officinate	82.75 ±0.74	87.58 ±1.02	82.38 ±0.53	90.85 ±1.12	86.86 ±0.74	92.47 ±1.02	63.71 ±0.99	75.96 ±1.01
Momordica charantia	76.93 ±1.45	79.43 ±0.76	85.33 ±1.51	91.08 ±1.05	59.63 ±0.94	76.41 ±1.98	61.45 ±1.10	70.71 ±0.91
Caralluma fimbriata	69.91 ±1.69	74.09 ±0.90	67.62 ±0.96	70.45 ±1.07	61.39 ±1.79	78.86 ±1.93	66.95 ±1.02	71.65 ±0.85

Table III: Antioxidant activity of four plants extracts (water, acetone, methanol and ethanol) by DPPH.

Plants	% Inhibition (IC 50)							
	Water		Acetone		Methanol		Ethanol	
	1mg/ml	2mg/ml	1mg/ml	2mg/ml	1mg/ml	2mg/ml	1mg/ml	2mg/ml
Allium. sativum	68.16 ±0.17	73.87 ±1.77	73.26 ±1.60	85.54 ±0.87	72.24 ±0.62	88.97 ±0.17	67.57 ±1.56	71.61 ±1.10
Zingiber. officinate	77.34 ±0.98	83.77 ±1.40	74.72 ±0.65	85.51 ±0.88	73.35 ±1.02	80.15 ±1.65	68.23 ±0.98	75.64 ±0.52
Momordica charantia	79.74 ±1.26	89.72 ±0.79	85.82 ±0.10	93.43 ±0.44	80.03 ±0.61	86.25 ±0.10	69.47 ±0.54	77.95 ±0.84
Caralluma fimbriata	71.29 ±0.13	82.55 ±0.31	79.21 ±1.02	88.57 ±0.15	79.69 ±0.50	90.61 ±0.46	76.85 ±0.68	85.61 ±0.15

DISCUSSION

The present studies are conducted to compare and correlate the antiglycation and antioxidant activities of aqueous, acetone, methanol and ethanol extracts of plants based food *Alliums sativum* (Garlic), *Zingiber officinal* (Ginger), *Momordica charantia* (Bitter Gourd), and *Caralluma fimbriata* (Choung). Prominent among them were *Allium sativum* and *Momordica charantia*, while almost all of them showed significant inhibition activity against glycation and oxidation. On the other hand, the antiglycation and antioxidation activities were less than rutin and quercetin (both were reference standard). Previous study for antiglycative effect of garlic scales showed inhibition by the increase of fluorescence intensity from glycated protein products. It also has good radical scavenging and metal chelating capacities (Tan et al., 2015). We reported in this study that aged garlic inhibited more AGEs as compared to fresh garlic extracts as aged garlic has more phenolic contents (Elosta et al., 2017). Previously, *Momordica charantia (MC)* fruit extract has been reported to have promising potential for use as an alternative tropical medication for diabetic wounds (Hussan et al., 2014). Many other studies also suggested that extracts of MC fruit had potent inhibition activity against glycation (Thent et al., 2018). The polysachaarides from MC possesses significant dose dependent anti-diabetic and anti-glycation properties (Xu et al., 2015).

The mechanism of glycation has not been fully defined yet; however, the correlation of AGEs and free radicals with diabetic complications (cataract, neuropathy, nephropathy and major cardiovascular diseases) has been relatively better understood now (Nowotny et al., 2015). Therefore, the inhibitors for AGEs formation and free radical scavengers can inhibit the major diabetic complications. In many other reports, the correlation between the free radical scavenging activity and antiglycation activity for diabetic disease and its complication reduction management was explored by experimentally and strong positive correlation ($r= 0.98$, $p\leq0.001$) was found (Price et al., 2001, Kazeem et al., 2012, Chinchansure et al., 2015, Shen et al., 2017). Similarly , the antiglycation activity to a certain extent positively relates to free radical scavenging activity (Wu et al., 2011, Hafsa et al., 2018). Our study confirmed that our extracts had antioxidant activity that correlated with the antiglycation activity and had a synergetic effect on diabetes control and inhibition of protein complication. Advantages of plant extracts having both antioxidant and antiglycation activity have been reported but extent of their effects depend on the inhibition pathway, dose and solvent extract used (Ramkissoon et al., 2012).

Advantages of using folk plants are that they have no adverse effects on other parts of the body and they are routinely used as food material and in folk medicines. Many studies suggested that dietary agents are the best sources for diabetes control (Ramkissoon et al., 2012). All extracts in the present study had antiglycation and antioxidant activity. Low molecular weight polyphenolic compounds and flavonoid which can inhibit or reduce the AGEs formation and have antioxidant activity had also been reported elsewhere (Prisilla et al., 2012). In the present study, It was also found that *B. gourd* exhibited higher antioxidant activity among all other extracts, especially with acetone extract. As shown in Table I, Garlic has been exhibiting the maximum inhibition of glycation activity (80.54%±1.30) with aqueous extract at 2 mg/ml concentration. Ginger showed its maximum

inhibition (75.83%±1.79) with methanolic extract at the 2 mg/ml concentration. The highest antiglycation among all the plant extracts was shown by *B. gourd* (89.80%±1.37) at 2mg/ml concentration with acetone extract. Cactus showed the maximum effect (74.05%±1.76) with the methanol extract at 2mg/ml concentration. All the observations of plant extracts of percentage inhibition were relatively less than the Rutin used as a standard for the inhibition of glycation (93.45%±0.91). However, the highest percentage of antiglycation by acetone extracts of *B. gourd* is comparatively near to standard on 7^{th} day of incubation and almost the same pattern of percentage of inhibition was seen at 14^{th} day of incubation with increased intensity of glycation reaction.

The antioxidant properties of four plants with four solvents showed some contrasting results as compared to antiglycation in Table. III. It was observed the acetone extract of *B. gourd* displayed the highest antioxidant activity. The garlic showed maximum antioxidant activity in methanol but previously it gave the maximum antiglycation activity in aqueous extract. The ginger showed the maximum antioxidant activity in acetone extract. The Cactus showed highest antioxidant and antiglycation activity in methanolic extracts. Therefore, it is established and suggested that the antioxidant properties of plant extracts showed significant effects of antiglycation activity.

CONCLUSION

In the present study, the methanolic and acetone extract of *A. sativum* (garlic), *Z. officinale* (ginger) and C*. fimbriata* (Cactus) showed highest inhibitory activities, which showed an increase with an increase in concentration. Moreover, the inhibition of glycation increases with increase in incubation duration, however, in case of *M. charantia* (B. gourd) it decreases. The extracts with more antioxidant activity are found to inhibit the formation of AGEs more efficiently compared to the extracts with less antioxidant activity, due to the free radicals produced during glycation may be scavenged by antioxidants. To the best of our knowledge, the *C. fimbriata* has never been used for such study before and it showed very promising results in the present study. The garlic extract with ethanol exhibited poor antioxidant activity. All compounds were found to be strong inhibitors of glycation and significant scavengers of free radicals.

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