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Original article

Antiglycation potential of metal ions and polyphenolic extract of chickpea on thiol-protease inhibitor: A management for diabetic complications

Mohd Shahnawaz Khan [a,*], Sheraz Ahmad Bhat [b], Monnera Saud Albagmi [a], Mohammed Arshad [c], Mohammad Tarique [d], Bilqees Bano [e]

[a] Department of Biochemistry, College of Science, King Saud University, Riyadh, 11451, Saudi Arabia
[b] College of Science, Cluster University Srinagar, INDIA
[c] Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia
[d] Department of Child Health, School of Medicine, University of Missouri, Columbia, MO 65201, USA
[e] Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, INDIA

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ABSTRACT

Glycation is the non-enzymatic adduct formation between reducing sugars or dicarbonyls with proteins and is a crucial molecular event under hyperglycaemic conditions of diabetes. The accumulation of advanced glycation end products (AGEs) due to glycation of proteins has been implicated in several diseases associated with ageing and diabetes. Thus, investigating the antiglycation potential of some trace metal ions (Manganese; Mn^{2+}, and Zinc; Zn^{2+}) and polyphenolic extract of chickpea seeds (PEC) on the methylglyoxal (MGO) induced glycation of a phytocystatin isolated from chickpea was taken up to find an inexpensive and non-toxic therapeutic means of medicating protein glycation and associated diabetic complications. The current study focused on the comparative analyses of these micronutrients and herbal extracts in inhibiting protein glycation and AGEs formation in a quest to develop nutraceuticals for managing diabetes. The effect of metals (Mn^{2+}, Zn^{2+}) and PEC on protein glycation was assessed by different techniques, i.e., glycation-specific AGE fluorescence and absorbance, thiol protease inhibitory activity assay, and conformational alterations by spectroscopic assays. This study revealed the significant anti-glycation potencies of Mn^{2+}, Zn^{2+}, and PEC against the MGO-induced glycation of CPC, which might pave the way for resolving pathological complications of diabetes by combining higher levels of efficacy, selectivity, and safety in humans. Moreover, characterization and identification of different AGEs formed during the glycation process in diabetics was done to apply the same for determining the onset of glycation at the early stage so that appropriate steps be taken to address the menace of diabetic complications.

1. Introduction

Dynamic equilibria exist within healthy human bodies and get altered under diseased conditions. One such imbalance is persistent blood sugar increase or hyperglycemia during diabetes (Li et al., 2019). Various therapeutic and home remedies are available to manage diabetes through diet, medications, insulin infusion, and pump and islet transplantation. Still, diabetes, with long-term consequences, continues to remain one of the significant causes of death (Li et al., 2019). These complications directly result from protein structure alterations due to glycation/Maillard reaction resulting in irreparable tissue damage (Kikuchi et al., 2003).

The Maillard reaction between protein amino groups and carbonyl groups on reducing sugars leads to the formation of stable end-stage adducts called advanced glycation end products (AGEs), via Schiff base formation and rearrangement to Amadori products (Van Nguyen, 2006). AGEs are also formed by the reactive dicarbonyl species (RDS) or α-oxoaldehydes such as methylglyoxal (MGO) and glyoxal (GO) and that too much rapidly than the reducing sugars as there is no formation of intermediates such as Schiff base (Van Nguyen, 2006). MGO and GO are

Abbreviations: AGE, Advanced Glycation End-product; CPC, Chickpea Cystatin; CE, Catechin Equivalent; GAE, Gallic Acid Equivalents; LDL, Low Density Lipoproteins; PEC, Polyphenolic Extract of Chickpeas; PC, Proanthocyanidin Content; MGO, Methylglyoxal; Mn, Manganese; RDS, Reactive Dicarbonyl Species; TPC, Total Phenolic Content; TFC, Total Flavonoid Content; UV, Ultra Violet; Zn, Zinc.

* Corresponding author.
E-mail address: moskhan@ksu.edu.sa (M. Shahnawaz Khan).

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the endogenous metabolites that increase in diabetes and are also considered to be key intermediates in this reaction (Han et al., 2007). Pathological changes to the proteins are brought on by glycation and AGE modifications (Ahmed, 2005). Glycation-induced structural and functional changes in enzymes have been reported in aldose reductase (Eble et al., 1983; Khan et al., 2020) and superoxide dismutase (Srivastava et al., 1989). Also, the binding of sugars to LDL increases its atherogenicity (Taniguchi et al., 1995). In addition, basement membrane permeability also increased during glycation reactions (Lyons, 1992). Notably, glycated insulin receptors decreased their affinity and binding with insulin (Cochrane and Robinson, 1995). All these changes aggravated diabetic complications such as atherosclerosis, retinopathy, nephropathy, and other neurological diseases (Ahmed, 2005). Moreover, AGEs also led to protein cross-linking and aggregation of various proteins (Goldman and George, 1990). Protein aggregation and misfolding are central contributors to neurodegenerative diseases which can lead to unfolded protein response (UPR) (García et al., 2018). The UPR is the main transduction pathway that maintains protein homeostasis under conditions of protein misfolding and aggregation (García-González et al., 2018). The UPR is an adaptive reaction controlled by three ER-located signal transducers: inositol requiring enzyme 1 (IRE1) α and β, protein kinase R-like ER kinase (PERK) and activating transcription factor 6 (ATF6) alpha and beta (Hetz and Papa, 2018). Thus, UPR mediators have a pivotal role in the progression of various protein misfolding diseases, which could be used as therapeutic targets in neurodegeneration (Hetz and Saxena, 2017; Scheper and Hoozemans, 2015; Valenzuela et al., 2018).

Inhibition of early glycation and AGEs is a crucial therapeutic target for treating diabetic complications (Rowan et al., 2018). Aminoguanidine and pyridoxamine are well-studied antiglycation agents and have been verified in clinical trials (Rowan et al., 2018). Aminoguanidine is reported to inhibit protein glycation by competition, scavenging α-dicarbonyls, and antioxidant activity by virtue of its guanidino group (Edelstein and Brownlee, 1992). It has been demonstrated that aspirin, acetaminophen, and ibuprofen reduce lens protein glycation and shield rats from developing diabetic cataracts (Robert and Harding, 1992). However, there have been some toxicity issues with aminoguanidine in clinical trials and other synthetic drugs (Rasheed et al., 2018). Thus, there is a need for an alternative treatment that would pass the test of time in terms of toxicity or adverse effects. Particularly interesting are micronutrients (trace metals like Mn^{2+} and Zn^{2+}) and herbal preparations such as chickpea polyphenol extract (PEC). Pyridoxine and ascorbic acid have been reported to inhibit protein glycation (Shenkin, 2006; Singh et al., 2001). Even though a bunch of literature exists regarding the pathogenic importance of AGE proteins, surprisingly, little is known about how micronutrients affect AGE formation and protein decarbonylation. Owing to their high antioxidant activity and potential to operate as an anti-AGE forming agent, chickpeas could be an appropriate option for researching herbal supplements for diabetes management.

Chickpea (*Cicer arietinum*) is one of the legumes with considerable importance in human nutrition for their high content carbohydrates, proteins, fiber, and minerals. Additionally, their secondary metabolites such as phenolics provide health benefits beyond basic nutrition (de Camargo et al, 2019). This important legume is currently cultivated on around 14.5 million ha, with annual production of 14.8 million tons globally as reported elsewhere (de Camargo et al, 2019). The geographical distribution includes India, Australia, Pakistan, Turkey, and Mexico, and Chile . Chickpeas are rich in bioactive substances such phenolic, flavonoid, and proanthocyanidin compounds. These bioactive components such as Biochanin A, Biochanin glucoside, Formononetin, Genistein, and Daidzein have been already identified and isolated (Gao et al., 2015), which made this legume suitable choice of investigation for the present study.

As reported earlier, Chickpea cystatin (CPC) is a proteinaceous thiol proteinase inhibitor isolated from chickpea seeds (Sheraz et al., 2017). This inhibitor was found to have a molecular mass of about 25.3 kDa (Sheraz et al., 2017). Glycation of CPC has also been studied earlier, but studies are scarce regarding side effect-free antiglycation of proteins in general and CPC in particular (Bhat et al., 2014). Thus, in the present study, glycation of CPC was performed by incubating it with MGO, a well-known potent glycating agent. Methylglyoxal (MGO) is a highly reactive endogenous metabolite that results from a variety of non-enzymatic and enzymatic processes, which include sugar-mediated glycation of proteins (Thornalley et al., 1999) apart from the degradation of triose phosphates (Phillips And Thornalley, 1993), and peroxidation of lipids (Abordo et al., 1999). To look for antiglycation therapeutic alternatives with minimum/no adverse effects, Mn, Zn, and CPE were screened for their AGEs inhibiting activities. Micronutrients and herbal preparations could be attractive agents for preventing AGEs formation. The positively inhibitory action against AGEs formation and glycation of CPC was assessed by monitoring the changes in structure, function and AGEs formation, that could well serve as a step forward for overcoming diabetic complications.

2. Materials and methods

2.1. Materials

Methylglyoxal, ethylene diamine tetra acetate (EDTA), L-cysteine, Sephacryl S-100 HR, and papain were purchased from Sigma (ST. Louis, MO). Salts were purchased from SRL (India). The study's other chemicals and reagents were of the highest analytical grade.

2.2. Methods

2.2.1. Purification of chickpea cystatin

Chickpea cystatin was purified in our lab by the method reported earlier (Sheraz et al., 2017).

2.2.2. Glycation protocol

0.5 mg/ml of CPC was prepared in 50 mM sodium phosphate buffer, pH 7.5, containing 0.05 % sodium azide. Next, it was filtered through a 0.22 μm syringe filter and incubated at 37 °C with or without methylglyoxal (5 mM final concentration) for 3 days. Additionally, glycated samples were incubated with chickpea extracts, PEC (5–50 μl of 100 mg/ml stock), or Mn^{2+}/Zn^{2+} (5–20/0.5–2 ppm) under similar conditions. Finally, samples were dialyzed against the same buffer and frozen at −80 °C until further analysis.

2.2.3. Preparation of phenolic extracts of chickpea seeds (PEC)

Chickpea extract was prepared by the modified method of Tarzi (Tarzi et al., 2012). Chickpea seeds were ground in a miller, and the resulting flour underwent phenolic component extraction using acetone. The extraction was done with 50 % acetone (1:10 w/v flour: acetone) for 24 h with constant stirring at room temperature. Suspensions were filtered through Whatman No. 41 filter paper to retain the clear solution. The solvent was removed by evaporation at 40 °C under vacuum. The extract was dried using a vacuum oven at 40 °C and then stored in a dry clean black glass bottle at 4 °C for further studies.

2.2.4. Assay of total phenolic, flavonoid, and proanthocyanidin contents in chickpea extract

The total phenolic content (TPC), total flavonoid content (TFC), and proanthocyanidin content (PC) of the chickpea extract were measured. TPC was estimated as per the method described by Singleton and Rossi (Singleton and Rossi, 1965). This technique is based on the Folin-Ciocalteu reagent's color reaction with hydroxyl groups, and the product was measured at 765 nm absorbance using a spectrophotometer (Shimadzu, Japan). The results were expressed as mg Gallic acid equivalents (GAE) per gram of extract. By measuring the absorbance at 510 nm, the TFC of the extract was determined using Kim's aluminum

Table 1
Purification results of chickpea cystatin (CPC).

Step	Volume (ml)	Total Protein (mg)[a]	Total Activity (units)[b]	Specific Activity (units/mg protein)	Fold Purification	Percent Yield
Crude Extract	200	5,463	147	0.027	1	100
Ammonium Sulphate Fractionation (40–60 %)	18	864	56	0.06	2.23	38.1
Sephacryl S-100 HR Chromatography	15	15.56	31	2.13	78.89	21.09

REPRODUCED/ ADOPTED from our earlier publication (Sheraz et al., 2017).
[a] Protein concentration was determined by the method of Lowry method (Classics Lowry et al., 1951).
[b] One unit of enzyme inhibitory activity is defined as the amount of inhibitor bringing about 0.001 change in OD/ml/min.

chloride colorimetric test method (Kim et al., 2003). The total flavonoid content of chickpea extract was expressed as mg of catechin equivalent (CE) per gram of extract. The PC of legume extract was determined using the vanillin-HCl colorimetric method, as described by Chandrasekara and Shahidi (Chandrasekara and Shahidi, 2010). Using a standard curve created for catechin, the amount of proanthocyanidins in the extract was determined and expressed as mg of CE per gram of extract.

2.2.5. Evaluation of glycation/AGEs inhibition activity

The effects of trace metals and PEC on the prevention of the production of advanced glycation end products were assessed using the CPC-MGO model system. During incubation, three glycation/AGEs-specific fluorescent measurements (excitation wavelength/emission wavelength: 325 nm/395 nm; 370 nm/440 nm; 485 nm/530 nm) were recorded at regular intervals of 1, 2, and 3 days. The following equation determined the percentage of AGE formation inhibition caused by each treatment.

$$\{1 - (\textit{fluorescence of the solution with inhibitors} / \textit{fluorescence of the solution without inhibitors})\} \times 100\%$$

AGE-specific absorbance was also monitored at 330 nm and 360 nm, and the formula calculated the percent inhibition of glycation by each agent as above.

2.2.6. Analysis of protein conformation changes

Fluorescence experiments were carried out to keep track of the inhibitor's overall structural alterations. Shimadzu RF-5301 PC Spectrofluorometer with HyperRS 1.57 software was used to excite the samples at 280 nm and analyze the results to detect the intrinsic fluorescence quenching by glycation. Aliquoted samples were diluted before fluorescence analysis.

The UV–Visible absorption characteristics of control and treated CPC were also determined as an additional index of conformational alterations. Using a cuvette with a 1 cm path length and a double beam Shimadzu UV–vis spectrophotometer (UV-1700), absorption spectra between 200 and 400 nm were obtained. Appropriate dilutions, if necessary, were made.

2.2.7. Measurements of cysteine protease inhibitory activity

Thiol protease inhibitory activity of chicken pea cystatin (CPC) in the absence and presence of methylglyoxal (MGO) was measured using method described by Kunitz (Kunitz, 1947). The effect of Zn, Mn, and polyphenolic extract of chickpea (PEC) were also determined using the same protocol as above. The effect of both varying time as well as concentration on CPC's cysteine protease inhibitory activity was monitored.

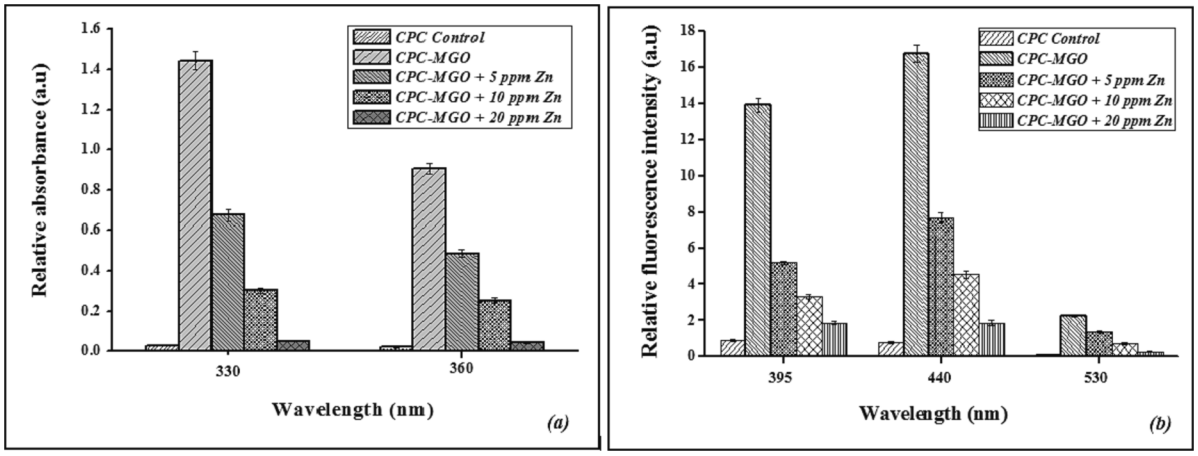

Fig. 1. Antiglycation effects of Zn on MGO incubated CPC: Relative AGE-specific *(a)* absorbance and *(b)* fluorescence measurements were recorded as an index of the extent of glycation. Each bar represents the mean ± SEM of three independent experiments performed in duplicates, and differences in glycation inhibitory activities of Zn-treated CPC samples from CPC-MGO control carry statistical significance at $P < 0.05$.

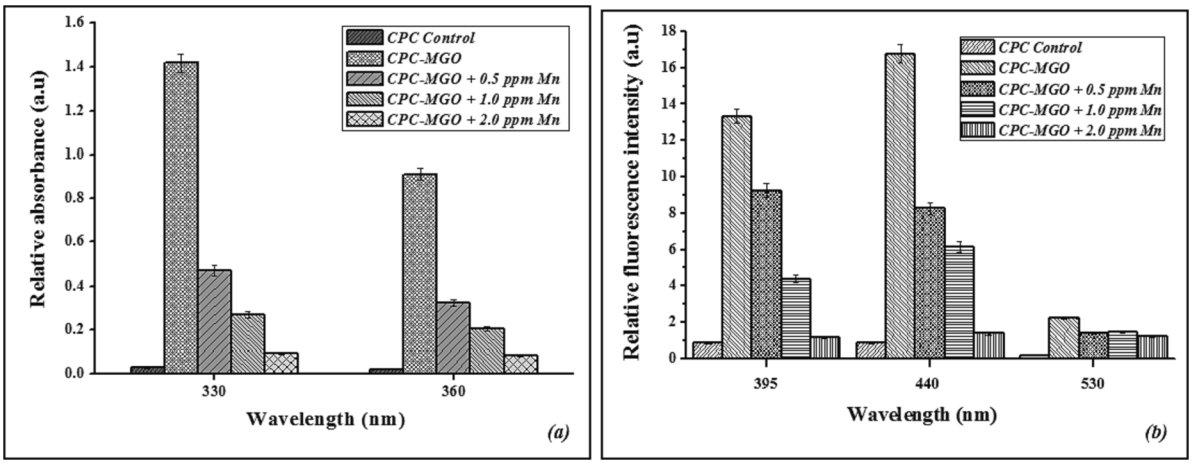

Fig. 2. Effect of manganese on MGO-induced modifications of CPC as evaluated by Effect of manganese on MGO-induced modifications of CPC as evaluated by relative glycation-specific absorbance and relative AGEs-specific fluorescence ***(a):*** Glycation-specific absorbance and ***(b):*** Relative fluorescence emissions. Data are mean ± SEM (n = 3). The results are different from the control at $p < 0.05$ and were considered statistically significant.

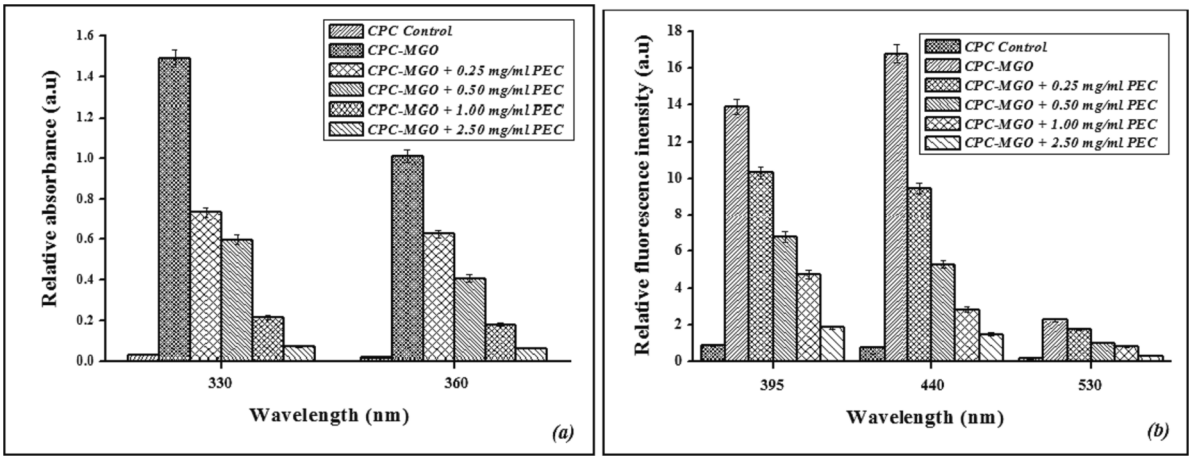

Fig. 3. Dose-dependent preventive effects of a polyphenolic extract of chickpea (PEC) on MGO-mediated modification of CPC: The antiglycation potential of PEC was evaluated by monitoring the effects on glycation-specific (a) absorbance and ***(b)*** AGEs specific fluorescence measurements. Each bar is mean ± SEM (n = 3). The results different from the control at $p < 0.05$ were considered statistically significant. [**CPC:** Chickpea cystatin; **MGO:** Methylglyoxal; **PEC:** Polyphenolic extract of chickpea].

2.2.8. Statistical analysis

All the experiments were carried out in replicates of 4. All the data were expressed as Mean ± SEM. Depending on the circumstance, one-way or two-way ANOVA was used to assess the significance of the mean value difference. The probability level chosen to denote statistical significance was $p < 0.001$. Controls were appropriately defined.

3. Results

3.1. Purification of chickpea cystatin

Chickpea cystatin (CPC) was purified from chickpea seeds to ensure homogeneity. Following homogenization, the method included two steps: gel filtration chromatography on a Sephacryl S-100 HR column and ammonium sulphate precipitation (40–60 %). The process produced a percent yield of 21.09 and a fold purification of 78.89. The progress of the present purification scheme is outlined in Table 1.

3.2. Assay of total phenolic, flavonoid, and proanthocyanidin contents in chickpea extract

Chickpea extract was prepared as described in the methods section. After drying using a vacuum oven at 40 °C, the extract was stored in a dry clean black glass bottle at 4 °C and assayed for total phenolic, flavonoid, and proanthocyanidin contents before being used for further studies. The total phenolic content (TPC) of chickpea seeds was 1.24 ± 0.13 mg GAE per gram of extract. The content of total flavonoids per gram of fresh chickpea seed extract was 0.91 ± 0.032 mg CE. Low amounts of PC were detected in the defatted extract of chickpea seeds, the value being around 0.32 ± 0.001 mg CE per gram of the sample.

3.3. Evaluation of glycation/AGEs inhibition activity

Zinc and manganese considerably decreased the AGE specific absorption (at 330 nm and 360 nm) and AGE fluorescence (325, 370, and 485 nm) when compared to the glycated protein (CPC-MGO), showing that the degree of glycation was lowered. [Figs. 1 and 2]. Additionally, dose–response experiments showed concentration dependent antiglycation effects by these micronutrients. The data also revealed considerable disparities between zinc and manganese's ability to inhibit glycation, with manganese being able to do so at far lower amounts than zinc.

In vitro, the antiglycation activity of the chickpea seeds was examined by testing the ability of its polyphenolic extract (PEC) to inhibit the

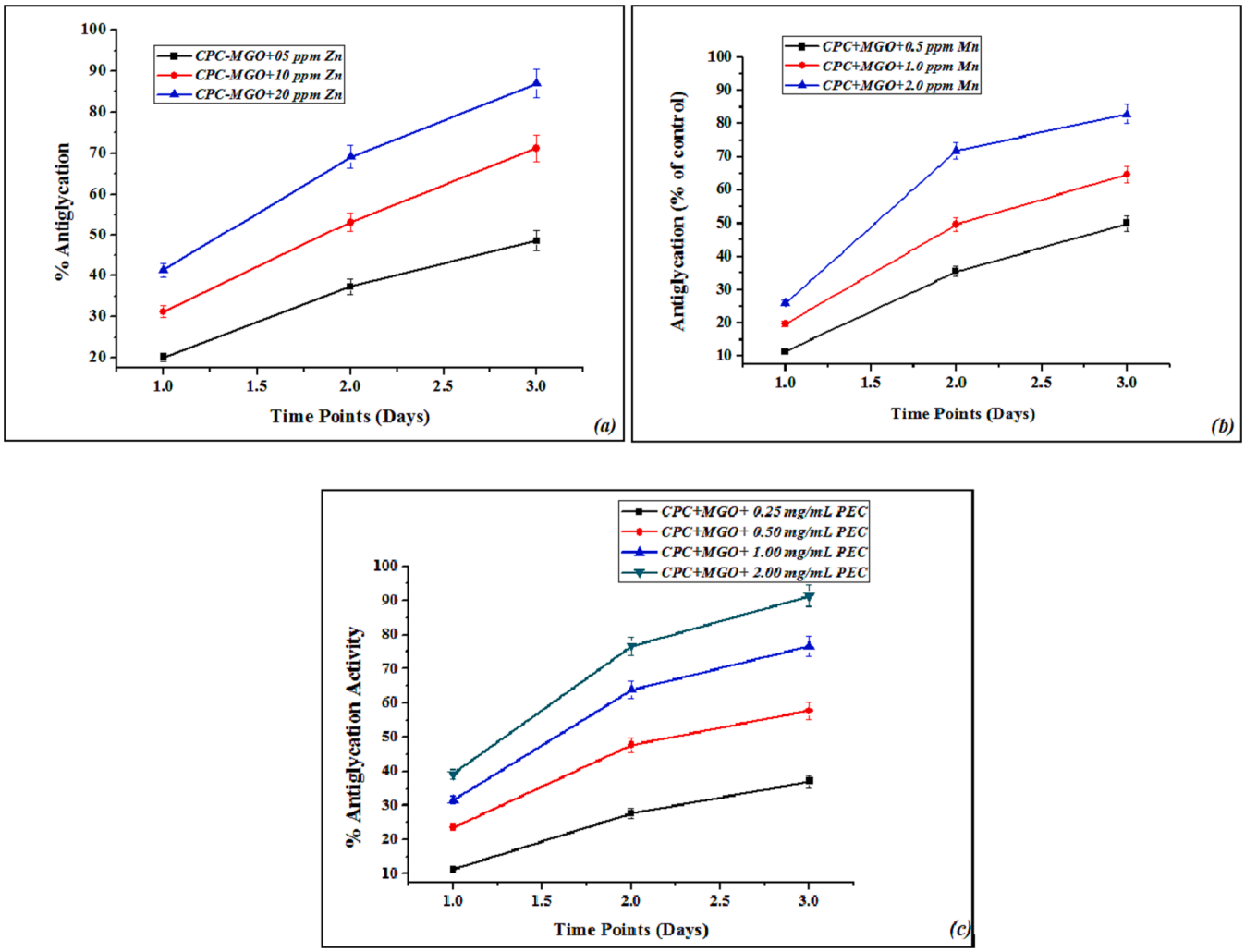

Fig. 4. Kinetic Study of the Inhibitory Effects on the Formation of AGEs by Zn, Mn, and PEC. Inhibitory effect of the formation of AGEs by (a) Zinc, (b) Manganese, and (c) PEC, in the CPC-MGO assay at different points of time (days). The data were generated from the three AGEs specific fluorescence and two absorption spectroscopic readings recorded against each concentration of putative antiglycation agent used at different points of time of the study. Data are presented as the means of these five experiments ± SEM of three replications.

MGO-mediated development of glycation-specific absorbance and AGEs fluorescence of CPC. Fig. 3 represents the antiglycation capacities of the increasing concentrations of polyphenolic extract of chickpea seeds, as evaluated by their inhibition towards the formation of AGEs in the CPC-MGO system. As depicted in Fig. 3, the PEC inhibited the MGO-mediated formation of chromophoric and fluorophoric AGEs in a dose-dependent manner. At 1.0 mg/ml, the extract exhibited high inhibition towards forming AGEs, while at 2.5 mg/ml, the extract inhibited the glycation almost entirely. The results suggest that chickpeas possess antiglycation activity which correlates with its phenolic content and thus could ameliorate diabetic complications.

3.4. Kinetic studies of the inhibitory effects on the formation of AGEs by trace metals and PEC

Our results suggest that Zn, Mn, and PEC could effectively and significantly inhibit the formation of AGEs in the CPC-MGO assay. Fig. 4 presents the antiglycation capacities of the trace metals and polyphenolic extract, as evaluated by their inhibition against AGEs formation in the MGO/CPC system using at least three types of AGEs (glycation) specific fluorescence and two types of absorption spectroscopic measurements as explained in the methods section. The results presented here are the mean of the three such AGEs' specific fluorescence and two absorption spectroscopic readings recorded against each concentration of putative antiglycation agent used at different points of time of the study. All the putative agents used showed glycation inhibitory effects, which varied with points of time as well as concentrations.

Fig. 4 **(a)** depicts the dose–response of zinc on the MGO-CPC model system at various time points (1, 2, and 3 days). On the final day of study (day 3), it was able to inhibit nearly 50 % glycation at 5 ppm concentration, while as the percent inhibition was around 70 % at 10 ppm and 20 ppm concentration, the trace metal could inhibit AGEs formation in MGO-CPC system by about 85 %. The results further showed that zinc followed the same inhibitory kinetics of glycation inhibition of MGO-CPC system at other time points as well. Moreover, there was also a saturation effect of zinc on day 3. Fig. 4 **(b)** shows the effects of manganese on the AGEs formation of CPC when incubated with 5 mM MGO for three concentrations (0.5, 1, and 2 ppm) at different time intervals. At just 0.5 ppm, Mn could inhibit glycation by about 50 % on day 3, while at a higher dose of 2 ppm Mn brought down glycation by about 80 %. It is noteworthy that manganese, inhibited glycation at a very low concentration compared to zinc. Thus, zinc and manganese considerably decreased the AGE fluorescence compared to the positive control (MGO + CPC), showing that the level of glycation was lowered over time and with dose. The probable mechanism of ameliorating glycation may

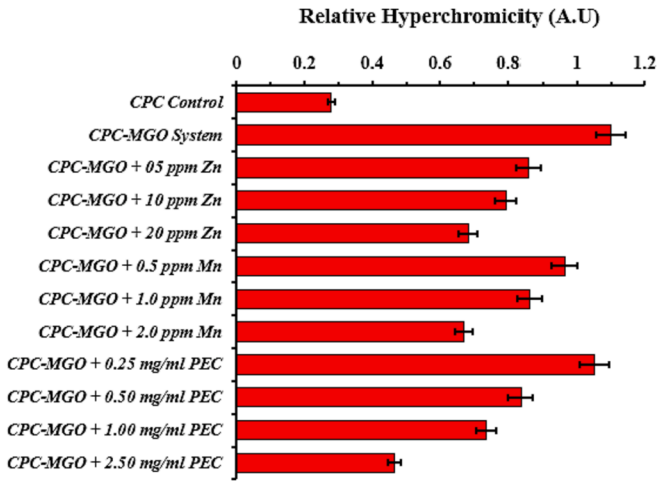

Fig. 5. Analyses of conformational changes by UV–Visible absorption spectroscopy. Relative hyperchromicity as a measure of tertiary structural change from control (CPC) incubated *in vitro* for 3 days at 37 °C in the absence or presence of 5 mmol/l methylglyoxal (MGO) or 5 mmol/l methylglyoxal and three concentrations of zinc or three concentrations of manganese or four concentrations of polyphenolic extract of chickpea seeds (PEC). The data have been generated from the spectra taken on the final day of incubation and measured in the wavelength range of 230–300 nm.

include either binding these trace metals to sites prone to non-enzymatic protein glycation or binding Zn/Mn with a glycating agent or both. The polyphenolic extract of chickpea seeds displayed good antiglycation ability, which increased with the increase in concentration and incubation time with the MGO-CPC system. There was a significant reduction in the formation of AGEs in the MGO-CPC model system in the presence of PEC on day 2 at all PEC doses, while the % inhibition of glycation on day 1 was about 20–40 % at higher extract doses of 0.5–2.0 mg/ml (Fig. 4c). On final day of incubation, the PEC incurred approximately 90 % of glycation reduction at 2 mg/ml concentration which can be attributed to its high polyphenol content.

Incubation of CPC with 5 mM MGO produced dramatic changes in intrinsic fluorescence that are prevented by all three putative antiglycation agents, though varying degrees. All the tested agents significantly affected these changes, which depicts meaningful participation as inhibitors in the first phase of the glycation cascade. However, it is impossible to completely rule out the possibility that the effect is attributable to suppressing the second stage of glycation processes, specifically the conversion of the Amadori products to AGEs by free radicals. These data suggest that Zn, Mn, and polyphenol extracts of chickpea seeds exert these effects through trapping of MGO and their antioxidant and free radical quenching capacity.

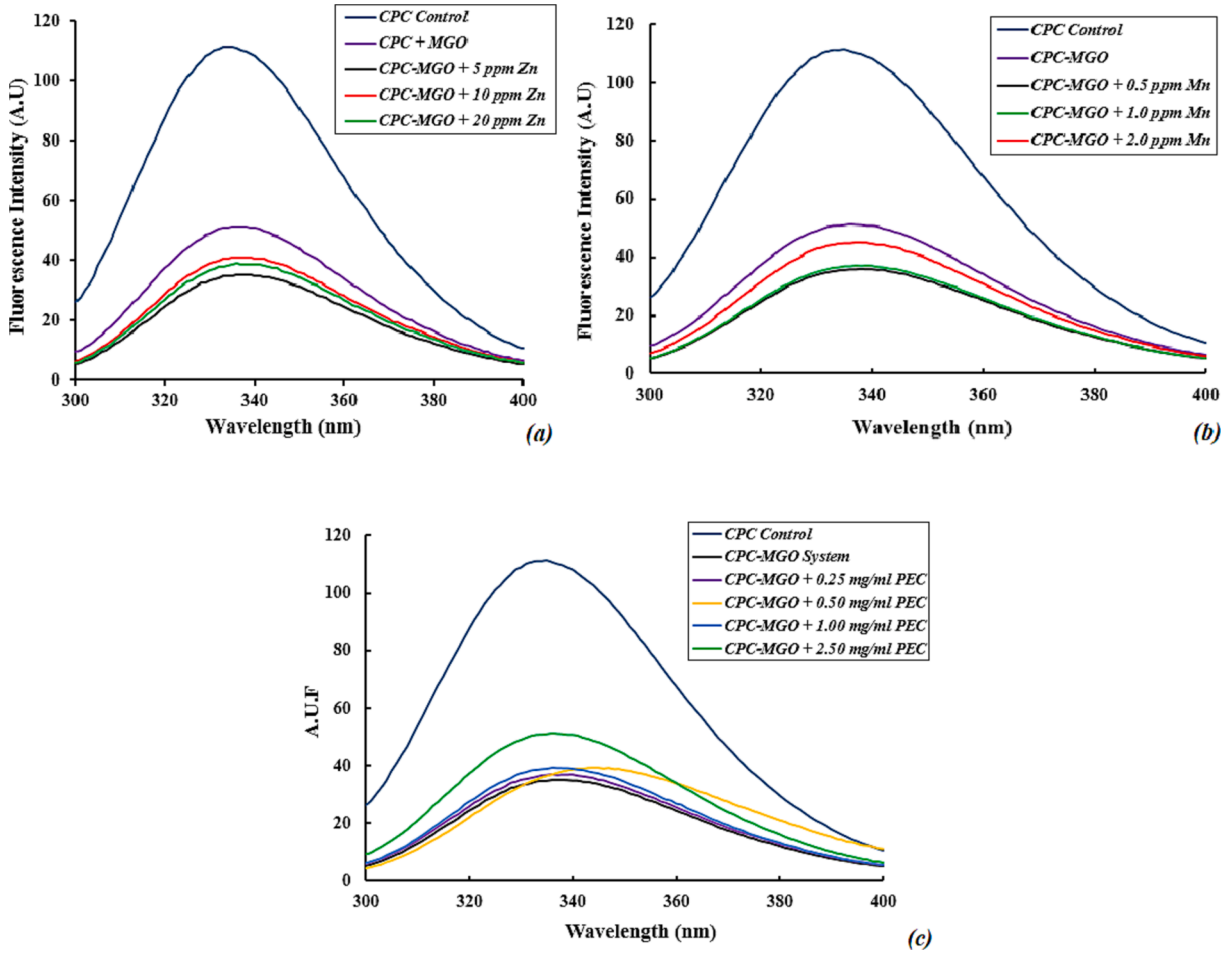

Fig. 6. Analyses of conformational changes by intrinsic fluorescence spectroscopy. Intrinsic fluorescence spectra from control (CPC) incubated *in vitro* for 3 days at 37 °C in the absence or presence of 5 mmol/l methylglyoxal (MGO) or 5 mmol/l methylglyoxal and (a) three concentrations of zinc or (b) three concentrations of manganese or (c) four concentrations of polyphenolic extract of chickpea seeds (PEC). Spectra were measured in the 300–400 nm wavelength range after excitation at 280 nm.

Table 2
Measurement of residual cysteine proteinase activity during incubation.

Sample	*% CPC Activity after an incubation time of*			
	0 days	*1 day*	*2 days*	*3 days*
CPC-MGO	89 ± 1.4	52.97 ± 1.3	27.72 ± 1.3	17.43 ± 0.7
CPC-MGO + 0.5 ppm Zn	90.98 ± 2.4	79.77 ± 2.1	63.68 ± 1.1	51.97 ± 1.2
CPC-MGO + 1.0 ppm Zn	93.41 ± 2.4	84.53 ± 1.2	72.64 ± 2.3	62.23 ± 1.1
CPC-MGO + 2.0 ppm Zn	96.54 ± 1.2	89.21 ± 1.1	78.54 ± 2.6	68.23 ± 2.4
CPC-MGO + 0.5 ppm Mn	90.21 ± 3.1	77.87 ± 3.3	61.43 ± 2.3	50.01 ± 1.7
CPC-MGO + 1.0 ppm Mn	92.64 ± 1.7	82.78 ± 2.5	70.88 ± 1.9	62.87 ± 3.4
CPC-MGO + 2.0 ppm Mn	97.42 ± 2.7	88.93 ± 4.2	79.03 ± 3.2	64.98 ± 2.2
CPC-MGO + 0.25 mg/ml PEC	92.64 ± 1.7	89.91 ± 3.2	79.79 ± 2.8	68.93 ± 3.1
CPC-MGO + 0.50 mg/ml PEC	94.31 ± 1.9	90.23 ± 2.8	82.71 ± 1.8	71.93 ± 2.7
CPC-MGO + 1.00 mg/ml PEC	97.49 ± 2.2	93.14 ± 4.1	85.11 ± 3.7	74.16 ± 3.2
CPC-MGO + 2.50 mg/ml PEC	98.01 ± 3.1	95.76 ± 3.9	88.31 ± 4.2	77.64 ± 2.7

Each value represents the average of three independent experiments performed in duplicates. The activity of native CPC in the absence of RDS was taken as 100 % for reference. Data are expressed as mean ± SEM (n = 3). [**CPC:** Chickpea cystatin; **PEC:** polyphenolic extract of chickpea; **ppm:** Parts per million **MGO:** Methylglyoxal; **Mn:** Manganese; **Zn**: Zinc].

3.5. Analysis of protein conformation changes

3.5.1. UV–Vis absorption spectroscopy

Using absorption spectroscopy, the MGO-induced changes in CPC structure and conformation were evaluated in relation to the chromophoric characteristics of the inhibitor. Upon modification with these MGO, an increase in absorbance over the whole range of spectrum was observed, the extent of which was dependent on the incubation period (Fig. 5**)**. The observed hyperchromicity in CPC can be attributed to a change in protein structure or conformation around aromatic groups. The variations observed in the hyperchromicity with MGO and Zn or Mn or PEC suggest significant differences in the extent of conformational alterations of CPC inflicted by reactive dicarbonyl species (MGO) and the micronutrients. The structural changes were significantly minimized in the presence of micronutrients which displayed concentration dependent effects as depicted in Fig. 5. The effects of Mn were more pronounced as compared to Zn, and similarly, PEC showed an excellent preventive effect, especially at higher doses of 2.5 mg/ml.

3.5.2. Intrinsic fluorescence spectroscopic measurements

Utilizing intrinsic fluorescence, which takes advantage of the proteins' aromatic amino acid residue content's fluorophore characteristics, is another intriguing method for monitoring protein conformations. Therefore, CPC samples' intrinsic fluorescence quenching (280 nm exc./ 335 nm em.) was assessed as another structure/conformation analysis indicator. As depicted in Fig. 6, incubation of CPC with MGO resulted in quenching of intrinsic fluorescence (around 70–75 %) relative to the native CPC control, the magnitude of which varied gradually with the time of incubation, with maximum quenching observed on the final day of incubation (day 3). The addition of micronutrients (Zn, Mn, and PEC) to the CPC-MGO system led to the retention and preservation of intrinsic fluorescence intensity, the effect of which was dependent on the concentrations of the respective micronutrient used. In the case of Zn, there was a slight but significant recovery of fluorescence intensity at a low dose of 5 ppm, which increased with the increase in the dose showing the highest curative effect at 20 ppm (Fig. 6a**)**. Similar pattern was observed in case of Mn which showed the resistive conformation changes at a small concentration of 0.5 ppm and peaking at 2 ppm as depicted in Fig. 6 **(b)**. The polyphenolic extract (PEC) also showed a good response toward the structural change resistance of CPC when incubated with MGO. The maximum effect was obtained with PEC concentrations of 1 and 2.5 mg/ml (Fig. 6c). Thus, the results suggest the structural changes in CPC induced by methylglyoxal are largely retained when co-incubated with micronutrients and the effects are concentration-dependently.

3.6. Measurements of cysteine protease inhibitory activity

The functional behaviour of the inhibitor isolated from chickpea seeds was also investigated during the study. The incubation of CPC with methylglyoxal led to its functional inactivation. Only 17.4 % activity was retained in the presence of MGO after 3 days of incubation. Co-incubation of CPC with increasing doses of Zn or Mn or PEC, protease inhibitory activity was attenuated, as shown in Table 2. Both micronutrients Zn and Mn, restored inhibitory activity in a concentration and time dependent manner. The residual activity of 68.23 and 64.98 % was measured for Zn and Mn, respectively after 3 days. Plant extract (PEC) also protected the functional inactivation caused by MGO and the activity was regained upto 77.64 %. The results also show that PEC was relatively more protective than Mn followed by Zn and could be related to its antioxidant or free radical scavenging potential.

4. Discussion

Flavonoids are the most researched class of polyphenolic chemicals and have been linked to a range of health benefits, including anti-inflammatory, anti-cancer, anti-allergic, anti-inflammatory, and gastroprotective effects (Tabrez, 2021; Ul Islam et al., 2021; Suhail et al., 2021). Similarly, proanthocyanidins (PC) are oligomeric or polymeric flavonoids consisting of flavan-3-ol units (Andersen-Civil et al., 2021). They have been shown to have anti-inflammatory, antiviral, antibacterial, and antioxidant activities in numerous *in vivo* tests (Andersen et al., 2021; Pandey and Rizvi, 2009).

Glycation is associated with the formation of glycoxidation products, cytotoxicity, and extensive cross-linking and polymerization of the proteins implicated in diabetic complications (Ahmed, 2005; Khan et al., 2020; Fournet et al., 2018). Therefore, inhibiting glycation is useful to avoid or lessen these complications. There are numerous natural compounds with antiglycation potential that might be suitable replacements for drugs that come with side effects. In this study, micronutrients such as Zn and Mn and polyphenol extract of chickpea seeds were examined for their glycation inhibitory effects using MGO-mediated glycation of thiol protease inhibitor (CPC) to get some crucial leads in developing inexpensive therapeutics for diabetic complications and neurological diseases . Although micronutrients are essential for metabolism and the preservation of tissue function, increasing intake can't be anticipated to have any impact on preventing or treating diseases that are not caused by micronutrient shortage. As shown in Figs. 1 and 2, both the micronutrients Zn and Mn were able to inhibit glycation induced structural and functional inactivation of CPC. Similar results were reported by Tarwadi and Agte against the glycation of bovine serum albumin (Tarwadi and Agte, 2011). The antiglycation potential of the polyphenol-rich extract of chickpeas (PEC) extract displayed good antiglycation

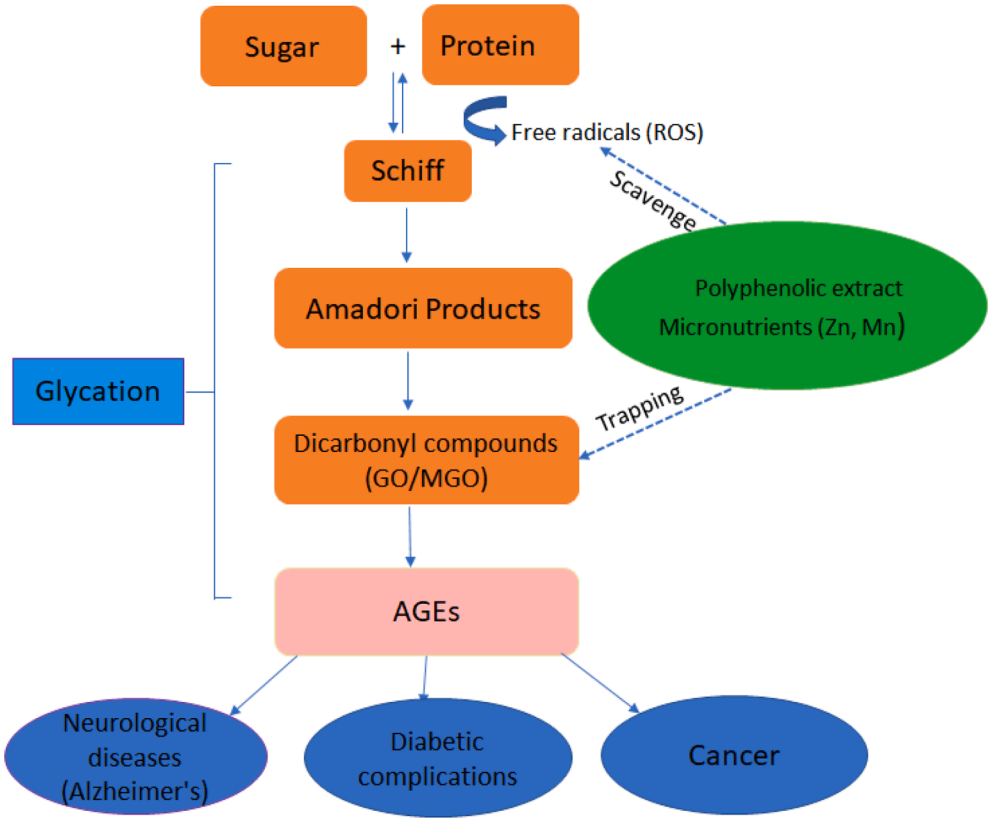

Fig. 7. Glycation reaction and their inhibition by plant extract and micronutrients (Zn/Mn).

ability by preventing CPC structure and inhibit the formation of advanced glycation end products (AGE) as shown in Fig. 3**.** Moreover, the protease inhibitory activity of CPC (cystatin) was also found to be preserved significantly upon coincubation with these nutraceuticals (Table 2). It is noteworthy to mention that polyphenols (e.g., proanthocyanins) are known to precipitate proteins and consequently inhibit enzymes (Griffiths, 1986). However, since we did not observe such inhibitory effect, we strongly assume that in the system of methylglyoxal-protein-polyphenols, the polyphenols might be interacting with methylglyoxal both being more reactive leaving much of the protein/enzyme intact. Moreover, low concentration of micronutrients (ppm) and PEC (0.25–2 mg/ml) was used to minimize the interference of pigments and small molecules. Furthermore, futuristic studies need to be conducted to explore this aspect of polyphenols-cystatin interaction activity. Overall, these results point towards the therapeutic potency of Zn, Mn, and PEC as antiglycation agents.

Oxidative processes plays major role in the formation of AGEs (Fu et al., 1994). This process can take place by two mechanisms: (1) through autoxidation of free sugars in the presence of oxygen to form reactive dicarbonyl compounds, which reacts with proteins forming highly reactive ketoamines, (2) oxidation of Amadori products, which gives rise to highly reactive protein enediols and protein dicarbonyls generating AGEs (Tupe et al., 2017). The antiglycative action of the phenolic compounds present in the plant extract has been credited to trapping capacity of dicarbonyls (Glyoxal and methylglyoxal) and antioxidant action over the scavenging of free radical and metal ion chelation (Peng et al., 2011; Srey et al., 2010). Studies have shown that zinc and manganese can reduce AGE formation by inhibiting the glycation reaction and scavenging free radicals (Tarwadi et al., 2011). Furthermore, a decrease in glycosylated hemoglobin by zinc supplementation in type 2 diabetic patients has been reported (Refaat & Shatha, 2006). Also, human cystatin cystatin C (CysC), a type of thiol protease inhibitor is co-deposited in amyloid plaques together with Aβ (Kaeser, et al., 2007). Increased CysC expression leads to protection in AD by inhibiting cysteine proteases, inducing autophagy and cell division and preventing amyloidogenesis (Kaur & Levy, 2012).

5. Conclusion

Diabetes is known to be characterized by hyperglycemia, a condition that leads to the development of AGEs. Thus, one of the main therapeutic targets for the management of diabetes complications is glycation. Although micronutrients are essential for metabolism and the preservation of tissue function, increasing intake cannot be anticipated to have any impact on preventing or treating diseases that are not caused by micronutrient shortage. The results suggest that Mn, Zn, and chickpeas extract possess antiglycation activity which helps retain the structure and function of physiologically important thiol protease inhibitor (CPC). Glycation of proteins and the formation of advanced glycation end products (AGEs) have been linked to a number of diseases, including Alzheimer's disease, Parkinson's disease, and other age-related neurodegenerative disorders. Furthermore, thiol protease inhibitor like cystatin C protect misfolding of Aβ proteins implicated in neurological disease like Alzheimer's. Thus, protecting the structure of thiol protease inhibitor against glycation by nutraceuticals like Mn, Zn and PEC could help to reduce diabetic complications and neurological diseases. Mechanistically, it could be correlates with antioxidant potential, free radical scavenging activity as well as dicarbonyl trapping activity of polyphenols and micronutrients (Fig. 7). Finally, this study should be viewed as a model for demonstrating the importance of healthy eating in diabetes and the potential for low-cost, comparatively non-toxic therapy for diabetes and amyloidosis. However, the outcomes of the current experiments are mostly suggestive, so further investigation is necessary in this field of study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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