Comparative study of antioxidant, metal chelating and antiglycation activities of *Momordica charantia* flesh and pulp fractions

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Abstract: *Momordica charantia* is commonly used as a vegetable and folk medicine in most parts of South Asia. This study aims to determine and compare the antioxidant, metal chelating and antiglycation activities of aqueous extracts of *M. charantia fruit* flesh (MCF) and fruit pulp (MCP) fractions. Our results show that MCP has pronounced DPPH and ABTS radical scavenging potential compared to MCF. In the antiglycation assay both fractions illustrated considerable inhibitory activities against the formation of AGEs induced by glucose with an efficacy of 75 and 67% with 150 µl of MCP and MCF extracts respectively, almost equal to 0.3mM amino guanidine. Results for metal catalysed protein fragmentation and autoxidative and glycoxidation assays demonstrate that MCF and MCP inhibited metal catalysed protein fragmentation. The percentage of relative standard deviation for three replicate measurements of 150 µl of MCF and MCP was ≤ 3.0% for antiglycation. The antioxidant assays with regression values of MCP (0.981 and 0.991) and MCF (0.967 and 0.999) were also recorded. We conclude that both extracts possess high antioxidant and antiglycation activities and are equally good sources of antioxidant and antiglycating agents.

Keywords: Fruits, antioxidant activity, antiglycation, SDS PAGE, *Momordica charantia.*

INTRODUCTION

Diabetes mellitus is a growing health burden worldwide. Its occurrence being doubled every 10 to 15 years and by 2010 around 250 million people were likely to be affected with diabetes worldwide (Mandrup-Poulsen, 2003). Diabetes is a metabolic disorder, characterised by hyperlipidaemia and chronic hyperglycaemia. Even though there have been important achievements in the control of hyperglycaemia through diet, hypoglycaemic drugs, the insulin pump, insulin and islet transplantation, the long-term worries of diabetes are still principal causes of death (Krolewski *et al*., 1985). Diabetic complications such as nephropathy, cataract, proliferative retinopathy, vasculopathy and atherosclerosis are direct results of accumulation of cross-linked advanced glycation end products (AGEs), created by non-enzymatic protein reactions with glucose and other reducing sugars (Gugliucci, 2000). The precise mechanism, by which AGE formation leads to these complications are not fully understood. However, the glycation process is accompanied by auto-oxidation of glucose and metal catalysed glycoxidation of Amadori products. The relationship between AGEs and diabetic complications has provoked a search for compounds proficient of inhibiting their formation (Rahbar and Figarola, 2003). Although some pharmacological compounds have strong inhibitory effects against the formation of AGEs such as aminoguanidine, aspirin, acetaminophen, and ibuprofen, but none have proved to be successful. Natural products are relatively safe for human consumption as compared to synthetic compounds. Therefore, plants might antiglycating agents. In this stare, in recent years the effect of plant extracts have been experienced on the formation of AGEs (Babu *et al*., 2008; Ardestani and Yazdanparast, 2007), but still vast botanical sources need to be explored for the control of diabetic complications**.** The herb *Momordica charantia* (MC) is cultivated right through the world for its use as a remedy (Chaiyasut and Chansakaow, 2007) and is frequently used as a vegetable in most Asian countries. The health benefits of MC have been documented in hundreds of studies in the last few decades (Satyawati and Gupta, 1987). *Momordica charantia* holds biologically active chemicals viz. saponins, glycosides, triterpenes, alkaloids, steroids and proteins (Raman, 1996).

Momordica charantia has been reported to possess antibacterial, antibiotic, antidiabetic, anti-inflammatory, antileukaemic, antimicrobial, antimutagenic, antioxidant, antitumor, antiulcer, antiviral, hypocholesterolaemic, hypotensive, hypotriglyceridaemic, hypoglycaemic, and insecticidal, properties (Omara *et al*., 2007; Das *et al*., 2006; Grover and Yadav, 2004). However, the role of MC in diabetes is of great importance as this plant lowers blood sugar, delays diabetic complications and is anti-infective as diabetics are recognized to be more vulnerable to infections (Grover and Yadav, 2004). The constituents believed to be responsible for these effects are proteins, steroids, and phenolic compounds (Ansari *et al*., 2005). Whilst the antioxidant activity of MC is relatively well known (Budrat and Shotipruk, 2009; Kubola and Siriamornpun, 2008; Semiz and Sen, 2007), literature on its antiglycation activity is scant and this has provided us a scope to explore its antiglycation properties. In this study, we have selected the fruit part of the herb

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and focused on the comparative activity study of aqueous extracts of the inner (pulp and seeds) and outer (green) parts.

MATERIALS AND METHODS

Plant material

Fresh green unripe plant *viz*., *Momordica charantia* was purchased from super market of Muzaffarabad, Azad Kashmir, Pakistan.

Preparation of extracts

Whole fresh, unripe MC fruit was thoroughly flushed under tap water and rinsed with distilled deionised water. The fruit was cut into half and the pulp (inner tissue and immature seeds) was scooped out with a spatula. The fruit flesh (green part) was cut into small pieces with a knife. Both fractions (7.5g each) were blended in a food processor using 100 ml of distilled deionised water for 15 min, filtered twice with Whatman No.1 filter paper and centrifuged at 20,000 rmp for 30 min. The supernatants were collected and stored at 4^{o}C till further use.

Evaluation of total antioxidant potential

DPPH free radical scavenging activity

DPPH free radical scavenging activity of *M. charantia* extracts was determined with slight modifications (You *et al*., 2006). 0.5 ml of MeOH (0.1 mM) solution of DPPH was mixed with 20-150µl of extracts, mixed with vortexed vigorously and left for 30 min in an incubator at 37°C and the volume was made up to 2 ml by the addition of methanol. Methanol used as a baseline control. The absorbance was recorded at 517 nm. 20-150µl water was used in place of the extracts as a control and the %age scavenging activity was calculated by equation: % =[(Ao-Ai)/Ao]*100; Where Ao is absorbance of control and Ai is absorbance in the presence of extracts.

ABTS radical cation decolorisation assay

Assay described by Re *et al*., was adopted after slight modifications (Re *et al*., 1999). ABTS (2,2- azinobis[3-ethylbenzothiazoline-6-sulfonate]) in water (3 mM final concentration) was oxidized *via* (2.5 mM) potassium persulfate for at least 12 h in the dark. $ABTS^{\cdot+}$ solution was diluted with distilled deionised water to an absorbance of 2.51 ± 0.05. Absorbance of $ABTS^{\cdot+}$ (1 ml) as a control with 2ml of distilled deionised water (Ao) was recorded at 734 nm. For percent scavenging activity of the extracts 1ml of $ABTS^{\cdot+}$ and 20-100 µl of extracts were mixed and incubated for 10 min at room temperature followed by the addition of water to make the volume of reaction mixture up to 3ml. The absorbance (Ai) of the reaction mixture was recorded. Percent scavenging potential (%) of the extracts was calculated by the equation; %=(Ao-Ai)/Ao*100.

Total phenolic contents

Phenolic contents (mg/100ml of extracts) were determined using the Folin-Ciocalteu reagent method expressed by Zhou and Yu with slight modifications (Zhou and Yu, 2006). The reaction mixture contained extracts (100µl), Folin-Ciocalteu reagent (100µl), and 20% sodium carbonate (3 ml). Reaction mixture was incubated at room temperature for 1h and the absorbance of deep blue complex was measured at 765 nm. Phenolic contents (mg/100ml) of the extracts were determined form the standard curve of gallic acid constructed in the range of 0.01-1.0 mg/ml.

Total flavonoid content

Estimation of total flavonoid contents of the extracts was established by the method illustrated by (Zou *et al*., 2004). The reaction mixture cocktail containing extracts (500 µl), distilled water (2ml) and 5% $NaNO_2$ (0.15 ml) was incubated at room temperature for 6 min. After incubation 10% $AlCl_3$ (0.15ml) solution was added, placed for a further 6 min at room temperature, followed by the addition of 2ml of 4% NaOH solution. Immediately, after the addition of water to the sample to bring the final volume to 10 ml, the mixture was mixed and stands for another 15 min. The absorbance of the reaction mixture was measured at 510 nm. Rutin was used as a standard reference. The calibration curve for rutin standard solutions ranged from 0.066-0.166 mg/ml and was used to calculate the flavonoid content of the extracts (mg/100ml).

Metal Chelating Activities

Evaluation of Iron (II) chelating activity

The Fe^{2+} chelation of the extracts was investigated by the modified protocol as described by Dinis and his co workers (Dinis *et al*., 1994). 50-150 µl extracts were added to a 1 ml of ferrous sulphate (2 mM). The reaction was started by the addition of 1 ml of ferrozine (0.25 mM), mixture was shaken vigorously and left at room temperature for 10 min. Ferrozine reacts with divalent iron to form a stable magenta complex. In controls, 1 ml of distilled deionised water was used to quantify the reaction mixture. Absorbance of the mixture in the presence (Ai) and in the absence of the extracts (Ao) was recorded at 517 nm. The chelating activity was calculated by the formula: Chelating rate (%) = Ao-Ai/Ao*100

Evaluation of Copper (II) chelating activity

Metal chelating activity of the extracts was also investigated by the described method of Wettasinghe, (2002). A solution of copper sulphate (0.5mM) was prepared in hexamine-HCl buffer (10 mM) containing calcium chloride (10mM: pH 5.0). 1mM tetramethylmureoxide solution was also prepared in the same buffer. 0.5ml extracts/controls were mixed followed by the addition of 0.1ml of tetramethylmurexide. Absorbance of the mixtures was recorded at 460 nm and 530 nm, and the ratio of A_{530} to A_{460} nm was calculated. These absorbance ratios were then transformed to equivalent free copper (II) concentrations via standard

curve of free copper (II) concentration/absorbance ratio. The concentration of chelated copper (II) was measured by calculating the difference between the free copper (II) and the total copper (II) concentrations as shown in following formula: % copper (II) chelation capacity = (concentration of chelated copper (II)/concentration of total copper (II)*100

In vitro glycation of proteins

In vitro protein glycation and antiglycation activity of the extracts was measured using 10 or 15% sodium dodecyl polyacrylamide gel electrophoresis (Ahmad *et al*., 2007). Lysozyme (10 mg/ml) was dissolved in 50 mM phosphate buffer (pH 7.4) with sodium azide (0.02%) to inhibit the bacterial growth and was mixed with 0.5 M glucose in the absence and presence of the extracts (50-150 µl). A blank was prepared by mixing 0.5ml of lysozyme solution with 1ml of buffer. Control reaction, in the absence of extracts (protective inhibitors) was also set up using 0.3 mM final concentration of aminoguanidine (AG). All reaction mixtures were incubated at 37°C for four weeks. All samples were kept frozen at -20°C until further analysis. Protein samples were diluted with 4X SDS loading dye and then boiled for 5 min at 100°C. Bromophenol blue (3 µl) was loaded into wells followed by glycated protein samples (10 µl) and then subjected to electrophoresis using the mini-Protean3 apparatus (Bio-Rad Laboratories, UK). Gels were stained and photographed were taken using a GBOX Chemi HR16 and gel documentation and a Syngene UK analysis system was used for the image analysis. Band density for each sample was compared within the same gel. Percent inhibition was calculated by the formula: % inhibition= (Do-Di)/Do*100. Where Do and Di are the densities of the bands in the absence and in the presence of inhibitors respectively?

Inhibition of metal catalysed protein fragmentation and autoxidative glycation and glycoxidation reactions

The assay was performed as described by Ahmad *et al*. (2007). Bovine Serum Albumin (10 mg/ml) dissolved in 50 mM phosphate buffer (pH 7.4) containing sodium azide (0.2 %), was pre-incubated with 0.5 M glucose, 20 µM Cu^{2+} in the presence and absence of extracts (100µl MCF and MCP extracts) for 8 weeks. Blank samples

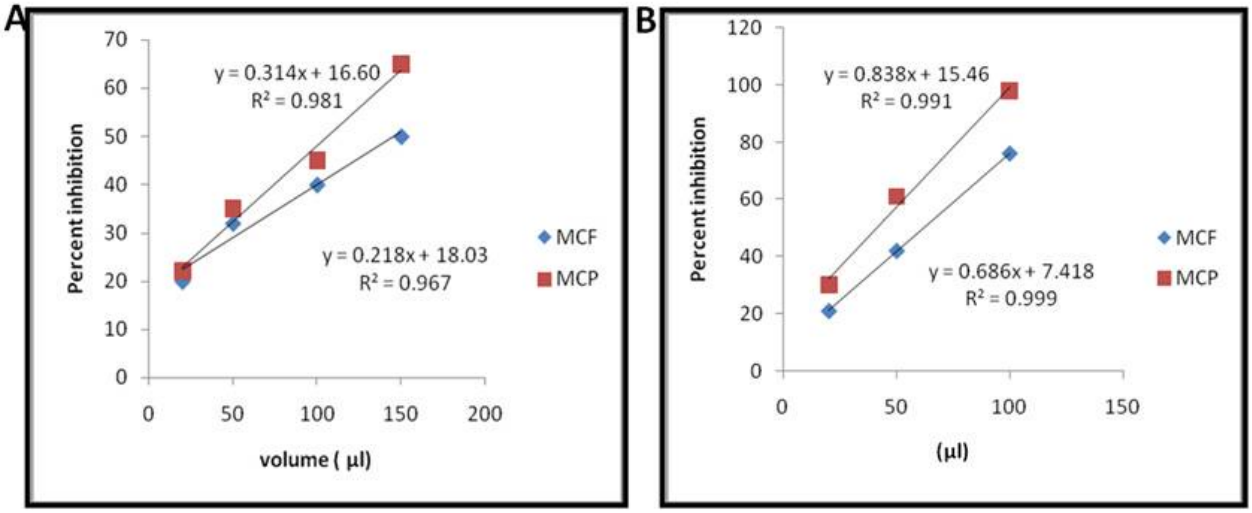

Fig. 1: Comparison of DPPH and ABTS free radical scavenging capacity for MCF and MCP (20-100µl). (A) Indicates DPPH activity of MCF and MCP; (B) indicates ABTS radical scavenging capacity for MCF and MCP.

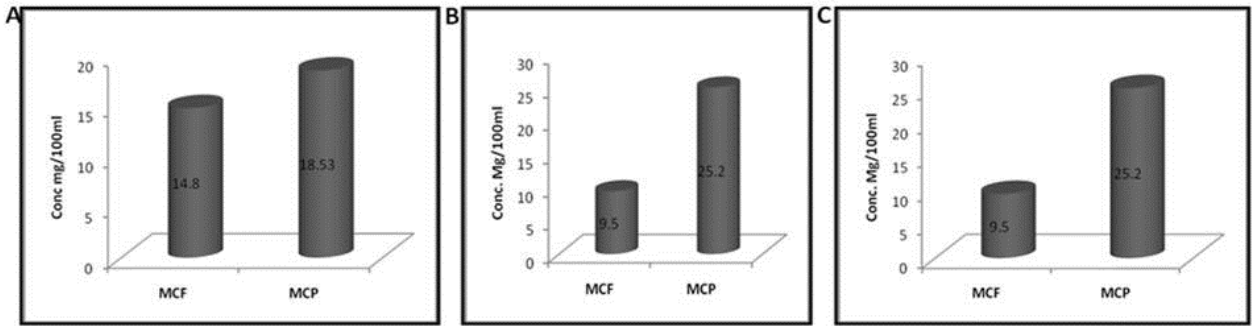

Fig. 2: Phenolic, flavonoid and iron contents in 1mg/100 ml of MCF and MCP extracts. (A) Gallic acid equivalent phenolic content (B) Rutin equivalent flavonoid content (C) Iron content

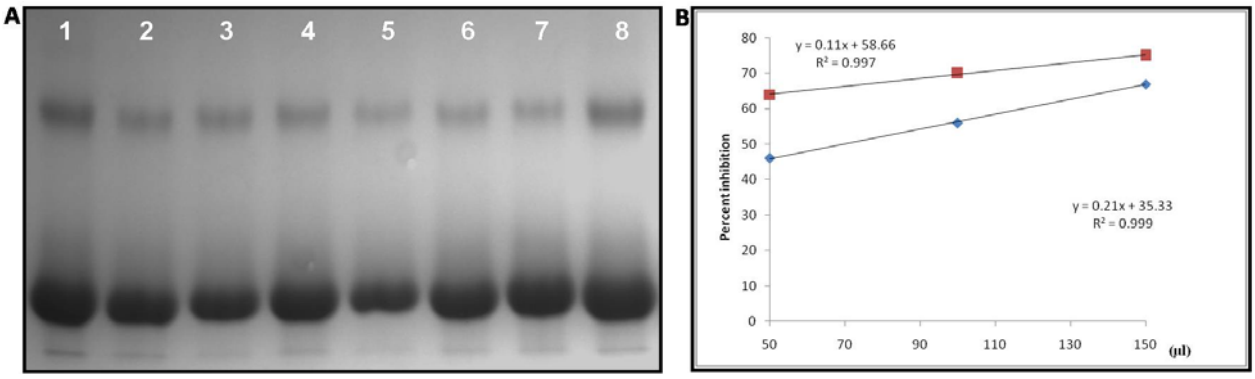

Fig. 3: (A) Gel showing lysozyme incubated in the presence of 0.5 M glucose (lane 1-8). Lane 8 shows lysozyme incubated with 0.5 M glucose, effect of 0.3 mM AG is shown in lane 7 and lane 1-6 show effect of different volumes of MCF (lane 4-6) and MCP (lane 1-3), (B) Image analysis of gels to show percentage inhibition of crossed linked AGE formation with different concentrations of MCF and MCP and AG as a positive control.

containing BSA alone and control with BSA and glucose were also incubated at the same time. Sample analysis was carried out using sodium SDS-PAGE as described above.

STATISTICAL ANALYSIS

Each experiment was repeated in triplicates and Mean ± Standard Deviation (M±SD) from absolute data were measured through on line Standard deviation calculator http://easycalculation.com/statistics/standard-deviation.php. The percentage of relative standard deviation was also calculated to express the precision.

RESULTS

Both extracts (MCF and MCP) of *M. charantia* in the range of 20-150 µl were analysed for DPPH free radical scavenging activity. Experimental study depicted in fig. 1.A, shows that DPPH scavenging activity increases in a dose dependent manner. *Momordica charantia* pulp, often discarded in Asian cooking, showed pronounced DPPH scavenging activity compared to MCF. The study demonstrates that MCP could be a significant food product with antioxidant activity like MCF. The scavenging activity of 0.1 mg/100 ml of a known antioxidant such as ascorbic acid was used as a positive control and was 87% (results not shown).

The antioxidant activity of the extracts (20-100 µl) was also measured by $ABTS^{\cdot+}$ decolourization assay. Results of the study presented in fig. 1.B show that both extracts acquire significant antioxidant potential. However, MCP again showed stronger action compared to MCF.

In this study we have determined concentration of phenolic contents (mg/100ml of extract) using the gallic acid calibration graph. The phenolic content was higher in MCP (18.5 mg/100 ml) then MCF (14.0mg/ 100ml). Results have been shown in fig. 2.A. The potent antioxidant activities may be endorsed to the phenolic content of the extracts.

Flavonoid contents were evaluated as mg/100ml of extracts. Under described experimental conditions, standard curve for rutin was plotted and found to be linear with the range of 0.024-0.4 mg/ml. As shown in fig. 2.B, a reasonable quantity of flavonoids was found in both extracts (MCF, 0.48 and MCP 3.8 mg/100 ml). In present study both extracts have shown less quantity of flavonoids compared to phenolic contents. However, the presence of flavonoids contributed towards the antioxidant potential of the extracts.

The results of the study showed that both extracts contained significant amount of Fe^{2+} (MCF, 9.5 and MCP, 25.1 mg/100ml). Results are presented in fig. 2.C. We believe that the metal selected for the metal chelating activity was not suitable. Therefore, we have used Cu^{2+} and determined the metal chelating potential of both extracts by the second method. Results of the study demonstrate that Cu^{2+} chelating potential MCP and MCF was 80 and 40% respectively with 10 µl extracts.

To the best of authors' knowledge, this is the foremost study signifying the ability of MC fruit fraction extracts to inhibit formation of crossed linked AGEs. Glucose was used as reducing sugar and lysozyme was selected as a protein model as it forms oligomers more readily and can easily be detected by SDS-PAGE. Samples were incubated for four weeks at 37°C because reaction between glucose and lysozyme is quite slow. Both extracts (50-150µl) have shown significant inhibition comparable to 0.3mM of AG. Percent inhibition for both extracts was in a dose-dependent fashion. This inhibitory effect may due to the antioxidant potential of the extracts, which could be due to the presence of total phenolic and flavonoid contents in the extracts. Results are shown in

fig. 3.A and 3.B. fig. 3.A gel showing lysozyme incubated in the presence of 0.5 M glucose (lane 1-8). Lane 8 shows lysozyme incubated with 0.5M glucose, effect of 0.3 mM AG is shown in lane 7 and lane 1-6 show effect of different volumes of MCF (lane 4-6) and MCP (lane 1-3). These molecules can damage biomolecules such as proteins, lipids and nucleic acids and eventually lead to the formation of AGEs. Metal ion catalysed autoxidative glycation and glycoxidation reactions play significant role in diabetic complications. In this study effect of MCF and MCP extracts (100 µl) was explored on the inhibition of metal catalysed protein fragmentation and autoxidative glycation and glycoxidation reactions. Results demonstrate that glycation of BSA in the presence of Cu^{2+} ions generates free radicals that cause protein fragmentation (fig. 3.A lane 3). This fragmentation was inhibited in the presence of MCF and MCP extracts (fig. 4, lane 4 and 5 respectively). Current research also showed that BSA incubated in the presence of glucose produces a small amount of fragmentation (fig. 3.A lane 2) not visible for BSA incubated alone (fig. 3.A, lane 1).

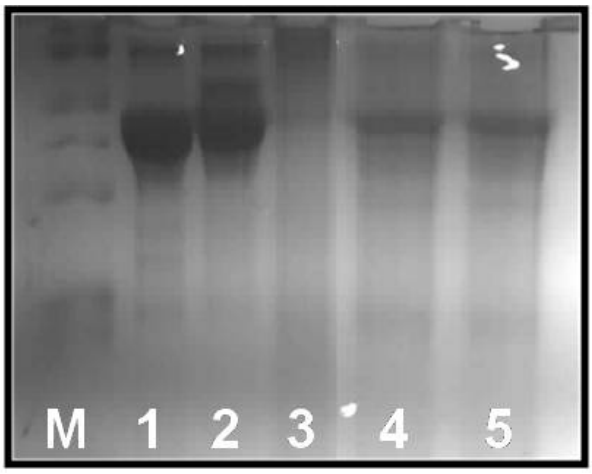

Fig. 4: Gel showing BSA incubated for 8 weeks alone (lane 1) or in the presence of 0.5M glucose (lane 2). BSA was also incubated in the presence of 0.5 M glucose and 20 µM Cu^{2+} and MCF and MCP (lane 3, 4 and 5 respectively).

DISCUSSION

Free radicals can be generated by biochemical reactions within body tissues but can also be derived from external sources like food, drugs, or from environmental pollution. Free radicals catalysed oxidative reactions are important contributors in the AGEs formation (Takagi *et al*., 1995)**.** Since the AGEs formation is facilitated in the presence of oxidative reactions, antioxidants or metal-chelators may retard the process of AGE formation by avoiding further oxidation of Amadori product and metal-catalyzed glucose oxidation. Screening of natural antioxidants has received considerable attention lately with the aim of discovering novel antioxidants from natural sources that may offer therapeutic potential. In current research the antioxidant potential of both fruit fractions (MCF and MCP) were studied by different methods commonly used to evaluate total antioxidant potential. Recently, attention has focused on natural products and herbal extracts for their antioxidant activity and some have proven to be effective to different extents (Kim and Kim, 2003). Antioxidants are classified as chelators of metal ions, free radical terminators or oxygen scavengers that react with oxygen in closed system. Phenolic antioxidants are integrated in the class of free radical terminators. Natural antioxidants are mainly polyphenolic and plant phenolics compounds that may crop up in all parts of the plant. Flavonoid and phenolic compounds are the core antioxidant components of vegetables and fruits (Huang *et al*., 1998). Investigation of natural phenolic compounds from plants is a substitute to synthetic antioxidants. Natural products with metal chelating potential could have beneficial effects on metal catalysed biochemical reactions like protein fragmentation and auto oxidative glycation and glycoxidation reactions. A foremost outcome of hyperglycaemia is excessive glycation of proteins consequential in various protein-protein crosslink's and non-cross linked structures. However, mechanism of AGE formation is quite complex making it hard to recognize specific chemical products (Hatfield, 2005). Different inhibitors can suppress AGE formation at different glycation stages and are classified as: sugar competitors**,** protein competitors (Argirova and Ortwerth, 2003), Amadorins (Metz *et al*., 2003) antioxidants (Abou-Seif and Youssef, 2004), transition metal chelators (Price, Rhett, Thorpe, and Baynes, 2001), reactive carbonyle scavengers (Yamagishi *et al*., 2008), and AGE cross-link breakers (Cooper *et al*., 2000). Almost all studies regarding inhibition of AGE formation are designed to target either specific or multiple steps of glycation. Although, the synthetic glycation inhibitors offer capable therapeutic approaches to prevent diabetic complications, their complete success in this regard is still a challenge for researchers. In current decades, attention has been focussed on natural plant sources preventing protein glycation and its ultimate consequences. Natural products with antioxidant and metal chelating activity are often strong antiglycating agents. Based on the antioxidant and metal chelating potential of MC extracts, we studied the antiglycation activity of the MC fruit fraction extracts. In addition to the direct glycation reaction involving sugar and protein, glucose and Amadori products are slowly oxidised by transition metal-catalysed reactions to form ketoaldehydes and dicarbonyl compounds respectively.

CONCLUSIONS

This study shows that both MCF and MCP extracts can effectively protect glucose mediated protein glycation *in vitro* and also inhibited metal catalysed protein

fragmentation and glucose autoxidation. Additional studies are needed to characterise the bioactive compounds responsible for the observed activity. The present findings suggest that aqueous extracts of MCF and MCP may be beneficial for preventing diabetic complications. The evidence that MCF and MCP can avoid protein glycation required to be investigated at the clinical level to conclude whether supplementation can reduce oxidative stress, lower levels of AGEs, and thereby reduces the incidence of diabetic problems in the diabetic patient population.

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