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Sri Lankan black tea (*Camellia sinensis* L.) inhibits the methylglyoxal mediated protein glycation and potentiates its reversing activity *in vitro*

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ABSTRACT

Objective: To evaluate inhibitory activity of methylglyoxal (MGO) mediated protein glycation and ability to potentiate its reversing activity and range of antioxidant properties of Sri Lankan low grown orthodox orange pekoe grade black tea.

Methods: Freeze dried black tea brew (BTB) was used as the sample in this study. Anti-glycation and glycation reversing activity was studied in bovine serum albumin (BSA)-MGO model. Antioxidant properties were studied using total polyphenolic content, total flavonoid content, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, 1,1-diphenyl-2-picrylhydrazine and ferric reducing antioxidant power *in vitro* antioxidant assays.

Results: The results demonstrated significant ($P < 0.05$) and dose dependant inhibition of BSA-MGO glycation [IC_{50}: (164.30 ± 4.85) μg/mL], potentiating of its reversing activity [EC_{50}: (235.39 ± 5.37) μg/mL] and marked antioxidant properties [total polyphenolic content: (119.55 ± 9.97) mg gallic acid equivalents/g BTB; total flavonoid content: (6.04 ± 1.26) mg quercetin equivalents/g BTB; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, 1,1-diphenyl-2-picrylhydrazine and ferric reducing antioxidant power: (3.29 ± 0.06), (1.95 ± 0.15) and (1.31 ± 0.19) mmol Trolox equivalents/g BTB, respectively]. No correlations were observed between antioxidant activity and BSA-MGO glycation.

Conclusions: The novel properties observed for Sri Lankan orange pekoe grade black tea indicate its usefulness as a supplementary beverage in managing MGO and advanced glycation end products related diseases and ailments.

1. Introduction

Glycation, non-enzymatic glycosylation of proteins by glucose is a relatively slow and complex series of endogenous reactions occurring in the body, giving rise to various different glycotoxins which are known as advanced glycation end products (AGEs) [1-5]. Accumulation of AGEs is now implicated as a major factor in the pathogenesis of long term complications of diabetes such as retinopathy, nephropathy and end stage renal disease[1-5]. AGEs are also linked to mechanisms responsible for development and progression of non-diabetic nephropathy, macro-vascular disease, cancer, rheumatic arthritis, osteoarthritis, atherosclerosis, cataract, neurodegenerative diseases including Alzheimer's disease, Parkinson disease and amyotrophic lateral sclerosis and age-related cognitive decline[2,4]. Further, the formation of AGEs is known to progressively increase with normal ageing, even in the absence of disease[4].

Low molecular weight carbonyl compounds such as glyoxal and methylglyoxal (MGO), most importantly MGO behave as precursors of AGEs[4-6]. They form adducts on proteins, inducing cellular dysfunctions leading to long term diabetes complications and other age related diseases[2,4-6]. Thus, pharmaceutical agents (natural and/

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or synthetic) that inhibits the formation of AGEs or AGEs reversing agents is of great value in the treatment and/or management of various seemingly unrelated diseases[2,4]. Few pharmaceuticals are reported to act as AGEs inhibitors through attenuation of glycoxidations and/or carbonyl trapping: aminoguanidine, metformin, carnosine, homocarnosine, pyridoxamine (vitamin B_6), thiamine pyrophosphate and aspirin[2,4]. However, some of these agents have low potency and often associated with undesirable side effects[4]. Accordingly, there is a renewed interest, in recent years, to identify and develop novel safe drugs and dietary beverage supplements preferably from plant sources which can inhibit the formation of AGEs and/or their subsequent breaking (reversing activity) to mitigate the complications in AGEs associated diseases/ disorders.

In this regard, we previously showed that Sri Lankan low grown orthodox orange pekoe (OP) grade black tea possess marked *in vitro* antiglycation and crosslink breaking activities when evaluated on bovine serum albumin/glucose system using fluorescence spectroscopy[7]. However, in this study, it was not possible to ascertain whether this variety of Sri Lankan black tea possess the ability to inhibit the MGO mediated protein glycation and to potentiate its reversing activity. Essentially, the glycosylation process occurs in three stages: early stage, which leads to the production of Amadoric compound/ products; middle stage, which involves in the production of reactive dicarbonyl compounds/species such as MGO, glyoxal and glucosone via auto oxidation of glucose and glycoxidation of Amadori compounds; and last stage, where dicarbonyls undergo further glycoxidation to form AGEs and their subsequent cross-linking[2,4]. Thus, this study was initiated to investigate the presence or absence of MGO mediated protein glycation, its reversing activity and antioxidant potential of Sri Lankan low grown orthodox OP grade black tea using series of *in vitro* assays.

2. Materials and methods

2.1. Sources of tea and processing technique

Top most immature leaves and buds of *Camellia sinensis* (L.) O. Kunty plucked from the plantation of St. Jochim's tea estate of the Tea Research Institute, Hedalla, Ratnapura, Sri Lanka (29 m above mean sea level; low grown) were used to process OP grade black tea by orthodox rotovane technique[8] at the estate factory.

2.2. Chemicals and reagents

BSA, MGO, Trolox, quercetin, rutin, gallic acid, potassium persulfate, Folin-Ciocalteu phenol reagent, trichloroacetic acid, 2,4,6-tripyridyl-s-triazine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 1,1-diphenyl-2-picrylhydrazine (DPPH) were obtained from Sigma-Aldrich, USA. All the other chemicals used for the preparation of buffers and solvents were of analytical grade.

2.3. Sieve analysis

The composition of true to size particles defined for the OP grade black tea[9] was determined by using a sieve shaker (Retsch AS 200, Retsch ambtt, Germany) with a standard set of sieves (shaking time: 10 min and starting speed: 50 vibrations/min).

2.4. Organoleptic analysis

Typical characteristics (aroma, flavor, liquor, colour and taste) belonging to the grade and agro- climatic elevation were assessed by well experienced professional tea tasters of the Tea Tasting Unit, Sri Lanka Tea board, Colpetty, Sri Lanka.

2.5. Preparation of black tea brew (BTB) sample

BTB was prepared according to International Organization for Standardization standards[10] by adding 2 g of OP grade black tea to 100 mL of boiling water and brewed for 5 min[10]. BTB was squeezed through a muslin cloth and freeze dried. The freeze dried product was stored in a brown coloured air tight container at 4 ℃ and used for the following bioassays.

2.6. BSA-MGO glycation inhibitory activity of BTB

The MGO mediated protein glycation inhibitory activity of BTB was performed according to the method of Lunceford and Gugliucci[11] with some modifications. Reaction volume of 1 mL containing 1 mg BSA, 5 mmol/L MGO and different concentrations of BTB (12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 μg/mL; n = 6) in 0.1 mol/L phosphate buffer pH 7.4 was incubated at 37 ℃ for 6 days. The test solution also contained 0.2 g/L NaN_3 to assure an aseptic condition. Rutin was used as the positive control (6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 μg/mL; n = 6). After the incubation, florescence intensity of samples was measured using a spectroflurometer (Amino-Bowman®, Thermo Spectronic, USA) with excitation and emission wave lengths of 370 nm and 440 nm respectively. The following equation was used for calculation of BSA-MGO glycation inhibitory activity of BTB and rutin.

$$\text{Inhibition } (\%) = [(F_c - F_b) - (F_s - F_{sb}) / (F_c - F_b)] \times 100$$

Where, F_c is the florescence of the control (incubated BSA and MGO), F_b is the florescence of the blank (incubated BSA alone), F_s

is the florescence of the positive control (rutin) or BTB incubated with BSA and MGO and F_{sb} is the florescence of the sample blank (incubated BSA with BTB or rutin).

2.7. BSA-MGO glycation reversing activity of BTB

The MGO mediated protein glycation reversing activity of BTB was performed according to the method of Premakumara *et al.* and Lunceford and Gugliucci with modifications[11,12]. Reaction mixture containing 1 mg BSA and 5 mmol/L MGO in 1 mL of 0.1 mol/L phosphate buffer pH 7.4 was incubated at 37 ℃ for 6 days. The test solution also contained 0.2 g/L NaN_3 to assure an aseptic condition. Then, 600 μL of aliquots and 60 μL of 100% (w/v) trichloroacetic acid were added to 1.5 mL eppendorf tubes. The reaction mixture was stirred, centrifuged (15000 r/min) at 4 ℃ for 4 min and supernatants were removed. The precipitates were dissolved in 0.1 mol/L phosphate buffer (pH 7.4) and the reaction volume of 1 mL was maintained with the addition of different concentrations of BTB (25, 50, 100, 200 and 400 μg/mL; n = 6). Then, samples were incubated at 37 ℃ for 6 days. After the incubation, florescence intensity of samples were measured using a spectroflurometer (Amino-Bowman®, Thermo Spectronic, USA) at an excitation and emission wave lengths of 370 nm and 440 nm, respectively. The following equation was used for calculation of percentage BSA-MGO glycation products reversing activity of samples.

BSA-MGO glycation products reversing activity (%) = $[(F_c - F_b) - (F_s - F_{sb}) / (F_c - F_b)] \times 100$

Where, F_c is the florescence of incubated BSA and MGO (control), F_b is the florescence of incubated BSA alone (blank), F_s is the florescence of the incubated BSA, MGO and BTB and F_{sb} is the florescence of incubated BSA with BTB.

2.8. Antioxidant properties of BTB

2.8.1. Total polyphenolic content

The total polyphenolic content (n = 6) of BTB was determined according to the method of Singleton *et al.* using 96 well micro plates[13]. A reaction volume of 200 μL containing 20 μL of BTB (0.25 and 0.5 mg/mL), 110 μL of ten times diluted freshly prepared Folin-Ciocalteu reagent and 70 μL of 10% sodium carbonate were incubated at room temperature [(25 ± 2) ℃] for 30 min and the absorbance was recorded at 765 nm. Gallic acid was used as the standard (12.5, 25.0, 50.0, 100.0 and 200.0 μg/mL) and TPC was expressed as mg gallic acid equivalents per gram of freeze dried BTB.

2.8.2. Total flavonoid content

Total flavonoid content of BTB was determined by aluminium chloride method Siddhuraju and Becker[14] using 96 well micro plates. One hundred micro liters of 2% aluminium chloride in methanol solution was added to 100 μL of BTB in methanol (0.25 and 0.5 mg/mL). The reaction mixture was incubated for 10 min at room temperature [(25 ± 2) ℃] and the absorbance was measured at 367 nm. Quercetin was used as the standard (7.81, 15.62, 31.25, 62.50 and 125.00 μg/mL) and TFC of BTB was expressed as mg quercetin equivalents per gram of freeze dried BTB.

2.8.3. Ferric reducing antioxidant power

Ferric reducing antioxidant power of BTB was carried out with slight modifications to the method described by Benzie and Szeto[15] in 96 well micro plates. The ratio of 10:1:1 of 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tripyridyl-s-triazine solution (prepared in 40 mmol/L HCl) and 20 mmol/L $FeCl_3.6H_2O$ was used in preparation of working FRAP reagent. Reaction volume of 200 μL containing 20 μL of BTB (0.125, 0.250 and 0.500 mg/mL), 30 μL acetate buffer and 150 μL working FRAP reagent were incubated for 8 min at room temperature [(30 ± 2) ℃]. Then, the absorbance was measured at 600 nm. Trolox was used as the standard and FRAP of samples were presented as mmol Trolox per gram of freeze dried BTB.

2.8.4. DPPH radical scavenging activity

The DPPH radical scavenging activity of BTB was performed in 96 well micro plates. The method of Blois[16] with some modifications was used. Reaction volumes of 200 μL, containing different concentrations (12.5, 25.0, 50.0, 100.0 and 200.0 μg/mL) of BTB and 125 μmol/L of DPPH radical were incubated at (25 ± 2) ℃ for 15 min. Then, the absorbance was measured at 517 nm. Trolox was used as the standard and different concentrations (3.125, 6.250, 12.500, 25.000 and 50.000 μg/mL) were used in the assay. Results were expressed as Trolox equivalents antioxidant capacity in mmol Trolox per gram of freeze dried BTB.

2.8.5. $ABTS^+$ radical scavenging activity

The $ABTS^+$ radical scavenging activity of BTB was performed in 96 well micro plates and the method described by Re *et al.*[17] was used with some modifications. A reaction volume of 200 μL containing different concentrations (2.5, 5.0, 10.0, 20.0 and 40.0 μg/mL) of BTB and 40 μmol/L of $ABTS^+$ radical was incubated for 10 min at (25 ± 2) ℃ and the absorbance was recorded at 734 nm. The ABTS radical was produced with the reaction of 10 mmol/L ABTS in potassium persulfate at 37 ℃ for 16 h in dark condition. Trolox was used as the standard and different concentrations (3.12, 6.25, 12.50, 25.00 and 50.00 μg/mL) were used in the assay. Results were expressed as Trolox equivalents antioxidant capacity in mmol Trolox per gram of freeze dried BTB.

2.9. Statistical analysis

Data presented as mean ± SD. SAS version 6.12 was used in analysis of data. One way ANOVA was used and mean separation was performed using Duncan's multiple range test. Correlation analysis was carried out with the Pearson's correlation coefficient and $P < 0.05$ was considered as significant.

3. Results

Sieve analysis showed that 83.5% of tea particles were of true size (2000 – 4000 μm) for OP grade of Sri Lankan orthodox black tea. Organoleptic analysis revealed that the tea sample possessed typical characteristics of low grown OP grade black tea.

The results obtained with the *in vitro* MGO mediated protein glycation inhibitory activity of BTB of OP grade black tea and reference drug rutin are summarized in Tables 1 and 2. As shown in Table 1, BTB induced profound *in vitro* MGO mediated protein glycation inhibitory activity with an IC_{50} = (164.30 ± 4.85) μg/mL. This effect was dose dependent (r^2 = 0.98). The reference drug, rutin, exhibited significant ($P < 0.05$) and dose dependent (r^2 = 0.97) MGO mediated protein glycation inhibitory activity which was 2.5 fold more potent [IC_{50} = (63.36 ± 0.67) μg/mL] than BTB.

Table 1
BSA-MGO glycation inhibitory activity of BTB.

Concentration (μg/mL)	% Inhibition
12.5	6.38 ± 1.26
25.0	13.53 ± 1.02
50.0	22.90 ± 1.10
100.0	39.75 ± 0.45
200.0	55.62 ± 0.62
400.0	68.84 ± 1.84

Data presented as mean ± SD (n = 6). IC_{50}: (164.30 ± 4.85) μg/mL.

Table 2
BSA-MGO glycation inhibitory activity of rutin.

Concentration (μg/mL)	% Inhibition
6.25	4.78 ± 0.60
12.50	12.24 ± 0.40
25.00	20.53 ± 0.74
50.00	46.36 ± 0.41
100.00	63.70 ± 1.17
200.00	75.48 ± 0.34

Data presented as mean ± SD (n = 6). IC_{50}: (63.36 ± 0.67) μg/mL.

BTB also possessed dose dependent (r^2 = 0.99) MGO mediated protein glycation reversing activity (Table 3). But this reversing activity was approximately 1.5 fold lower IC_{50} of (235.39 ± 5.37) μg/mL than that of MGO mediated protein glycation inhibitory activity of BTB.

Table 3
BSA-MGO glycation reversing activity of BTB.

Concentration (μg/mL)	% Reversing
25	5.87 ± 1.27
50	10.64 ± 2.23
100	23.39 ± 1.43
200	42.14 ± 1.04
400	61.46 ± 1.47

Data presented as mean ± SD (n = 6). EC_{50}: (235.39 ± 5.37) μg/mL.

Results on antioxidant activities of BTB showed that TPC, TFC, ABTS, DPPH and FRAP antioxidant properties were (119.55 ± 9.97) mg gallic acid equivalents, (6.04 ± 1.26) mg quercetin equivalents, (3.29 ± 0.06) mmol Trolox equivalents, (1.95 ± 0.15) mmol Trolox equivalents and (1.31 ± 0.19) mmol Trolox equivalents/g of BTB, respectively. The BTB had considerable *in vitro* antioxidant activity when assessed by five different antioxidant assay models. However, surprisingly there was no significant ($P > 0.05$) linear correlation between MGO mediated protein glycation inhibitory activity and antioxidant activity of BTB.

4. Discussion

The potential of BTB of Sri Lankan low grown orthodox OP grade black tea to inhibit the MGO (an important carbonyl intermediate precursor of AGEs) mediated protein glycation and its reversing ability were evaluated using two *in vitro* techniques which are validated, reproducible, sensitive and reliable[7,11,12]. Further, these models produce very stringent glycation conditions, particularly its detection[7,11,12]. The tea sample used was unblended, garden fresh, typical and representative to the OP grade black tea in terms of sieve analysis, agro-climatic elevation and organoleptic properties (aroma, flavor, liquor, color and taste [9,18]. On the other hand, most of other studies on bioactivities of black tea have used blended teas of multiple origin with no specification on agro-climatic elevation[19,20]. BTB was made according to International Organization for Standardization specifications employing a 5 min brewing time[10]: because extraction of most of the water soluble phytoconstitutents including flavones, flavanones, flavonols, catechins, theaflavins and thearubigins are completed within 4 min[21].

The results demonstrated for the first time, that BTB of Sri Lankan low grown OP grade black tea can inhibit the MGO mediated protein glycation, one of the most important reactive intermediate carbonyl species of AGEs[2,4] and potentiates its reversing activity markedly *in vitro* as determined by florescence spectroscopy. Most importantly, as yet, presence of MGO mediated protein glycation reversing activity has not been shown to any

variety of tea (black, oolong, green and white). However, the ability of BTB to inhibit BSA-MGO glycation was significantly low ($P < 0.05$) compared (2.5 fold low) to rutin, the reference drug used in the study. On the other hand, there is no reference drug currently available to be used as a MGO mediated protein glycation reversing ability. Nevertheless, the presence of these two bioactivities of BTB of OP grade black tea indicates its ability to ameliorate carbonyl stress which leads to increased modification of proteins, followed by debilitating oxidative stress and tissue damage[2,4]. What is more, these two important findings of this study would likely to create positive financial impacts to Sri Lanka and health benefits to black tea drinks worldwide: currently tea is the most consume beverage beside water[8] and black tea accounts for 80% global tea consumption[8]. Sri Lanka is the main exporter of orthodox black tea[22] which is drunk in more than 138 countries, accounting for 20% of global tea consumption[23]; and MGO as the most important reactive carbonyl intermediate of AGEs[4,5,24] which is one of the major causative agent of pathogenesis of long term complications of diabetes and other age related diseases[2,4].

Several studies have indicated that MGO, one of the major reactive carbonyl intermediate precursor of AGEs[4,5,24] can be suppressed by carbonyl trapping mechanisms. Black tea[8] including OP grade black tea is an excellent source of natural phenolics including the dimeric form, theaflavin[8,25]. Black tea polyphenolics possess strong MGO trapping abilities, even higher than the major green tea catechin, epigallochatechin gallate[5,26]. For example, one molecule of theaflavin-3-3'-digallate can trap two molecules of MGO under simulated physiological conditions[5,26]. Accordingly, it is presumed that this carbonyl quenching action of black tea is mainly responsible, if not solely, for the observed impairment of MGO mediated protein glycation inhibitory activity. Interestingly, AGEs inhibitors like aminoguanidine, metformin, pyridoxamine act by sequestering reactive dicarbonyls, the important precursors of AGEs[4].

Black tea[8] including Sri Lankan low grown OP as revealed from our previous studies[18] and from this study, is an excellent source of antioxidants which is attributed to its phenolics such as catechins, flavonols, thearubigins, theaflavins, meracetin, quercetin and rutin[8]. Antioxidants are implicated as MGO mediated protein glycation inhibitors[4,27,28]. Thus, the antioxidant activity of OP grade tea is likely to play a crucial role in inhibiting the MGO mediated protein glycation in this study. Several studies have revealed that a strong relationship exists between phenolic content and MGO mediated protein glycation inhibitory activity[28,29]. However, surprisingly in this study, no strong positive linear correlations were evident between MGO mediated protein glycation inhibition and TPC, TFC (indirect measures of antioxidant activity), or ABTS, DPPH and FRAP (direct measures of antioxidant activity) antioxidant activities. This indicates that antioxidant mechanism is unlikely to be the key determinant for MGO mediated protein glycation inhibitory activity evident in this study. However, an absence of a positive linear correlation between antioxidant activity and MGO mediated protein glycation inhibition could be due to few data points that were close to each other[30]. Alternatively, a lack of a relationship between these two parameters may be due to its unique phenolic composition and/or concentration and/or their structural architecture as reported with some herbal infusions[11].

There is evidence to show that transition metal ions, particularly copper and iron, are involved in the production of AGEs via MGO[4,27]. Black tea is known to possess the ability to chelate and sequester metal ions[4,8]. As such, this metal ion trapping activity of black tea may also play a crucial role in the BSA-MGO glycation inhibitory ability evident in this study. In this context, it is noteworthy that carnosine, a commercially crucible drug and its analogues act as antiglycation agents because of their ability to chelate transitional metal ions[4].

In addition to its BSA-MGO glycation inhibitory activity, Sri Lankan OP grade black tea also exhibited BSA-MGO glycation reversing ability. Mechanisms underlying this activity remains unclear, at present. Nevertheless, this is the first study to demonstrate this activity in any variety of tea.

The results demonstrate that Sri Lankan low grown orthodox OP grade black tea possess BSA-MGO glycation inhibitory activity, facilitation of BSA-MGO glycation reversing activity and considerable antioxidant activity. This is the first study to show BSA-MGO glycation inhibitory activity and facilitation of its subsequent reversing activity for any variety of Sri Lankan tea and BSA-MGO glycation reversing ability for any variety of tea worldwide. The novel properties observed for Sri Lankan OP grade black tea indicate its usefulness as a supplementary beverage in managing MGO and AGEs related diseases and ailments.

Conflict of interest statement

We declare that we have no conflict of interest.

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