

Original Research Article

In vitro Antiglycation and Cross-Link Breaking Activities of Sri Lankan Low-Grown Orthodox Orange Pekoe Grade Black Tea (*Camellia sinensis* L)

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Abstract

Purpose: To investigate the antiglycation and cross-link breaking activities of Sri Lankan low-grown orthodox Orange Pekoe grade black tea (*Camellia sinensis* L)

Methods: Five concentrations (6.25, 12.5, 25.0, 50.0 or 100.0 µg/ml) of Black tea brew (BTB) were made using Sri Lankan low-grown Orange Pekoe (O.P.) grade tea. Antiglycation and advanced glycation end products (AGEs) cross-link breaking activities of BTB as well as the antiglycation activity of rutin were determined *in vitro* on bovine serum albumin/glucose system using fluorescence spectroscopy.

Results: BTB induced significant ($p < 0.05$) antiglycation activity (IC_{50} , 19.04 ± 5.18 µg/ml) and AGEs cross-link breaking activities (IC_{50} , 82.89 ± 3.44 µg/ml). These effects were dose-dependent. Further, the antiglycation activity of BTB was comparable to rutin, a well-known antiglycation agent (IC_{50} , 21.88 ± 2.82 µg/ml).

Conclusion: These results show that Sri Lankan low-grown O.P. grade black tea possesses both antiglycation and AGEs cross-link breaking activities *in vitro* and thus provides scientific justification for the use of black tea in Sri Lankan indigenous medicine for the management of diabetic complications.

Keywords: Diabetic complications, Antiglycation, Glycotoxin, Black tea, Cross-link breakers, Sri Lankan tea, Orange Pekoe tea

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INTRODUCTION

It is well known that diabetic complications are the major causes of morbidity and mortality among patients with type 2 diabetic mellitus. Accumulation of advanced glycation end products (AGEs), also known as glycotoxins, is now implicated as a major factor in the pathogenesis of long-term complications of diabetes and other health disorders [1-5]. Since AGEs are involved in the pathogenesis of

complications of diabetes and other disorders [1-5], several synthetic and natural products have been tested as inhibitors of AGEs formation and AGEs breakers as potential therapeutics for management of diabetic complications. Although, these pharmaceuticals have shown promise, was associated with undesirable side effects. Thus, there is a need for the development of new efficacious drugs, with minimal or no side effects, preferably from plant origin, to minimize the incidence and severity of diabetic complications.

Interestingly, several natural products have been tested as potential inhibitors of AGEs formation [5,6]. In this regard, if Sri Lankan black tea can attenuate diabetic complications as is claimed in indigenous medicine [7], it should possess potent antiglycation (inhibiting activity against AGEs formation) and/or cross-link breaking properties. This has not been tested previously; antiglycation activity of black tea is largely unknown [5,6] as compared with those of green tea [5,6,8]. Thus, in this study, we investigated the antiglycation and AGEs cross-link breaking potential of Sri Lankan black tea, which is the objective of the study.

Tea, which is manufactured from the top most immature leaves and the buds of the plant *Camellia sinensis* (L) O. Kuntz is the most consumed beverage in the world beside water [9]. There are three major types of tea: black, green and oolong [9]. Of these, black tea is the most popular type.

The antiglycation and AGEs cross-link breaking potential was tested *in vitro* (in bovine serum albumin/glucose system) using garden fresh, unblended Sri Lankan low grown (< 600m above mean sea level) Orange Pekoe (O.P.) grade orthodox black tea. O.P. grade was selected as it is one of the main grades of black tea exported from Sri Lanka: Sri Lanka is the second main exporter of black tea which is consumed in 138 countries worldwide [9].

EXPERIMENTAL

Source of tea and processing technique

Topmost immature leaves and buds of *C. sinensis* plucked from the plantation of St Jochims' tea estate of the Tea Research Institute, Hedalla, Ratnapura, Sri Lanka (29 m above mean sea level: low grown) during November – December, 2011 were used to process O.P. grade black tea by orthodox-rotovane technique [9] at the estate's factory. The tea leaves were identified and authenticated by Dr (Mrs) S Ranwala, Department of Plant Science, University of Colombo, Sri Lanka. A voucher specimen (cs/01/2011) was deposited in the museum of Department of Zoology, University of Colombo. The composition of true to size particles defined for the O.P. grade black tea was determined using sieve shaker (Retsch AS 200, Retsch GmbH, Haan, Germany) with standard set of sieves (shaking time: 10 min and shaking speed: 50 vibrations/min). Typical characters belonging to elevations were

assessed organoleptically by professional tea tasters of the tea testing unit, Sri Lanka Tea Board. Tea samples were packed in triple laminated aluminium foil bags (1 kg each) and stored at -20°C until use.

Preparation of black tea brew (BTB) samples

BTB was made according to ISO standards (ISO 3103) by adding 2 g of O.P. grade black tea to 100 ml of boiling water and brewed for 5 min [10]. The tea brew was squeezed through a muslin cloth and freeze-dried. The freeze-dried product was stored in air-tight container at 4°C until use.

Evaluation of antiglycation activity

The antiglycation activity of black tea extract against Maillard reaction on Advanced Glycation End products (AGEs) formation was performed according to Matasuura *et al* [11] with some modifications. Two milligrams of freeze-dried tea extract was dissolved in 40 µl of DMSO (dimethyl sulfoxide). Reaction volume of 1 ml containing 800 µg BSA (bovine serum albumin), 400 mM glucose and different concentrations of tea extract (6.25, 12.5, 25.0, 50.0, and 100.0 µg/ml) in 50 mM phosphate buffer (pH 7.4) containing 0.02% sodium azide (w/v) were incubated at 60°C for 40 h (N = 6/concentration). After cooling, aliquots of 600µl were transferred to 1.5 ml eppendorf tubes and 60µl of 100% w/v trichloroacetic acid (TCA) was added and stirred. Supernatant was removed after centrifugation at 15000 rpm at 40°C for 4 min and the AGEs-BSA precipitate was dissolved in 3 ml of phosphate buffer saline (pH 10). Fluorescence intensity of the samples was measured at an excitation wave length of 370 nm and emission wave length of 440 nm using spectrofluorometer (Amino-Bowman®, Thermo Spectronic, USA). Rutin was used as the standard anti-glycating agent. Antiglycation activity (% inhibition) was calculated using Eq 1.

$$\text{Inhibition (\%)} = \{(F_c - F_b) - (F_s - F_{sb}) / (F_c - F_b)\} 100 \dots (1)$$

where F_c is the fluorescence of incubated BSA, glucose and DMSO (control), F_b is the fluorescence of incubated BSA alone (blank), F_s is the fluorescence of incubated BSA, glucose and tea extract or rutin and F_{sb} is the fluorescence of incubated BSA with the tea extract or aspirin. The concentration which inhibits glycation activity by 50% (IC_{50}) was determined.

Evaluation of AGEs cross-link breaking capacity of O.P. grade black tea

Reaction mixture containing 800 µg BSA and 400 mM glucose in 1ml of 50 mM phosphate buffer (pH 7.4) containing 0.02% sodium aside (w/v) was incubated at 60°C for 40 h. After cooling, aliquots of 600 µl were transferred to 1.5 ml eppendorf tubes and 60 µl of 100% (w/v) TCA was added and stirred. AGEs-BSA precipitate was dissolved in 50 mM of phosphate buffer (pH 7.4) and 6.25, 12.5, 25.0, 50.0, and 100.0 µg/ml tea extract were added (reaction volume of 1 ml) and incubated at 60°C for 40 h (N = 6/concentration). After cooling, 60 µl of 100% w/v trichloroacetic acid (TCA) was added and stirred. Supernatant was removed after centrifugation at 15000 rpm at 40°C for 4 min and the AGEs-BSA precipitate was dissolved in 3 ml of phosphate buffer saline (pH 10). Fluorescence intensity of the samples was measured at an excitation wave length of 370 nm and emission wave length of 440 nm using spectrofluorometer (Amino-Bowman®, Thermo Spectronic, USA). AGEs cross-links breaking (Ac) was calculated according to Eq 2.

$$\text{Ac (\%)} = \{(F_c - F_b) - (F_s - F_{sb}) / (F_c - F_b)\} \times 10 \dots \dots (2)$$

where F_c is the florescence of incubated BSA, glucose and DMSO (control), F_b is the florescence of incubated BSA alone (blank), F_s is the florescence of incubated BSA, glucose and tea extract and F_{sb} is the florescence of incubated BSA with the tea extracts. The concentration which breaks AGEs cross-links by 50% (IC_{50}) was determined.

Statistical analysis

Data are presented as mean \pm SEM (standard error of mean) and IC_{50} values were calculated using Microsoft Excel 2007 package. Dose-dependency was determined by regression analysis using Minitab 14.0 statistical software. Significance level was set at $p < 0.05$.

RESULTS

Sieve analysis revealed that 83.5% of the tea particles were of true size (2000 – 4000 µm) for O.P. grade black tea. This indicates that the tea sample used in the study was typical of O.P. grade black tea. Organoleptic testing by professional tea testers showed that the sample can be considered well-made high quality low grown O.P. grade Sri Lankan black tea.

The results of the tests carried out are summarized in Tables 1, 2 and 3. As shown, BTB induced marked in vitro antiglycation action (Table 1) with an $IC_{50} = 19.04 \pm 5.18$ µg/ml. This effect was dose dependent ($r^2 = 0.62$; $p < 0.05$). Further, the antiglycation activity of BTB was comparable to rutin ($IC_{50} = 21.88 \pm 2.82$ µg/ml) the reference drug, a well known glycation inhibitor, which also exhibited a dose dependent ($r^2 = 0.818$; $p < 0.05$) antiglycation action (Table 2). BTB also provoked a dose dependent ($r^2 = 0.997$; $p < 0.05$) cross-link breaking activity (Table 3) with an $IC_{50} = 82.89 \pm 3.44$ µg/ml.

Table 1: Effect of Black tea brew (BTB) of Sri Lankan low-grown orthodox Orange Pekoe grade black tea on antiglycation activity in vitro (mean \pm SEM)

Concentration of BTB (µg/ml)	Inhibition (%)
6.25	29.18 \pm 1.52
12.5	45.17 \pm 2.94
25.0	71.21 \pm 3.07
50.0	83.85 \pm 3.18
100.0	90.01 \pm 2.15

Table 2: Effect of rutin on antiglycation activity in vitro (mean \pm SEM)

Concentration of rutin (µg/ml)	Inhibition (%)
3.12	9.27 \pm 0.75
6.25	27.99 \pm 0.60
12.5	42.54 \pm 2.73
25.0	51.74 \pm 3.09
50.0	64.12 \pm 2.42
100.0	87.77 \pm 2.11

Table 3: Effect of Black tea brew (BTB) on Sri Lankan low-grown orthodox Orange Pekoe grade black tea on AGEs cross-link breaking activity in vitro (Mean \pm SEM)

Concentration of BTB (µg/ml)	AGEs cross-link breaking (%)
6.25	10.35 \pm 2.18
12.5	16.05 \pm 1.96
25.0	20.90 \pm 1.45
50.0	34.07 \pm 0.85
100.0	59.09 \pm 1.25

DISCUSSION

This study examined the antiglycation and cross-link breaking potential of BTB made from Sri

Lankan low grown orthodox O.P. grade black tea using an in vitro assay technique. This technique is validated, sensitive, reliable and widely used in assessing these activities [5,6,11]. The BTB was made using typical and representative sample (as determined by organoleptic and sieve analysis) of garden fresh and unblended O.P. grade tea. Further, for the preparation of BTB, five minutes brewing time was used as indicated in ISO standards [10], as extraction of water soluble flavanoids (flavanols, catechins, theaflavins or thearabugins) is almost complete within 4 min [12]. In contrast, previously reported limited number of studies on antiglycation activity of black tea has used blended tea of multi-origin or unknown origin purchased from supermarkets [5,6]. We believe that, providing this information, as in this study, is important as it is known that bioactivity of black tea varies with country of origin, grade of tea, particle size, brewing time and agroclimatic elevation [13].

The results show, for the first time, that BTB of Sri Lankan low grown orthodox O.P. grade black tea possesses both potent and stable in vitro antiglycation and cross-link breaking activities as determined by fluorescence spectroscopy. Further, most importantly, this is the first study to demonstrate the AGEs cross-link breaking activity of any type of tea. Both these effects were dose dependent. This indicates that the effects are genuine, intrinsic, causal and specific. Antiglycation activity of BTB was greater than its AGEs cross-link breaking capacity and comparable (in terms of IC_{50} values) to the reference drug, rutin, which is a well known antiglycation agent. Unfortunately, currently there is no accepted drug to be used as a reference drug in studies on AGEs cross-link breaking activity.

Ever increasing evidence indicate that all reported natural [5,6,8] and synthetic [1-4] antiglycation agents act via multiple mechanisms. As mentioned in the introduction, reactive oxygen species are generated in the classical pathway of AGE formation and autoxidative pathway and these contributes to its own synthesis and its cross-links [3-5]. It is well recognized that antioxidants, agents which scavenge reactive oxygen species, inhibit AGEs production both in vitro and in vivo [1-6,8]. Further, the antiglycation activity of all the reputed pharmaceuticals is mediated primarily via anti-oxidation [1-6,8]. Black tea is an excellent natural antioxidant [5,9] due to its catechins, flavanols, thearabugins, theaflavins, meracetin, quarecetin or rutin contents [5,11]. Thus, it is presumed that antiglycation activity observed in this study is mediated mainly, if not

solely by this mechanism as reported with other glycation inhibitors [1-6,8] although the mechanisms of AGEs formation are extremely complex. Tea catechins (both simple and oxidized) are also reported to have ability to effectively trap carbonyl species [6,8]. Carbonyl species are formed during early stages of glycation and these participate in the AGEs formation [2-6]. Accordingly, this mode of quenching action of carbonyl species could also contribute to the BTB induced antiglycation action. Recent investigations have shown, theanine, the unique amino acid present in tea has glycation inhibiting activity [8], which could play a pivotal role in inducing inhibition of AGEs formation in this study. There is also evidence to show that transition metal ions, particularly copper and iron, and products of lipid peroxidation are involved in the AGEs formation [3-5,8]. Black tea possess both metal ion trapping action and lipid peroxidation inhibition activity due to its flavanoids [5,6,8,9] and quarecetin [9] respectively, which could also play a crucial role in the BTB elicited antiglycation activity. In addition, metal ion trapping activity of black tea can prevent/impair glucose and Amadori products from self oxidation, leading to the inhibition of AGEs formation [5]. Anti-inflammatory drugs such as ibuprofen and indomethacin have been shown to possess antiglycation activity [1-5]. Sri Lankan black tea also has marked anti-inflammatory activity [14]. Accordingly, this mode of action too may have contributed to the antiglycation activity of BTB in this study.

Our previous studies have shown that Sri Lankan black tea has both hypoglycaemic [15,16] and anti-diabetic [15,16] activities. These activities would be an additional advantage in consuming black tea since hyperglycaemia promotes AGEs formation [1-5].

In complete contrast, as yet, the exact underlying mechanism of cross-link breaking activity is not clearly understood even with thiozolum salt-based cross-link breakers [1-5]. Accordingly, it is premature to confidently speculate the mode of AGEs cross-link breaking activity of BTB. However, anti-oxidative, trapping of reactive species and metal ion chelating activities may play a substantial role as indicated for other synthetic AGEs cross-link breakers [1-5].

CONCLUSION

These results show that Sri Lankan low grown orthodox O.P. grade black tea possess both in vitro antiglycation and AGEs cross-link breaking

activities and scientifically rationalize the use of black tea in Sri Lankan indigenous medicine in the management of diabetic complications.

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