Research Journal of Chemical Sciences ISSN 2231-606X
Vol. **7(7),** 11-15, July **(2017)** *Res. J. Chem. Sci.*

In vitro anti-collagenase activity of Sri Lankan low grown orthodox Orange Pekoe grade black tea (*Camellia sinensis* L.)

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Available online at: www.isca.in, www.isca.me
Received 17th May 2017, revised 9th July 2017, accepted 16th July 2017

Abstract

Anti-collagenase activity of tea brew of Sri Lankan low grown orthodox Orange Pekoe grade black tea (Camellia sinensis L.) was evaluated using five concentrations (25, 50, 75, 100 and 200 µg/ml) of black tea brew (BTB) made according to ISO specifications. Anti-collagenase activity of BTB and tea catechin, epigallocatechingallate (EGCG) were ascertained in vitro using collagenase enzyme from Clostridium histolyticum and a synthetic substrate (FALGPA) using spectroscopy. BTB induced marked and significant ($P < 0.05$) anti-collagenase activity (IC_{50} = 80.04 ± 2.34 µg/ml). This effect was dose dependent. Moreover, anti-collagenase activity of BTB was superior to EGCG (IC_{50} = 112.12 ± 0.93 µg/ml), a well known anti-collagenase phytoconstituent of green tea. The results convincingly show that Sri Lankan low grown orthodox Orange Pekoe grade black tea possesses remarkable anti-collagenase activity in vitro and display its promise to be developed as a potent anti-aging skin nutraceutical.

Keywords: Anti-aging, Anti-collagenase, *Camellia sinensis,* Orange Pekoe tea, Sri Lankan tea.

Introduction

Every person likes to have a wrinkle free, smooth, non sagging, firm, glowing and healthy looking young skin[1,2]. However, like any other organ of the body, skin is subjected to a natural or cellular or intrinsic, inevitable aging process which ultimately results in wrinkling[1]. Asides this natural aging process the skin can be subjected to photo ageing or extrinsic aging which is mainly due to over exposure to solar radiation (UVA and UVB rays)[1]. Photo ageing is however, largely preventable since it is under volitional control of a person.

Tight and firmness of the human skin is due to elastin and collagenous fibers present in its dermis[3,4]. Collagen is synthesized and secreted by the fibroblast cells of the dermis[3] and its rate of synthesis decreases with ageing[3,4]. Collagen is the primary structural component of the dermis and accounts for 80% of the skin dry weight[4]. With increase in age, and particularly due to over exposure to sunlight, collagen deteriorates resulting in skin wrinkling[1,3].

Today, one of the most frequent dermatological concerns is skin ageing[2]. As a result, there are several anti-ageing procedures and topical skin care cosmetics (creams and lotions) available in the market which are designed to suppress the ageing process of the skin[1,2]. In addition, anti-ageing oral supplements are also available[2]. Some of topical anti-ageing formulations are synthetic and others are herbal[2]. Synthetic anti-ageing formulations often contain varying amounts of vitamins C and E, coenzyme Q10 (ubiquinone), ferulic acid, idebenone, epidermal growth factor, estrogen, α-hydroxyl acid, glycolic acid, retinol or sylimarin[1,2]. Several of these ingredients are antioxidants[1,2]. Unfortunately, most of these anti-ageing cosmaceuticals induce unpleasant side effects such as contact dermatitis, allergies, skin irritations and even skin cancer[1,2].

In contrast, anti-ageing herbal cosmaceuticals are claimed to be less harmful and more user friendly. Green tea and/or its constituents (catechins and epigallochatechingallate) are often being incorporated in these topical anti-ageing cosmetics[2,5,6]. Recently, extracts of white tea, a special kind of black tea, manufactured solely from buds of *Camellia sinensis* L. plant is shown to possess marked anti-collagenase activity *in vitro* and is claimed as a potential ingredient to topical skin anti-ageing formulations[4]. However, very little white tea is produced and it is highly expensive. On the other hand, so far, potential of oolong and black tea as an anti-ageing cosmaceutical acting via inhibition of collagenase activity has not being assessed. Nevertheless, we have tested the ability of Sri Lankan low grown orthodox Orange Pekoe (O.P) grade black tea as a skin anti-ageing phytoconstituent acting through inhibition of elastase activity[7].

The aim of this study was to investigate the anti-collagenase activity of Sri Lankan low grown orthodox O.P grade black tea *in vitro* with a view to assess its potential to be incorporated into

herbal anti-ageing skin formulations. Currently, Sri Lanka is the main producer and exporter of orthodox black tea[8].

Materials and methods

Source of tea: Top most immature leaves and buds of *C. sinensis* plucked from the plantation of St. Jochims tea estate of the Tea Research Institute, Hedallana, 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 at the estate factory. The leaves were identified and authenticated by Dr. S. Ranawala, Department of Plant Sciences, University of Colombo, Sri Lanka. A voucher specimen (CS/01/2011) is deposited in the museum of Department of Zoology, University of Colombo, Sri Lanka. Sieve analysis of the tea sample has revealed that it has tea particles true for the grade (83.5% between 2000-4000 µm) and organoleptic properties (tested by professional tea tasters of the Tea testing unit, Sri Lanka Tea Board) reflecting well made high quality low grown Sri Lankan orthodox black tea[7]. Tea samples were packed in triple laminated aluminium foil bags (1 kg each) and stored at -20^{x}C until use.

Investigation of physical parameters of tea sample: The moisture[9], total ash[10], water soluble ash[11], acid insoluble ash contents[12], alkalinity of water soluble ash (relative to KOH)[13], amount of extractable solids in tea brew (water extract)[14] and crude fibre content[15] of the sample was determined as described by the International Standard Organization (ISO, Geneva, Switzerland). The results are expressed as % (w/w).

Analysis of major organic phytoconstituents in tea sample: Total polyphenols, major catechins [Epigallocatechingallate (EGCG), Epigallocatechin (EGC), Epicatechingallate (ECG), and Epicatechin (EC)], theaflavins, thearubigins and caffeine were determined using high performance liquid chromatography and UV visible spectrometer measured at 765 nm[16,17].

Preparation of Black Tea Brew (BTB): BTB was made according to the ISO standards adding 2 g of O.P grade black tea to 100 ml of boiling water and brewed for 5 min[18]. This contained 36.1% (w/w) tea solids in water. The tea brew was squeezed through a muslin cloth and freeze dried. The freeze dried product was stored in an air tight container at 4^{x}C until used.

Evaluation of anti-collagenase activity of BTB of O.P grade black tea: The anti-collagenase activity of BTB was evaluated *in vitro* as described by Van Wart and Steinbrink[19], with some modifications, using a 96 well microtiterplate. Freeze dried BTB was dissolved in Tricine buffer (0.5 mg/ml) to obtain different concentrations (25, 50, 75 and 200 µg/ml). Fifty microliters of 50 µg/mL *Clostridium histolyticum* collagenase enzyme (EC 3.4.23.3) and different concentrations of BTB (n=4 per concentration) in 50 mM Tricine buffer with 10 mM $CaCl_2$ and 0.4M NaCl, pH 7.5 were incubated at 25^{x}C for 10 minutes. Twenty microliters of synthetic substrate, N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) dissolved in Tricine buffer was added to the reaction mixture to start the reaction and decrease of optical density (OD) at 324 nm was measured continuously for 10 minutes using SpectraMax384 microplate reader. Different concentrations of (12.5, 25, 50, 100 and 200 µg/ml) epigallocatechingallate (EGCG) was used as the reference anti-collagenase agent. Inhibition of collagenase activity was expressed as the percentage decrease in Vmax. Collagenase inhibition (%) was calculated using the formula

$$\text{Inhibition } (\%) = \frac{A-B}{A} \times 100$$

Where: A is V_{max} without the BTB/EGCG, B is the V_{max} with the BTB/EGCG.

Statistical Analysis: Data is represented as Mean ± standard error of mean (SE) and IC_{50} values were calculated using Microsoft Excel 2007 package. Dose dependencies were determined using regression analysis with Minitab 14.0 statistical software. Significant level was set at $P < 0.05$.

Results and discussion

O.P. grade black tea sample used in this study was garden fresh, unblended and prepared according to ISO specializations[18] before freeze drying. The physical parameter data of O.P grade black tea is summarized in Table-1. The results revealed that the parameters determined are within the ISO 3720 recommended range for black tea. Further, the tea sample used here is typical to the O.P. grade black tea and agro climatic elevation in terms of sieve analysis and organoleptic properties[7]. Hence, the results obtained are valid to this grade of tea and can be meaningfully interpreted[20].

Table-1: Some physical parameters of Sri Lankan low grown orthodox orange pekoe grade black tea with ISO 3720 standard.

Parameter	(O.P) grade black tea	ISO 3720 requirement
% Moisture content (w/w)	6.17 ± 0.18	Not established
% Total ash content (w/w)	5.62 ± 0.15	4.0-8.0
% Water soluble ash content (w/w)	50.77 ± 1.06	≥ 45.0
% Acid insoluble ash content (w/w)	0.21 ± 0.02	≤ 1.0
% Alkalinity of water soluble ash (as KOH) (w/w)	1.69 ± 0.3	1.0-3.0
Water extract (w/w)	38.98 ± 0.44	≥ 32.0
Crude fiber content (w/w)	9.58 ± 0.34	≤ 16.5

Data represented as mean ± SE (n=3).

This study examined *in vitro* anti-collagenase potential of Sri Lankan orthodox low grown O.P grade black tea with a view to extrapolate the data to skin anti- ageing properties: collagen in the dermis plays pivotal role in maintaining a healthy texture of skin[3,4] and its deterioration results in wrinkling which is a visible sign of ageing[3,4]. Collagen degradation is primarily mediated via activity of collagenase enzyme which zinc is containing metalloproteinase found in the matrix of dermis[4]. The *in vitro* assay used employed *Clostridium histolyticum* collagenase, which is analogous with human collagenase and a synthetic substrate, FALGPA[4]. Further, the assay used is simple, rapid, convenient, validated, sensitive, reliable, and reproducible[4,21]. Also, usage of this assay avoided ethical issues associated with *in vivo* testing.

Anti-collagenase activity of Sri Lankan orthodox low grown O.P grade black tea is summarized in Table-2. As shown, BTB induced a marked *in vitro* anti-collagenase activity ranging from 2.46 to 75.19% inhibition (Table-2) with an IC_{50} value of 80.04 ± 2.34 µg/ml. Further, this effect was dose dependent (r^2= 0.96). The reference agent, EGCG, also displayed substantial anti-collagenase activity ranging from 12.07 to 88.66% inhibition with an IC_{50} value of 112.12 ± 0.93µg/ml (Table-3). This effect too was dose dependant (r^2 = 0.99). The results unequivocally demonstrated for the first time that Sri Lankan low grown orthodox O.P. grade BTB possesses remarkable anti-collagenase activity (ranging up to 75% inhibition) *in vitro.* Interestingly, anti-collagenase activity of BTB was superior to the reference agent (EGCG) used: EGCG is a well-known anti-collagenase agent[4,21]. Further, observed anti-collagenase activity of BTB was almost similar to white tea and was superior to green tea (by 1.5 fold)[4]. As anti-collagenase activity of BTB was dose dependent the results indicate the effect was genuine, intrinsic, causal and specific. So far, there are no published reports of clinical toxicity from daily consumption of black tea[22]. The results of this study, taken together with other reported studies[23-26] points out the high promise of developing a safe anti-ageing skin formulation based on Sri Lankan O.P grade black tea. Possibility also exists that this grade of Sri Lankan black tea can function as a supplementary anti-ageing herbal beverage. After all, tea is the most consumed beverage besides water[22].

Table-2: *In vitro* anti-collagenase activity of Sri Lankan low grown orthodox orange pekoe grade black tea brew.

Concentration (µg/ml)	% Inhibition
200	75.19 ± 5.84
100	64.04 ± 1.01
75	43.30 ± 1.65
50	26.20 ± 1.49
25	2.46 ± 0.95
IC_{50}	80.04 ± 2.34

Data represented as mean ± SE (n=4).

Table-3: *In vitro* anti-collagenase activity of epigallocatechin gallate (EGCG).

Concentration (µg/ml)	% Inhibition
200	88.66 ± 1.72
100	41.76 ± 1.93
75	20.86 ± 0.85
50	15.12 ± 0.97
25	12.07 ± 0.93
IC_{50}	112.12 ± 0.93

Data represented as mean ± SE (n=5).

The individual flavanol, polyphenol and caffeine content BTB is given in Table-4. Results showed that BTB contained large amount of polyphenols and thearubigins, and small amounts of catechins and theaflavins (Table-4) as reported with other black teas[22,27], although the composition was different. These phytoconstituents are known to inhibit collagenase activity, by primarily binding zinc ions within the enzyme, thereby preventing it from binding to triple halide region of collagen and its subsequent hydrolisation[4,21]. Anti-collagenase activity evident in this study may be attributed to this mechanism. However, currently, it is unknown whether the collagenase inhibition of O.P grade tea is competitive or noncompetitive. Alternatively, phytoconstituents of O.P tea could have attach to the enzyme at sites other than the active site as proposed to anti-collagenase activity of aloe gel[21]. Further, flavonoid metal complexes have shown possess the potential to act as superoxide dismutase (SOD) mimetics and novel SOD mimetics are being developed as anti-ageing agents[4]. This mode of action may also operate *in vivo* if a formulation containing O.P grade black tea is applied to the skin as a skin anti-ageing agent or when consumed daily as a supplementary beverage.

Anti-collagenase activity is not the sole mechanism through skin anti-ageing effects can be mediated as skin ageing is a multifunctional process[1,28]. Reactive oxygen species are linked with skin aging[1,5,6] and antioxidants are shown to suppress it[1,2,28]. Black tea, including Sri Lankan varieties[22] and its phytoconstituents[22] are shown to function as powerful antioxidants. In fact, black tea is one of the strongest botanical antioxidants, known, as yet[4,22]. Further, thearubigins and theaflavins, which are unique polymerized polyphenols of black tea, can impair lipid peroxidation markedly[22]. Lipid peroxidation is involved in ageing[1,2,28]. Therefore, this mechanism can also contribute to the proposed anti-ageing activities of O.P grade tea. Further, we have recently shown that Sri Lankan O.P grade tea has mild anti-elastase activity *in vitro*[7].

It is well recognized that elastase enzyme is responsible for fragmenting elastin fibers in dermis[1,3] which results in wrinkling of skin[1,2,3,28]. Obviously, this anti-elastase mechanism too can confer anti-ageing properties to O.P grade tea.

Table-4: Individual flavanol, polyphenol and caffeine content of Sri Lankan low grown orthodox orange pekoe grade black tea.

Compound	Content (% on W/W basis)
Epigallocatechingallate (EGCG)	0.58 ± 0.00
Epigallocatechin (EGC)	0.01 ± 0.01
Epicatechingallate (ECG)	0.82 ± 0.02
Epicatechin	0.13 ± 0.005
Catechins	0.1 ± 0.005
Total polyphenols	17.04 ± 0.42
Theaflavins	0.68 ± 0.02
Thearubigins	10.64 ± 0.29
TR/TF	15.60 ± 0.74
Caffeine	3.83 ± 0.06

Data represented as mean ± SE (n=3). Thearubigins/ Theaflavins: TR/TF.

Accumulation of advanced glycation end products (AGEs) is considered to accelerate skin ageing [28], and inhibition of AGEs production and rapid breaking down of them are reported to suppress skin ageing[28]. We recently showed that this variety of black tea has strong anti AGEs activity (both anti-glycation and glycation reversing activities) *in vitro*[24]. Certainly, presence of these two activities in O.P tea would enhance its anti-ageing properties considerably.

In addition to these bioactivities, we recently showed that, Sri Lankan O.P grade tea exhibits anti-hyaluronidase[26] sun screening[23] and skin whitening and lightening (in terms of tyrosinase inhibition)[25] activities *in vitro* which are desirable properties expected of an anti-ageing skin formulation. Yet another mechanism via O.P tea could produce anti-ageing properties are by inhibiting inflammation. Inflammation is now implicated as a causative factor in premature ageing[1,2,28] and anti-inflammatory agents are considered as another integral approach to impair skin ageing [1,2,28]. As Sri Lankan black tea has marked anti-inflammatory activity[29] such a mode of action could contribute substantially to skin anti-ageing potential of Sri Lankan O.P grade tea.

Conclusion

In conclusion, the results of this study conclusively show that Sri Lankan low grown orthodox O.P grade black tea possesses remarkable anti-collagenase activity *in vitro*, and display it promise to be developed as a potent anti-aging skin neutraceutical.

Acknowledgement

This investigation received financial support from the National Science Foundation of Sri Lanka under the grant number NSF/Fellow/2011/01.

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