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In vitro anti-hyaluronidase activity of Sri Lankan low grown orthodox orange pekoe grade black tea (*Camellia sinensis* L.)

Wanigasekera Daya Ratnasooriya[1*], Walimuni Prabhashini Kaushalya Mendis Abeysekera[2], Chatura Tissa Dayendra Ratnasooriya[3]

[1]*Faculty of Allied Health Sciences, General Sir John Kothalawala Defence University, Ratmalana, 10390, Sri Lanka*

[2]*Herbal Technology Section, Industrial Technology Institute, 363, Bauddhaloka Mawatha, Colombo 07, Sri Lanka*

[3]*Faculty of Medicine, University of Colombo, Colombo-08, Sri Lanka*

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ABSTRACT

Objective: To access the anti-hyaluronidase activity of Sri Lankan low grown orthodox orange pekoe (OP) grade black tea with a view to develop an anti-aging skin formulation.

Methods: Five concentrations (0.125, 0.250, 0.500, 1.000 and 2.000 mg/mL) of black tea brew (BTB) were made using a freeze dried sample of Sri Lankan low grown orthodox OP grade black tea which was prepared according to international organization for standardization specification. Epigallocatechin gallate (EGCG) was used as the reference agent (concentrations tested: 0.012, 0.025, 0.050, 0.100 and 0.200 mg/mL). Anti-hyaluronidase activity of BTB and EGCG *in vitro* were ascertained spectrometrically using hyaluronic acid (from rooster comb) and bovine testicular hyaluronidase.

Results: The results revealed that BTB had moderate [IC_{50}=(1.09±0.12) mg/mL] and dose dependent (r^2=0.94) anti-hyaluronidase activity. EGCG also exhibited dose dependent (r^2=0.93, P<0.05) anti-hyaluronidase activity which was superior [IC_{50}=(0.09±0.00) mg/mL] to BTB.

Conclusions: Sri Lankan low grown orthodox OP grade black tea has promising anti-hyaluronidase activity *in vitro* and has the potential to be used as an anti-aging cosmaceutical. In addition, it may prove useful as a beverage in the management of allergy, some joint diseases and envenomation.

1. Introduction

Aging is an integral part of human life. It affects all systems of the body including the integumentory system, the skin[1]. Skin aging has two types. The first is referred to as natural, cellular or intrinsic aging which is inevitable and generally becomes evident around 30-40 years of age[2]. However, in some individuals, this natural aging process starts prematurely. The second type is known as photo aging or extrinsic aging which is mainly due to over exposure to solar radiation (ultraviolet A and ultraviolet B rays) which is under volitional control of the individual and is therefore largely preventable[2]. Irrespective of the type of aging process, aging usually results in a leathery, dry, dull, unsmooth, saggy, laxative skin with wrinkles (rhythides)[2,3]. Since skin is the outermost layer[1], these unpleasant and depressing signs become obviously visible. In fact, today, one of the most frequent dermatological concerns especially in women, is skin aging[2,4]. As such, there are several anti-ageing procedures and cosmaceuticals (creams, powders, lotions) available in the market which are alleged to diminish or delay the aging process[2,3,5]. In addition, now, anti-ageing oral supplements are available and

*Corresponding author: Prof. Wanigasekera Daya Ratnasooriya, Department of Zoology, University of Colombo, Colombo-03, Sri Lanka.
Tel: +94-11-2503399
E-mail: wdr@zoology.cmb.ac.lk

recommended by some dermatologists[3,5].

Some of these anti-ageing formulations are synthetic and others are herbal. The synthetic anti-ageing products often contain varying amounts of vitamins C and E, co-enzyme Q10 (ubiquinone), ferulic acid, idebenone, epidermal growth factor, pyenogoal oestrogen, hydroxy acid, glycolic acid, retinol or silymarin[2,4,5]. Unfortunately, most of these anti-ageing synthetic cosmaceuticals induce unpleasant side effects such as contact dermatitis, irritant contact dermatitis, phototoxicity, photoallergic reactions and even skin cancer[2,3]. Further, in some, claimed beneficial effects are not scientifically proven and validated and most are expensive. On the contrary anti-ageing herbal cosmaceuticals are supposed to be less harmful and more user friendly. Nevertheless, their long term safety, efficacy and modes of actions are not fully established. However, herbal products have been in long use as cosmaceuticals by women in several South Asian countries irrespective of their cultural variations[6].

Undoubtedly, there is an imperative need for the development of novel, potent, safe and cheap cosmaceuticals or supplementary beverages and/or food from herbal products as anti-ageing agents. In this context, we initiated a program of research to investigate the anti-ageing properties of Sri Lankan low grown orthodox orange pekoe (OP) grade black tea *in vitro* with a view to incorporate it into an anti-ageing skin formulation and/or to develop a novel herbal skin cosmaceutical. So far, many studies have examed its anti-elastase[7], anti-collagenase (unpublished) and antiglycation and advanced glycation end-products (AGEs) cross-link breaking activities *in vitro*[8]. These are potential mechanisms which can inhibit skin ageing[2,5]. Also, its *in vitro* sun screening activity and skin whitening and lightening properties were determined[9,10]. The aim of this study was to assess *in vitro* anti-hyaluronidase activity of Sri Lankan low grown orthodox OP grade black tea. Hyaluronic acid is a high molecular weight glycosaminoglycan, a mucopolysaccharide present in the ground substance of extracellular matrix of the dermis of skin[1,11]. They are extremely hydrophobic and hold water together and keep the skin moist, lubricant and smooth[11]. On the other hand, hyaluronidase is an enzyme present in the dermis which depolymerizes the hyaluronic acid[1,11], resulting in drying and wrinkling of skin[3,11].

2. Material and methods

Top most immature leaves and unopened buds of *Camellia sinensis* L. 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) (Latitude: 6042°57.96″, Longitude: 80022°46.2″) during November-December 2011 were used to process OP grade black tea by orthodox-rotovane technique at the estate factory.

The sieve analysis of the sample has shown that 83.5% of tea particles were true sized (1 400-2 000 μm) and typical for the grade[8]. Further organoleptic profile analysis made by the professional and experienced tea tasters at the tea testing unit of Sri Lanka Tea Board has confirmed that the used sample can be accepted as well to make high quality low grown OP grade Sri Lankan black tea[8]. Tea samples were packed in triple laminated aluminium foil bags (1 kg each) and stored at -20 ℃ until use.

2.1. Preparation of black tea brew (BTB)

BTB was made according to the international organization for standardization (ISO 3103): by adding 2 g of OP grade black tea to 100 mL of boiling water and brewed for 5 min[12]. This contained 36.1% (w/v) tea solids in water and BTB was then squeezed through a muslin cloth and was freeze dried. The freeze dried product was stored in air tight container at 4 ℃ until use.

2.2. Evaluation of in vitro anti-hyaluronidase activities

Hyaluronidase enzyme inhibitory activity of BTB was assessed spectrophotometrically as described by Reissig J.L et al[13]with some modifications by measuring the amount of N-acetylglucosamine formed from sodium hyaluronate. Type-1-S bovine (50 μL) testes hyaluronidase (Sigma Aldrich, USA) was dissolved in 0.1 mol/L acetate buffer (pH 3.5) and was mixed with 50 μL of different concentrations of BTB (2.000, 1.000, 0.500, 0.250 and 0.125 mg/mL) were incubated in a water bath at 37 C for 20 min. Enzyme was activated by adding 100 μL of 12.5 mmol/L calcium chloride and the mixture was incubated in a water bath at 37 ℃ for 20 min. Reaction was started by adding 250 μL of sodium hyaluronate (hyaluronic acid sodium salt from rooster comb, Sigma Aldrich, USA) (1.2 mg/mL) dissolved in 0.1 mol/L acetate buffer (pH 3.5) to the calcium activated hyaluronidase and was incubated in a water bath at 37 ℃ for 40 min. At the end of incubation period 100 μL of 0.4 mol/L sodium hydroxide and 100 μL of 0.4 mol/L potassium borate were added and the reaction mixture was incubated in a boiling water bath exactly for 3 min. Finally, mixtures were cooled to the room temperature and 3 mL of dimethyl benzaldehyde solution (4 g of *p*-dimethylaminobenzaldehyde dissolved in 350 mL of 100% acetic acid and 50 mL of 10 mol/L HCl) was added and the reaction mixtures were incubated in a water bath at 37 ℃ for 20 min. Absorbance was measured at 585 nm using 96 well micro plate reader (SPECTRAmaxPLUS384 Molecular Devices, Inc, USA). Epigallocatechin gallate (EGCG) was used as the reference agent. The percentage inhibition was calculated as:

$$\text{Inhibition}(\%)=(A_C-A_S)/A_C\times 100$$

Where A_C refers to the absorbance at 585 nm of the control, and

A_S means the absorbance at 585 nm of different concentrations of black tea extract.

2.3. Statistical analysis

Data is represented as mean±SD and IC_{50} values were calculated using Microsoft Excel 2007 package. Dose dependencies were determined using regression analysis with the software MINITAB version 14.0 (Minitab Inc, USA). Significant level was set at $P<0.05$.

3. Results

The results obtained are summarized in Tables 1 and 2. As shown in Table 1, BTB imparted a moderate *in vitro* antihyaluronidase activity (ranging from 4.42%-82.75%) with a IC_{50} value of (1.09±0.12) mg/mL. Moreover this anti-hyaluronidase activity was dose-dependent. (r^2 =0.94, $P<0.05$). As expected, EGCG shown in Table 2, exhibited a profound and dose–dependent (r^2=0.92, $P<0.05$) anti-hyaluronidase activity (ranging from 7.99% to 92.98%) with an IC_{50} value of (0.090±0.00) mg/mL.

Table 1

In vitro anti-hyaluronidase activity of Sri Lankan OP grade black tea.

Concentration (mg/mL)	Inhibition (%)
2.000	82.75±0.60
1.000	54.56±2.23
0.500	33.63±1.24
0.250	6.39±0.44
0.125	4.42±0.97

Data presented as mean±SD; n=6; r^2=0.94.

Table 2

In vitro anti-hyaluronidase activity of EGCG.

Concentration (mg/mL)	Inhibition (%)
0.200	92.98±0.54
0.100	62.52±0.47
0.050	42.31±0.81
0.025	9.82±0.73
0.012	7.99±1.31

Data presented as mean±SD; n=4; r^2=0.93.

4. Discussion

This study examined the *in vitro* hyaluronidase inhibiting potential of Sri Lankan low grown orthodox OP grade black tea which is a whole leaf variety. This is done with a view to develop safe, efficient and cheap plant based skin anti-aging formulation based on Sri Lankan OP grade black tea. The *in vitro* assay used to determine anti-hyaluronidase activity is a simple, validated, reliable, sensitive and widely used[13]. The tea sample used was unblended, garden fresh, typical and representative to the grade (in terms of sieve analysis, physical parameters, composition of flavonoids and organoleptic properties)[8,14]. Also, BTB was made employing 5 min brewing time as specified in the international organization for standardization[12], since extraction of water soluble flavonoids (flavanols, catechins, theaflavins and thearubigins) is almost completed within 4 min[15]. It is necessary to provide these information as it is known that bioactivity of black tea varies with country of origin, agroclimatic elevation, harvesting season, age of leaf, processing method, particle size, grade of tea, brewing condition, temperature of tea brew and tea brewing time[15-17]. Hence, the results obtained are valid to this grade of tea and can be meaningfully interpreted and compared. In contrast, most of the studies on bioactivity of black tea have used blended tea of multi origin or unknown origin.

The results clearly show, for the first time, that BTB of Sri Lankan grown orthodox OP grade black tea possesses marked anti-hyaluronidase activity *in vitro*. Further, this anti-hyaluronidase activity was dose dependent indicating a genuine, intrinsic, causal and specific effect. This is a therapeutically important finding because this result taken together with our previous studies indicates the promising development of a safe, effective and potent skin anti-aging cosmaceutical based on Sri Lankan low grown orthodox OP grade black tea[7-10]. Since tea is the most consumed day-to-day beverage besides water and is non toxic even with high regular consumption[18]. The results also indicate the possibility of developing this grade of Sri Lankan black tea as a supplementary anti-aging beverage. It is now known that hyaluronidase plays a crucial role in allergic reactions (by stimulating the expression of anti-inflammatory genes, granulation of mast cells and release of chemical mediators)[19,20], and envenomation of snake, honey bee, wasp, scorpion, stone fish or lizard toxins (by promoting systemic spreading of venom)[20,21]. Further, potent hyaluronidase inhibitors (such as disodium cromoglycates, translist, liquiritigenin) have been demonstrated to possess strong anti-allergic effects[19], and use of them is proposed as first aid agents in snake bite therapy to increase the survival time of victims[20]. Since hyaluronic acid forms the back bone of cartilage matrix[22], its degradation by hyaluronidase is link with pathogenesis of joint diseases such as, arthritis, osteoarthritis which is needed to search for potent hyaluronidase agents urgently[19]. Since OP grade black tea has marked anti-hyaluronidase activity, collectively these facts suggest that it may also offer a beneficial role in the management of allergies, envenomation of animal toxins and in cartilage degrading conditions (such as chronic arthritis)[19-21].

Hyaluronic acid is a high molecular weight glycosaminoglycan. A mucopolysaccharide is present as the main component of the extracellular matrix of dermis of human skin: about 15 g are present in a 70 kg individual[19,23]. It plays a vital role in maintaining structural and functional integrity and helps to preserve smooth and youthful appearance of human skin[23].

Hyaluronic acid is extremely hydrophilic and holds water together, and keeps skin lubricant and smooth due to its unique rheologic and viscoelastic properties[23]. The dermis also contains an hyaluronidase which depolymerizes hyaluronic acid, lowering the viscoelasticity of dermis, resulting in the loss of tension, drying and wrinkling of skin, leading to skin aging[1,19,23]. There are strong associations exists between degradation or alteration of hyaluronic acid and skin aging[24]. And impairment of hyaluronidase activity is considered as one of the main mechanisms of skin anti-ageing[5,23,24]. Further, flavonoids such as catechins, EGCG, quercetin, gallotannins, tannins, apigenin, kaempferol, ascorbic acid and some alkaloids are reported to be strong inhibitors of hyaluronidase[20,23,25,26]. In addition, high molecular weight bulky polyphenols are claimed to act as inhibitors of hyaluronidase[19]. The OP grade tea used in this study is shown to contain catechins, EGCG, quercetin, kaempferol, theaflavins, thearubigins and also high theaflavin ratio[14,18]. Thus, anti-hyaluronidase activity seen in this study can be attributed to these phenolic phytoconstituents. However, at present, it is unknown whether the hyaluronidase inhibition of OP grade tea is competitive or non competitive. Lineweaver-burk plots have to be undertaken to resolve this.

Anti-hyaluronidase activity is not the only mechanism which can be mediated via skin anti-ageing, as skin aging is a multifunctional and complex process[2,3,5]. Production of AGEs and their accumulation facilitate premature skin aging[4]. Further, it has been shown that reduced production of AGEs and rapid breakdown of AGEs cross links confer skin anti-aging properties[4]. This study has recently shown that Sri Lankan low grown orthodox OP grade black tea possesses remarkable anti-ageing activity (both antiglycation and AGEs cross-link breaking actions) *in vitro*[8], which was superior to many nutraceutical so far tested[8]. Surely, these two bioactivities namely anti-hyaluronidase and antiglycation activities would enhance the anti-ageing potential of Sri Lankan low grown OP grade black tea substantially. Anti-elastase activity is another mechanism which can delay aging process[2], since the degradation of elastin fibers in the dermis makes the skin sag, dry and wrinkled[2,5,27]. However, anti-elastase activity of Sri Lankan low grown orthodox OP grade black tea is weak[7]. Therefore its contribution to anti-ageing by this mechanism may be minimal. Reactive oxygen species are associated with aging of skin[2,5,27], and antioxidants retard aging[2,5]. Sri Lankan low grown orthodox OP grade black tea has been shown to have considerable antioxidant activities[18,28], superior to many herbal beverages so as to confer anti-aging activity.

Anti-inflammatory agents are now incorporated into anti-aging skin cosmaceuticals as inflammation also plays a key role in skin aging[2,5,27]. Sri Lankan black tea shows anti-inflammatory activity *in vivo*[29]. This tea can be used as a topical anti-aging skin formulation.

Another two interesting properties in Sri Lankan low grown orthodox OP grade black tea are likely to be used as effective sun screen[9], and anti-tyrosinase agents[10]. These characters obviously suppress photo aging[2], and promote skin whitening and lightening[10]. Thus, it can be developed as an effective, cheap and safe skin anti-ageing cosmaceutical.

In conclusion, this study conclusively demonstrates marked *in vitro* anti-hyaluronidase activity of Sri Lankan low grown orthodox OP grade black tea. This property with its wide range of anti-aging characteristics, namely, antiglycation and AGEs cross-link breaking[8], anti-collagenase (unpublished), antioxidant[14,28], anti-inflammatory[29], sun screening[9], and anti-tyrosinase[10] activities makes it an ideal candidate as an anti-aging, natural and herbal cosmaceutical. Further, this grade of black tea may prove usefully in the management of envenomation, allergic conditions and some forms of joint diseases such as osteoarthritis.

Conflict of interest statement

We declare that we have no conflict of interest.

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