



Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article

doi:10.12980/JCLM.3.2015J5-72

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In vitro pancreatic lipase, cholesterol esterase and cholesterol micellization inhibitory activity of Sri Lankan low grown orthodox Orange Pekoe grade black tea (*Camellia sinensis* L.)

Wanigasekera Daya Ratnasooriya^{1,2*}, Walimuni Prabhashini Kaushalya Mendis Abeysekera³, Chatura Tissa Dayendra Ratnasooriya⁴

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

²Department of Zoology, University of Colombo, Colombo-03, Sri Lanka

³Herbal Technology Section, Industrial Technology Institute (ITI), 363, Bauddhaloka Mawatha, Colombo 07, Sri Lanka

⁴Faculty of Medicine, University of Colombo, Colombo-08, Sri Lanka

ARTICLE INFO

Article history:

Received 5 Jun 2015

Accepted 10 Jun 2015

Available online 12 Jun 2015

KeyWords:

Black tea

Orange Pekoe

Antilipase

Anti-cholesterol esterase

Cholesterol micellization

Hyperlipidaemia

Camellia sinensis

ABSTRACT

Objective: To access the pancreatic lipase, cholesterol esterase and cholesterol micellization inhibitory activities of Sri Lankan low grown orthodox Orange Pekoe grade black tea made from uppermost tender leaves and unopened buds of *Camellia sinensis* L.

Methods: Black tea brew (BTB) was made according to International Organization for Standardization 3103 specifications and concentrations of BTB tested were 37.5, 75.0, 150.0, 300.0 and 600.0 µg/mL for antilipase and anti-cholesterol esterase assays and 0.25, 0.50 and 1.00 µg/mL for cholesterol micellization inhibitory assay.

Results: The results showed that BTB of Sri Lankan low grown orthodox Orange Pekoe grade black tea has marked and dose-dependent ($r^2 = 0.95$) cholesterol micellization inhibitory activity *in vitro* comparable to epigallocatechin gallate, the reference drug used. In contrast, BTB had only mild but dose-dependent ($r^2 = 0.94$) inhibitory activity against pancreatic lipase and weak inhibitory effect (up to 13.17%) on pancreatic cholesterol esterase.

Conclusions: It is concluded that consumption of BTB of Sri Lankan low grown orthodox Orange Pekoe grade tea as a beverage may be a useful strategy in the management of hyperlipidaemia.

1. Introduction

The prevalence of hyperlipidaemia, which is a metabolic disorder characterized by elevated levels of blood triglycerides and cholesterol, is increasing rapidly throughout the world[1].

This may be related to sedentary and stressful life styles, lack of physical exercise and consumption of high fat diets[1]. Hyperlipidaemia plays a central role in the pathogenesis of obesity, diabetes, hypertension with a consequent increased risk of cardiovascular diseases[2]. Currently, there are six categories of antilipemic drugs available to treat lipidaemia: 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins), e.g. lovastatin; cholesterol absorption inhibitors, e.g. ezetimibe; bile acid sequestrants (resins), e.g. colestipol; nicotinic acid, e.g. niacin; fibric acid derivatives (fibrates), e.g. fenofibrate; and

*Corresponding author: Prof. W.D Ratnasooriya, Faculty of Allied Health Sciences, General Sir John Kothalawala Defence University, Ratmalana, Sri Lanka.

Tel: +94-11-2503399

E-mail: wdr@zoology.cmb.ac.lk

Foundation Project: Supported by National Science Foundation of Sri Lanka (Grant No. NSF/Fellow/2011/01).

omega-3 fatty acids (fish oil), e.g. pulse[2]. Most, if not all, of these drugs are expensive and beyond the reach of many people in developing countries. Most importantly, they induce undesirable side effects such as myalgias, arthralgias, elevated liver enzymes, elevated blood glucose, dyspepsia and constipation[2]. Hence, there is an imperative need for search of novel agents (therapeutics, supplements, functional foods or beverages) preferably from natural sources to be used in prevention and treatment of hyperlipidaemia.

Inhibition of fat digestion and absorption by functional foods and beverages is now considered as an important strategy in the dietary management of hyperlipidaemia[3]. In this connection, very few studies have investigated the antilipemic ability of black tea (both *in vitro* and *in vivo*) made from *Camellia sinensis* L. (*C. sinensis*)[4,5]. However, unfortunately, none of these studies has given important information regarding the tea sample such as country of origin, agro-climatic elevation, grade (particle size), harvesting season, climatic changes, wind velocity, age of leaf, processing method, brewing time of tea brew and temperature of tea brew which are known to alter phytoconstituents and bioactivities of tea brew[6-11].

The aim of this study was to investigate the antihyperlipidemic potential (mediated via impairment of lipid digestion and absorption) of Sri Lankan low grown orthodox Orange Pekoe grade black tea by assessing its lipase, cholesterol esterase and cholesterol micellization inhibitory activities *in vitro*. In this connection, it is important to note that at present tea is the most consumed beverage besides water[12]; approximately 80% of the tea produced and consumed worldwide is black tea[12]; Sri Lanka is the leading provider and third largest exporter of orthodox black tea which is consumed in more than 150 countries[12,13]; Sri Lankan black tea accounts for 9%-10% of tea consumption in world and Orange Pekoe grade black tea is most widely used tea[13,14].

2. Materials and methods

2.1. Source of tea

The uppermost tender leaves and unopened buds of *C. sinensis* are plucked from the plantations of St. Jochims Tea Estate of the Tea Research Institute, Hedallana, Ratnapura, Sri Lanka (29 m above mean sea level: low grown) (latitude 6°42'57.96" N, longitude 80°22'46.2" E) during November–December 2011 which were used to process Orange Pekoe grade black tea by orthodox-rotovane technique at the factory estate. The sieve analysis of the tea sample has shown that 83.5% of tea particles were true size (1400-2000 µm) and typical for the grade. Moreover, organoleptic

profile analysis conducted by the professional tea tasters at the tea tasting unit of Sri Lanka Tea Board has confirmed that the sample used can be accepted as well made high quality Orange Pekoe grade Sri Lankan black tea[8]. Tea sample were packed in triple aluminum foil bags (1 kg each) and stored at -20 °C until use.

2.2. Preparation of black tea

Black tea brew (BTB) was made according to the International Organization for Standardization (ISO) standards (ISO 3103): adding 2 g of Orange Pekoe grade black tea to 100 mL boiling water and brewed for 5 min[15]. This contained 36.1% (w/v) tea solids in water and BTB was then squeezed through a muslin cloth and freeze dried. The freeze dried product was stored in air tight container at 4 °C until use.

2.3. Pancreatic lipase inhibition assay

Pancreatic lipase inhibitory activity of BTB was evaluated according to the method described by Kim *et al.* with minor modifications[16]. Porcine pancreatic lipase (Type II, Sigma Aldrich, USA) stock solution (2.5 mg/mL) was prepared in 0.1 mol/L Tris-HCl buffer with 5 mmol/L CaCl₂, pH 7.0 and was stored at -20 °C. Reaction volume of 200 µL, containing 30 µL of 2.5 mg/mL enzyme and 120 µL of different concentrations of BTB (37.5, 75.0, 150.0, 300.0 and 600.0 µg/mL) was pre-incubated at 37 °C for 15 min. Reaction was started by adding 5 µL of 10 mmol/L *p*-nitrophenyl butyrate (*p*-NPB) (Sigma Aldrich, USA) in dimethylformamide and was allowed to proceed at 37 °C for 30 min. Lipase inhibitory activity of BTB was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 405 nm using SpectraMax384 micro plate reader (SPECTRAMaxPLUS³⁸⁴ Molecular Devices, Inc, USA). Inhibition of lipase activity was expressed as the percentage decrease in optical density when pancreatic lipase was incubated with the BTB. The kinetic parameter V_{max} was used to calculate the % lipase inhibition according to the following formula:

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

Where, A is the V_{max} of the control (without the BTB); a is the V_{max} without enzyme and BTB; B is the V_{max} with BTB; b is the V_{max} of respective sample blank (reaction mixture with the BTB and without enzyme was used as the sample blank). Results were expressed as mean ± SEM ($n = 4$).

2.4. Cholesterol esterase inhibition assay

Pancreatic cholesterol esterase inhibitory activity of BTB was determined according to the method reported by Pietsch and

Gütschow with some modifications[17]. Reaction volume of 200 μL , containing different concentrations of BTB (37.5, 75.0, 150.0, 300.0 and 600.0 $\mu\text{g/mL}$) were pre-incubated with 50 μL of 24 mmol/L taurocholic acid, 5 μL of 8 mmol/L *p*-NPB in acetonitrile in 0.1 mol/L sodium phosphate buffer, 0.1 mol/L NaCl, pH 7.0 at 25 °C for 10 min. Reaction was initiated by adding 42.5 μL of cholesterol esterase enzyme (1.25 $\mu\text{g/mL}$) and enzyme reaction was monitored at 25 °C for 6 min by measuring the change in absorbance at 405 nm using SpectraMax384 micro plate reader (IC_{50} values were calculated from the linear steady state turnover of the substrate).

2.5. Cholesterol micellization assay

Artificial micelles were used as a model system for *in vitro* cholesterol solubilization, which contained predominantly uniform particles based on sodium taurocholate, egg lecithins, cholesterol and oleic acid to reflect the natural mixed micelle. Further, they were prepared according to the method described by Kirana *et al.* with minor modifications[18]. Briefly, the solution containing 2 mmol/L cholesterol, 1 mmol/L oleic acid and 2.4 mmol/L phosphatidylcholine were dissolved in methanol and dried under nitrogen before adding 15 mmol/L phosphate-buffered saline containing 6.6 mmol/L taurocholate salt, pH 7.4. The suspension was sonicated twice for 30 min using a sonicator (Bandelin SANOREX electronic, RK 510) and was incubated overnight at 37 °C. Different concentrations of BTB (0.25, 0.5 and 1.00 mg/mL) and phosphate-buffered saline as control were added to the mixed micelle solution and were incubated at 37 °C for more than 2 h. The solution was centrifuged at 16000 r/min for 20 min. The supernatant was collected and cholesterol concentration was determined using total cholesterol test kit (BXC 0261, Fortress diagnostics, UK). Epigallocatechin gallate (EGCG) was used as the positive control.

2.6. Statistical analysis

Data was expressed as mean \pm SEM. IC_{50} values were calculated by linear regression analysis computed using excel software.

3. Results

At the concentration range tested, BTB exhibited dose-dependent ($r^2 = 0.94$) but mild (up to 15.13%) inhibitory activity against pancreatic lipase *in vitro*. The IC_{50} value was (372.22 \pm 81.00) mg/mL. Besides, BTB showed an extremely weak inhibitory activity against pancreatic cholesterol esterase with maximum activity being 13.170%. Nevertheless, it appears that this inhibitory activity has a dose-dependent trend ($r^2 = 0.95$) (Table 1).

In contrast, BTB displayed a marked (up to 60%) and dose-dependent ($r^2 = 0.95$) cholesterol micellization inhibitory activity, at the concentrations tested, which was comparable to cholesterol micellization inhibitory activity exhibited by EGCG, the reference agent used (Table 2). The IC_{50} values for BTB and EGCG were (0.64 \pm 0.01) and (0.15 \pm 0.01) mg/mL respectively.

Table 1

Effect of Sri Lankan low grown orthodox Orange Pekoe grade black tea on pancreatic lipase and cholesterol esterase activity *in vitro*.

Concentration ($\mu\text{g/mL}$)	Inhibition (%)	
	Pancreatic lipase activity	Pancreatic cholesterol esterase activity
600.0	15.13 \pm 0.45	13.170 \pm 0.790
300.0	13.83 \pm 0.69	12.070 \pm 1.840
150.0	5.90 \pm 0.79	5.330 \pm 0.450
75.0	4.45 \pm 0.13	1.040 \pm 0.330
37.5	1.08 \pm 0.34	-0.802 \pm 0.490

Data are expressed as mean \pm SEM, $n = 4$.

Table 2

Inhibition effect of Sri Lankan low grown orthodox Orange Pekoe grade black tea and EGCG on cholesterol micellization *in vitro*.

Concentration (mg/mL)	Inhibition (%)	
	BTB	EGCG
1.00	60.32 \pm 1.61	96.75 \pm 1.08
0.50	46.35 \pm 0.64	69.78 \pm 1.16
0.25	30.54 \pm 1.01	55.16 \pm 0.58

Data are expressed as mean \pm SEM, $n = 6$ and 3 for BTB and EGCG respectively.

4. Discussion

This study examined the *in vitro* lipase, cholesterol esterase and cholesterol micellization inhibitory activities of Sri Lankan low grown orthodox Orange Pekoe grade black tea with a view to explore the use of this tea as a herbal beverage in the prevention and management of hyperlipidaemia, which is an emerging health problem worldwide[1]. In this connection, it should be noted that *in vivo* studies are much difficult and *in vitro* studies are more tractable. Orange Pekoe teas have a pleasant aroma, flavor and taste and are widely used in blending of black teas[12,14]. Tea sample used was factory fresh, unblended and typical to the grade (in terms of sieve analysis, organoleptic properties and phytochemical composition[8,14,19]). Also, BTB was made according to ISO 3103 specification before freeze drying and information of the parameters which can influence bioactivities of the BTB are detailed[15]. The three *in vitro* assays are widely used, well established, specific and validated techniques[16-18]. Thus, on the whole, the results obtained are genuine, reliable and meaningful to this grade of black tea.

The results showed, for the first time, that BTB of Sri Lankan low grown orthodox Orange Pekoe grade tea possesses weak

pancreatic cholesterol esterase inhibitory activity, mild to moderate pancreatic lipase activity and marked and promising cholesterol micellization inhibitory action *in vitro* at the concentration range tested. Impairments of lipase and cholesterol esterase activity observed in this study are direct effects of BTB and possibility exists that it could also act indirectly by modulating the expression of lipase enzyme. All the BTB induced inhibitory activities showed dose-dependent trend indicating genuine, intrinsic, causal and specific activities. Hence, this is an important finding which shows health benefits and therapeutic implications of Orange Pekoe grade tea; inhibition of fat digestion and absorption is now considered as a possible preventive and curative treatment for hyperlipidaemia as well as obesity acting through gastrointestinal mechanisms[2,3].

Dietary fats are digested into free fatty acids and glycerol mainly by the pancreatic lipase secreted into the duodenum[20]. In addition healthy human's gastric lipase secreted by stomach also plays an important role in fat digestion[20]. This study showed that BTB has mild to moderate pancreatic lipase inhibitory activity *in vitro* which would undoubtedly contribute to an antihyperlipidaemic activity in *in vivo* conditions. Indeed, some investigations have shown a suppression of post prandial hypertriacylglycerolemia with black tea polyphenols and theaflavins with galloyl moieties via inhibition of pancreatic lipase supporting this notion[21]. However, the *in vitro* pancreatic lipase inhibitory activity observed in this study with Orange Pekoe black tea is low compared to what has been already reported for black tea[4,21]. But, in those studies, the grade of the black tea used is not specified or high concentrations of tea extracts have been used, or the enzyme source used are not stated or different artificial substrates have been used[4,21]. This could be one possible explanation for the discrepancy observed between this study and other investigations[4,21]. Alternatively, there could be low theaflavin content in the Orange Pekoe grade tea though it has a high polyphenol content[19]. BTB could also provide an antihyperlipidaemic effect by inhibiting gastric lipase and it is worth examining this aspect too. In healthy humans, intra gastric lipolysis, even limited, initiate fat digestion and emulsification, and thereby facilitating lipolysis catalyzation by pancreatic lipase in the duodenum[21].

An inhibition of fat absorption and a suppression of lipid absorption can be mediated by three main mechanisms: inhibition of pancreatic cholesterol esterase activity[22]; impairment of cholesterol micellization[5,23], and inhibition of bile acid binding[3]. Dietary cholesterol consists of both free cholesterol and esterified cholesterol[22]. Esterified cholesterol are hydrolysed by pancreatic cholesterol esterase which has wide substrate specificity[22]. Because of this, it is also known as non-

specific lipase or bile salt activated lipase[22]. This study showed that BTB possesses *in vitro* weak pancreatic cholesterol esterase activity. However, inhibition of pancreatic cholesterol by any variety of tea (green, black or oolong) is poorly investigated compared to edible plants[3]. Recently a study has shown *in vitro* cholesterol esterase inhibitory activity of tea leaves (*C. sinensis*) using a methanolic extract and bovine cholesterol esterase enzyme[22]. But, in this study, we have used manufactured black tea instead of fresh tea leaves, and the tea brew was made according to ISO 3103 specifications which is more realistic[15]. Further, porcine cholesterol esterase enzyme was used. These could account for the evident low pancreatic cholesterol esterase inhibitory activity here. It is claimed/suggested that cholesterol esterase inhibitory activity is mediated via flavonoids in tea by irreversibly binding to its active fatty acid pocket at serine 194[22]. It is also suggested that flavonoids act as suicide substrate ahead of cholesterol esters[22]. Thus, it is rather surprising why BTB failed to show marked cholesterol esterase inhibitory activity in spite of its high flavonoid content[19]. In contrast, BTB exhibited marked *in vitro* cholesterol micellization inhibitory activity. This cholesterol micellization inhibitory activity of BTB can be attributed to theaflavins present in the Orange Pekoe grade tea[23]. Several studies have shown that incorporation of cholesterol into micelles are inhibited by theaflavin, which results in impaired intestinal cholesterol absorption[5,23]. Further, it has been shown by electron microscopic studies that theaflavins, in the presence of cholesterol, form insoluble multilamellar membrane structures entrapping cholesterol, thereby reducing the cholesterol available for absorption[23]. In addition to theaflavins it is possible that thearubigins which is present in high amounts in Orange Pekoe grade black tea could also play a role, at least partly, in the inhibition of cholesterol micellization process[12,19]. Evidence is now accumulating to suggest that thearubigins contribute to many bioactivities shown by black tea[11,12]. The third mechanism which impairs cholesterol absorption in the intestine is via inhibition of bile acid binding[3]. This mechanism is also likely to be operative with Orange Pekoe grade black tea since polyphenols have been shown to inhibit bile acid binding *in vitro*[3,5].

Oxidative stress is now known to be involved in hyperlipidaemia[2,3]; it is indeed an early event in the evolution of hyperlipidaemia[24]. Orange Pekoe tea has shown to possess antioxidant activity and this property is also likely to play a pivotal role in triggering antihyperlipidaemia and anti-obesity effects[19,25].

In conclusion, the results of this *in vitro* study showed, for the first time, antihyperlipidaemic and anti-obesity properties of Sri Lankan low grown orthodox Orange Pekoe grade black tea, which is mediated mainly via inhibition of cholesterol micellization. It is

suggested that regular consumption of Sri Lankan Orange Pekoe grade tea as a beverage may be valuable strategy in the dietary management of hyperlipidaemia (hypertriacylglycerolemia and hypercholesterolemia) and obesity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We acknowledge the National Science Foundation of Sri Lanka for funding (NSF/Fellow/2011/01).

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