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In-vitro and *In-vivo* Antioxidant Activity of High-Grown Sri Lankan Black Tea *(Camellia Sinensis L.)*

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ABSTRACT

The antioxidant potential of high-grown Sri Lankan black tea (BOPF grade, obtained from St Coombs Estate, Talawakelle) was measured in this study. Antioxidant activity was measured both *in vitro* (radical scavenging activity using DPPH assays and inhibition of lipid peroxidation using TBARS assays) and *in vivo* (using DPPH assays on rat serum, following oral administration for three months). The study also estimated total polyphenols, caffeine, theaflavin, thearubigin and total catechin levels, and individual levels of five major catechins (EGCG, EGC, ECG, EC and C).

The results show that Sri Lankan black tea possesses mild but dose-dependant antioxidant activity *in vitro*. The *in-vivo* antioxidant activity was both dose- and time-dependant. The antioxidant activity of serum was elevated only as long as the tea was administered to the rat.

Key words: *Camellia sinensis*, Sri Lankan tea, antioxidant activity.

INTRODUCTION

A hot, aqueous infusion of the processed leaves of the *Camellia sinensis L.* plant (black tea) is the most popular and widely-consumed beverage in the world (Lai *et al.*, 2001).

Black tea contains 15 – 30% antioxidant flavonoids, which are polyphenolic phytochemicals, on a dry weight basis (Hara, 1997). The predominant flavonoids in fresh tea leaves are flavanols. The major flavanols in fresh leaf are catechins or flavan-3-ols. Six types of major catechins have been identified: epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), gallocatechin (GC) and catechin (C) (Hilton *et al.*, 1973).

Most of the catechins in the fresh tea flush are oxidized to form thearubigins and theaflavins during black tea processing, the remainder being unchanged (Hara, 1997). Therefore, black tea could be a major dietary source of natural antioxidants (Modder and Amarakoon, 2002).

Antioxidants are defined as any substance that can delay or prevent the oxidation of a substrate, when present in small amounts relative to the amount of the substrate (Diplock, 1994). Substances with *in-vivo* antioxidant activity could prevent or delay damage to cells and tissues, by free radicals and other oxidizing substances. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are forms of activated oxygen and nitrogen which include unstable molecules such as superoxide ($O_2^{-\bullet}$, $OOH^\bullet$), hydroxide ($OH^\bullet$), peroxide ($ROO^\bullet$), nitric oxide ($NO^\bullet$) and peroxy nitrite ($ONOO^\bullet$) radicals (Halliwell and Aruoma, 1997). In an attempt to stabilize, ROS and RNS attack other molecules in the body, leading to cell damage and triggering the formation of other free radicals which result in a chain reaction. Free radicals are produced naturally in the body as a result of chemical and biochemical reactions during normal cellular processes. Flavonoids in tea would neutralize these radicals and reduce the risk of heart disease, stroke, atherosclerosis, dental caries, cataract and cancers (Aruoma, 1998).

"Ceylon tea" (produced in Sri Lanka) accounts for 20% of the tea consumed in the world (Anon., Sri Lanka Tea Board Annual Report, 2003). However, antioxidant activity, specially *in-vivo* antioxidant activity, in various types of Sri Lankan tea has been poorly investigated. This study examines the antioxidant potential of the high-grown, Broken Orange Pekoe Fannings (BOPF) grade of Sri Lankan black tea, both *in vitro* (DPPH assay and TBARS assay) and *in vivo* (DPPH assay), using rat blood serum. The BOPF grade has a high demand among Sri Lankan tea drinkers.

MATERIALS AND METHODS

Preparation of black tea

BOPF-grade black tea processed by the orthodox-rotorvane process during the second week of August, 2003, at St Coombs Estate tea factory, Talawakelle, was used for the present study.

Green leaf plucked at St Coombs Estate (1382 m above mean sea level) was used for processing the tea. The conditions of manufacture were: 18 h trough withering; precondition rolling using orthodox roller and four rotorvane passes; 2 h 30 min fermenting period; and 20 min firing period. Tea samples was packed in triple laminated, aluminum foil bags, 1 kg in each bag, and stored at -20 °C.

Experimental animals

Healthy adult, male albino rats of the Wistar strain (body weight: 225 – 250 g), purchased from the Medical Research Institute of Sri Lanka, were used. The rats were housed in the Department of Zoology, University of Colombo, Sri Lanka, under standard animal-house conditions with continuous access to food and water *ad libitum*..

Sieve analysis to determine tea grade

The particle composition of the tea sample was determined by sieve analysis using a sieve shaker (Retsch AS 200, UK), equipped with a standard sieve set, 850 – 350 μm. A 100 g tea sample was used for the sieve analysis. Tea particles retained over the 850 μm size sieve were regarded as the 'over size' fraction, while the particles which passed through the 350 μm size sieve was regarded as 'under size'. Particle sizes in between 850 and 350 μm were considered as the 'true size' composition of the BOPF grade. When this fraction was more than 75% (w/w) of the total quantity, the tea sample was considered as being true to the size of the BOPF grade.

Estimation of total polyphenols, caffeine, catechins, thearubigins and theaflavins in the tea samples

Total polyphenols were determined according to the method described by Anon (2004 a). Briefly, after adding Folin-Ciocalteu reagent, the absorbance of the sample solution was measured at 765 nm using a UV/visible spectrophotometer (Cintra 5 - GBC Scientific, Australia). The concentration of the total polyphenols was expressed as a percentage of the dry weights of the samples.

Caffeine and individual catechins were determined according to the method described by Anon (2004 b). Caffeine and individual catechins were estimated using high performance liquid chromatography (HPLC).

The HPLC conditions were:

HPLC: Perkin Elmar, Model LC 250, Australia

Column: Phenomenex Luna 5μm Phenyl-Hexyl®, 250mm x 4.6mm

Detector: UV/visible, 278 nm

Mobile phase

Mobile phase A: 9% v/v acetonitrile, 2% v/v acetic acid, with 20 μg mL^{-1} EDTA

Mobile phase B: 80% acetonitrile, 2% v/v acetic acid, with 20 μg mL^{-1} EDTA

Flow rate: 1 mL min^{-1}, mobile phase A for 1 min, then over 15 min a linear gradient to 68% mobile phase A, and 32% mobile phase B and hold for 10 min

Injection volume: 10 μL (standards and test samples)

Standard solutions of gallic acid, C, EC, ECG, EGCG and ECG, were used where appropriate. The results of the triplicate analysis were expressed as a percentage of the dry weight of the samples.

Theaflavins (TF) and thearubigins (TR) of the tea samples were determined according to the method previously described by Roberts and Smith (1963). Twenty-five millilitres of infusion (9.0 g tea in 375 ml boiling DW) were extracted with an equal volume of isobutyl methyl ketone (IBMK, 4-methyl-2-pentanone) in a separating funnel. The aqueous layers and IBMK layers were separated, and used to determine TF and TR colorimetrically using the UV/visible spectrophotometer at 380 nm and 460 nm. The results were expressed as a percentage of the dry weight of the samples.

Assessment of total *in-vitro* antioxidant activity in tea samples, using the DPPH assay

The DPPH radical-scavenging assay of Brand–Williams *et al.* (1995) was used, with minor modifications. A 5-minute incubation period was used instead of 20 minutes. In this method, 750 µl of freshly prepared 20 ppm 1-1-diphenyl–2-picrylhydrazyl (DPPH) solution was added to an equal volume of the sample solution. A sample solution at the low dose was prepared by adding 100 ml of boiling water to 1 g of tea (and allowing the mixture to stand for 5 min); the mid dose by adding 100 ml of the water to 2 g of tea; and the high dose by adding the water to 8 g of tea.

DPPH and the sample solutions were incubated at room temperature for 5 min. After incubation, absorbance was measured using the UV/visible spectrophotometer at 517 nm. The percentage of the DPPH radical scavenged by the tea extract was calculated, and the antioxidant activity was expressed as the Trolox equivalent in $\mu g l^{-1}$.

Assessment of total *in-vitro* antioxidant activity in tea samples, using the TBARS assay

The reduction by tea extract of thiobarbituric acid reactive substances (TBARS), produced in rat serum, was monitored as previously described by Lefevre *et al.* (1998). This technique measures the effect of tea extract on lipid peroxidation in serum, a major indicator of oxidative stress. Ten microlitres of rat blood serum, diluted with 40 µl distilled water (DW), were subjected to analysis with the TBARS assay (using egg-yolk), and the absorbance of the butanol layer was measured in a spectrophotometer (ANA 72 – UV/visible, Japan, at 532 nm.. Vitamin E (Torrent Pharmaceuticals Ltd., Mehsana, India) was used as the reference.

The antioxidant activity of the serum sample was estimated as an 'Antioxidant Index' (AI), using the following equation:

AI = (1 – T/C) x 100 (T = absorbance of the test sample, C = absorbance of the oxidized control.)

Assessment of total *in-vivo* antioxidant activity in rat serum, using the DPPH assay

Thirty-six male rats were divided randomly into four equal groups (n = 9/group). The rats in each group had either tea brew (three groups), or DW (one group), orally administered to them at the rate of 1 ml/100 g body weight, three times a day (0800, 1200 and 1600 h), for 12 consecutive weeks. The three groups administered tea brew received the brew at concentrations of 60, 120 and 480 µg tea solids/ml of DW, respectively.

The rats were fasted for 14 – 17 h, after which blood samples were collected from their tails under mild ether anaesthesia, using aseptic precautions, in the 4th, 8th and 12th weeks of treatment, and in the 6th week after the cessation of treatment. Serum was separated from the blood at room temperature (28 – 30 ^{0}C), and stored at –70 ^{0}C until use. The frozen serum was thawed at room temperature (28 – 30 ^{0}C) for 20 min, and immediately assessed for antioxidant activity using the DPPH free-radical scavenging assay (Brand-Williams *et al.*, 1995), but with minor modifications: 30 µl sample volume of blood plasma and 5 minutes incubation period, instead of 20 µl sample volume and 30 minutes incubation period. Trolox, a water-soluble analogue of vitamin E, at 25 ppm was used as the reference standard.

The reaction mixture was incubated at room temperature for 5 min, after which the discolouration of the stable DPPH solution (20 ppm) was evaluated by measuring the absorbance at 517 nm using a spectrophotometer (Cintra 5 – GBC Scientific, Australia). The total antioxidant activity was estimated using the calibration curve for Trolox.

The results are expressed as the percentage scavenged by the sample using the following equation on the control and each treated group:

$$\% \text{ Scavenged} = \frac{(A_{\text{Control}} - A_{\text{sample}})}{A_{\text{Control}}} \times 100$$

Statistical analysis of the results

The results are expressed as mean ± SEM. Statistical comparisons were made using the Mann-Whitney U-test. Differences in mean values with $P \leq 0.05$ were considered as significant. Linear regression analysis was performed to assess dose-dependencies.

RESULTS

Tea grading test

The 'true size' mean particle composition of the sample was 89.33 ± 1.29 % (w/w).

Total polyphenols, caffeine, catechins, thearubigins and theaflavins in the tea samples

The concentrations of the major constituents of the tea samples are summarized in Table 1. The total polyphenol content in the samples was 22.58 ± 0.21%. The caffeine in the sample was 3.38 ± 0.014%. As shown, epigallocatechin gallate (EGCG) was the predominant catechin in the sample (48% of the total catechins), followed by ECG > EC > EGC > C.

Table 1: Polyphenols and other extractable compounds in high-grown, Sri Lankan BOPF grade, black tea (2 g in 160 ml water)

Constituents	Content (mg/tea cup, means ± SEM)
Total polyphenols	451.6 ± 4.2
Caffeine	67.6 ± 0.28
Total catechins Epigallocatechingallate(EGCG) Epigallocatechin (EGC) Epicatechin gallate (ECG)) Epicatechin (EC)) Catechin (C))	102.4 ± 3.4 49.6 ± 2.0 6.4 ± 0.3 33.0 ± 0.6 10.4 ± 0.3 2.8 ± 0.3
Theaflavins	21.8 ± 0.32
Thearubigins	285.4 ± 2.2
Ratio: thearubigins: theaflavins	13.08 ± 0.18

The total catechin content in a tea cup (160 ± 10 ml) was 102.4 ± 3.4 mg, assuming that 2.0 g of black tea was used. The theaflavin and thearubigin content in the sample was 1.09 ± 0.016% and 14.27 ± 0.11%, respectively. The ratio, thearubigins: theaflavins, was found to be 13.08 ± 0.18%, which is 13 times higher than the ratio in the sample.

Total *in-vitro* antioxidant activity in tea samples, as determined by the DPPH assay

Antioxidant activity of the tea samples, obtained by the DPPH method and expressed as Trolox equivalents, is given in Table 2. The mean antioxidant activity of the tea brew (prepared by placing 2 g of tea in 100 ml boiling water for 5 min) was 2858.7 ± 37.8 μg Trolox equivalents per tea cup.

Table 2: *In-vitro* antioxidant activity in hot water extracts of high-grown, Sri Lankan BOPF grade, black tea (DPPH assay method)

Hot Water Extracts	Antioxidant Activity (μg/tea cup in Trolox (vitamin E) equivalents, means ± SEM)
Replicate 1	2860.6 ± 28.7
Replicate 2	2854.9 ± 45.3
Replicate 3	2860.6 ± 39.5

Total *in-vitro* antioxidant activity in tea samples, as determined by the TBARS assay

Antioxidant activity of the tea samples, obtained by the TBARS method, is given in Table 3. As shown, the tea infusions exhibited dose-dependant antioxidant activity ($r^2 = 0.8768$, $P \leq 0.02$) in terms of the Antioxidant Index. The Antioxidant Index of the tea infusions are compared with that of vitamin E.

Table 3: *In-vitro* antioxidant activity in hot water extracts of high-grown, Sri Lankan BOPF grade, black tea (TBARS assay method)

Concentration of extracted tea solids (μg / ml)	Antioxidant Index (AI) (mean ± SEM)
60.0	395.6 ± 70.9
120.0	839.0 ± 128.5
480.0	3987.0 ± 531.0
Vitamin E, 108.0 μg/ml	1403.0 ± 231.9

Total *in-vivo* antioxidant activity in rat serum

The total *in-vivo* antioxidant activity found in rat serum is presented in Fig. 1. The results (expressed as percentages of scavenged DPPH) show that the tea brew increased the serum antioxidant activity, both in a dose- and time-dependent fashion, in the 4th (r^2 = 1.0, $P < 0.05$), the 8th (r^2=0.9724, $P < 0.05$), and the 12th (r^2 = 0.9805, $P < 0.05$) weeks of treatment.

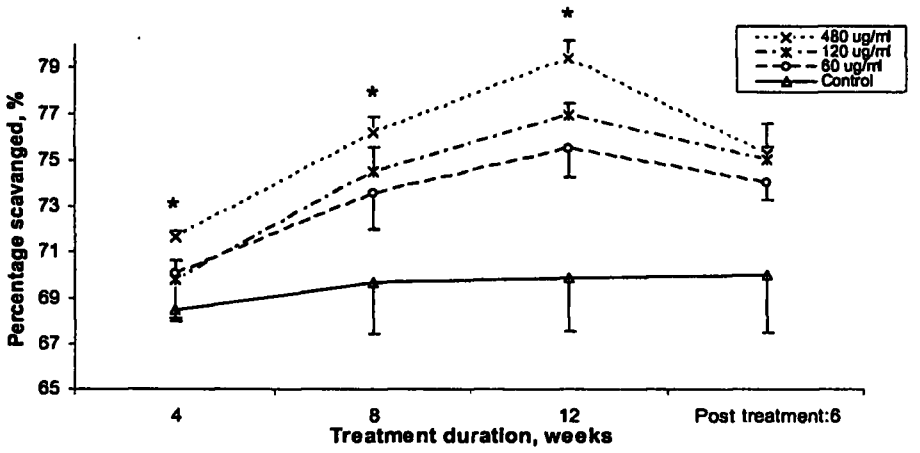

Fig. 1: Effect of three-months oral administration of different doses of hot water extracts of high-grown, Sri Lankan BOPF grade, black tea on total antioxidant activity in blood serum of rats (DPPH assay method) (mean ± SEM, N = 9/group).

* Values are significantly different to that of the controls ($P < 0.05$).

There was a small but significant increase ($P \leq 0.05$) in the antioxidant capacity of rat blood serum in the 4th week (an increment of 2.3 %), the 8th week (5.5 %), and the 12th week (8.0 %), in the low-dose group. In the mid-dose group, there was a significant increase ($P \leq 0.05$) in serum antioxidant capacity in the 4th week (an increment of 1.9 %), the 8th week (6.8 %), and the 12th week (10.1 %). With the high-dose group, a significant increase ($P \leq 0.05$) in serum antioxidant capacity in the 4th week (an increment of 4.6 %), the 8th week (9.2 %), and the 12th week (13.6%), was evident.

In contrast, six weeks after the cessation of treatment, there was no significant difference ($P > 0.05$) in the antioxidant activity of the serum of both the treated and the control groups.

DISCUSSION

This study examined the antioxidant potential of high-grown BOPF-grade, Sri Lankan black tea, manufactured at St Coombs Estate (elevation: 1382 m mean sea level), Talawakele, Sri Lanka, both *in vitro* and *in vivo*. The BOPF grade was selected as it is in high demand among Sri Lankan tea drinkers, probably owing to its particular taste, colour and flavour. Sieve analysis confirmed that the sample selected was true to the size of the BOPF grade.

The composition of total catechins in fresh, green leaves of tea is known to change with climate (Wickramasingha, 1978; Keegal, 1983). Therefore, it is planned to extend these studies to other types of Sri Lankan teas, namely mid-grown and low-grown teas, so that a complete picture of antioxidant activity in Sri Lankan teas could be obtained.

In this study, *in-vitro* antioxidant activity was assessed using assays based on DPPH (which measure the antioxidant activity in terms of hydrogen-donating or radical-scavenging ability), and on TBARS (which measure the antioxidant activity in terms of lipid peroxidation). Both these techniques are sensitive, reliable and widely used in the antioxidant-activity estimation of crude aqueous plant extracts (Brand-Williams *et al.*, 1995; Lefevre *et al.*, 1998).

The results show that BOPF tea possesses dose-dependant antioxidant activity, both *in vitro* (using DPPH and TBARS assays) and *in vivo* (using DPPH assays). With the DPPH technique, the antioxidant activity recorded was similar to what has been reported earlier for Sri Lankan BOPF-grade teas (TRI, unpublished). To our knowledge, the TBARS technique has not been used previously to assess the antioxidant activity of Sri Lankan teas. However, it is of interest to note that, in our experimental settings, the antioxidant activity (in terms of an 'antioxidant index') of the extracted tea solids was comparable to that of vitamin E, on a weight for weight basis. Tea polyphenols account for only 30 – 40% of the extracted solids (Harbowy and Balentine, 1997). Therefore, tea polyphenols would have higher antioxidant activity than vitamin E.

EGCG has been reported as being the most active antioxidant in tea (Matsuzaki and Hara, 1985; Hara, 1999). The EGCG content of the BOPF sample in the present study (48% of the catechin content) is not markedly different from that reported previously (50 – 60%, Guo *et al.*, 1999). However, the total catechins in the sample (102.4 ± 3.4 mg/cup) was lower than that reported previously (140 mg/cup, Lakenbrink *et al.*, 2000). The amount of total catechins in a sample of black tea depends on that initially present in the fresh leaf, and the time allowed for oxidation reactions to take place which is essentially the fermentation period during processing, when catechins are converted to theaflavins and thearubigins. The amount of catechins in a black tea sample could therefore vary, depending on the amount of catechins in the fresh leaf and the extent of the fermentation period.

In-vivo antioxidant activity was studied in rat blood serum using the DPPH assay. All the doses showed time-dependant and dose-dependant antioxidant activity. This antioxidant activity appeared to be present as long as tea was being administered to the experimental rats, since antioxidant activity fell markedly following cessation of the treatment (at six weeks, which is half the duration of treatment).

Other workers have reported higher antioxidant activity *in-vivo*, using the DPPH (Brand-Williams *et al.*, 1995; Hara, 1999) and the FRAP (ferric reducing antioxidant power) (Hansheng, 1999; Benzie and Szeto, 1999) techniques. This discrepancy in antioxidant activity could be due to the different techniques being used (DPPH and FRAP assays), and due to differences in the total amounts of polyphenols in the sample.

The amounts of tea solids received by rats in the low dose used in this study is similar to that received by humans when 1.5 cups of tea are consumed per day. Mid and high doses correspond to 3 and 12 cups per day, respectively. Therefore, the tea normally consumed by Sri Lankans (4-5 cups per day) would elevate their plasma antioxidant activity.

This study demonstrated antioxidant activity in high-grown BOPF-grade Sri Lankan black tea, both *in vitro* and *in vivo*. The tea sample used showed mild antioxidant activity both *in vitro* and *in vivo*.

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