EVALUATION OF IN VITRO ANTIOXIDANT POTENTIAL OF GREEN TEA (CAMELIA SINENSIS) INFUSIONS WITH LEAVES COLLECTED FROM DIFFERENT HEIGHTS OF DARJEELING HILL, WEST BENGAL

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ABSTRACT

Tea (Camelia sinensis) is the most consumed beverage and is also known for its medicinal value. The leaves are processed industrially for commercial usage or processed traditionally for preparation of infusions in different cultures of different countries. The present study was undertaken to find out whether the antioxidant activity and the content of tea bioactives change after processing by a local traditional process. Fresh tea leaves were collected from six different altitudes of Darjeeling hill, viz. 6900, 5800, 4500, 3600, 2500 and 500 feet. The assays performed included ABTS and DPPH radical decolorization assays and contents of total polyphenols, tannins, total flavonoid and ascorbic acid. It was observed that ABTS and DPPH radical scavenging abilities as well as ascorbic acid content were reduced in the leaves collected from higher altitudes, suggesting probable depletion of the antioxidant bioactives on exposure to extreme climatic conditions as well as elevated UV radiations. This was substantiated by the fact that total phenolic contents and flavonoid contents were higher in the higher altitude leaves. Tannin contents were almost unchanged. The study indicated that the medicinal value of the tea infusions remained almost unchanged irrespective of altitude.

KEY WORDS: Tea, Antioxidant, flavonoids, polyphenols, tannins.
INTRODUCTION

Tea is one of the most popular beverages worldwide and second most consumed beverage over coffee, soft drinks, beer or wine.\cite{1,2} It is cultivated in more than 30 countries, however mainly in China, India, Japan and Sri Lanka.\cite{3} The economic and social impact of tea consumption is vast and it is now a part of food habit of several populations as an everyday drink and as a therapeutic aid in many illnesses.\cite{4} Black tea is the primary choice in western countries and in south Asian countries such as India and Sri Lanka, whereas green and oolong teas are consumed mainly in East Asian countries such as China, Japan and Taiwan.\cite{5} However, tea cultivated and processed in Darjeeling hill in India is one of the most popular teas & widely commended for its satisfying aroma and high therapeutic potential.\cite{6}

Tea beverage is an infusion of the dried leaves of the shrub *Camellia sinensis*, a member of Theaceae family. The Indian variant, *Camellia assamica* is also consumed largely in many parts of this country.\cite{3} Green tea is prepared from the fresh tea leaf and widely consumed in Japan, China, Korea and Morocco. However, western cultures favour black tea which is prepared through the oxidation, curing process of maceration and exposure to atmospheric oxygen.\cite{7,8} Green tea is characterized by its high flavonoid content, mainly catechins (20-30% of the dry weight). Condensed tannins are also an important component of tea which are transformed products of flavan-3-ols or flavan-3,4-diols.\cite{9} Consumption of green tea is especially popular in different human cultures and races, and its association with anti-inflammatory, anti-proliferative and anti-atherosclerotic activities has led to the inclusion of green tea extracts in dietetic supplements, nutraceuticals and functional foods as well.\cite{10} Recent studies revealed that it might possess synergistic effect with antibiotics, anti-Alzheimer, anti-Parkinsonism and anti-viral effects.\cite{11} However, a few harmful effects of tea overconsumption (black or green) were observed, probably due to three main factors: (1) its caffeine content, (2) the presence of aluminum, and (3) the effects of tea polyphenols on iron bioavailability.\cite{12}

Plants grown at high altitude are subjects to enhanced oxidative stress due to high exposures to UV radiations.\cite{13,14} To combat such stress, plants have evolved effective cell protective mechanisms that retard cell damage, thus enabling plants to survive.\cite{6} In this context, flavonoids were found to be most relevant.\cite{15} In a study published previously from our laboratory, it was observed that antioxidant profile of the tea leaves change with the altitudes of collection of tea leaves and radical scavenging abilities were reduced at higher altitudes.
However, major bioactive components remained unchanged suggesting their unaltered medicinal values.\[16\]

Depending on the manufacturing process, teas are classified into three major types: (i) non-fermented green tea (produced by drying and steaming the fresh leaves), (ii) semi-fermented oolong tea (produced by partial fermentation before drying) and (iii) fermented black tea, which undergo a post-harvest fermentation stage before drying and steaming. The process for preparation of the green tea from fresh tea leaves involves a non-fermentative process which is employed by the locales in the region. In this process, almost all the bioactives are supposed to be preserved which could be manifested in the infusions prepared from them. The present study was conducted to found out the relationship between altitude variations and changes of antioxidant profile of the green tea infusions prepared from fresh tea leaves by the conventional process used by the locales. As we know tea as a popular beverage, this study will provide information about the changes in the contents of polyphenolics, especially tannins, in different green tea preparations, using tea leaves collected from different altitudes, thus providing the knowledge whether the quality of the tea remains same or not with change in altitudes. The present study reports the achievement of the aim through some common in vitro antioxidant assays.

**MATERIALS AND METHODS**

**Chemicals**

2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, were obtained from Sigma, USA. 2,2′-Diphenyl-1-picryl hydrazyl (DPPH) were obtained from Himedia, India. Folin-Denis reagent was a kind gift from Dr. Tapan Kumar Pal, Department of Biotechnology, Bengal Institute of Technology, Kolkata. All other reagents and chemicals used were of analytical grade procured from Merck, India and SRL, India. Deionized distilled water was used in the entire study.

**Collection of samples**

Young fresh leaves (twigs, from which processed teas are prepared) were collected from tea plants of first flush in the month of April, from 6 different altitudes of Darjeeling Hill region, viz. 6900, 5800, 4500, 3600, 2500 and 500 feet respectively. The tea gardens at different altitudes were randomly selected following the scheme – simple random sampling without replacement (SRSWOR).\[16\] The heights of the tea gardens from mean sea level (msl) were obtained from the respective offices of the tea gardens from where the samples were collected.
Preparation of non-commercial green tea leaves

½–1 lb of fresh tea shoots young tea shoots that have two leaves and a central, needle-like leaf were collected from the six different altitudes. The tea shoots were microwaved for approximately 2 minutes in an autoclavable plastic bag. The cooked tea leaves were then taken out from the bag, the shoots made separated, and spread on a muslin cloth for about 3 minutes. The tea leaves were then gathered into a loose ball within the muslin cloth and rolled with light pressure for 1 or 2 minutes. The shoots were broken apart and they were separated from each other carefully. This allowed a uniform moisture loss for each tea shoot prior to pan-frying. The shoots were then pan fried in a frying pan over low heat, by tossing them gently for about 1½ minutes until the surfaces of the leaves appear dry. The tea leaves were then spread on a muslin cloth to cool, after that the shoots again separated. The tea leaves were then gathered into a loose ball within the muslin cloth and the previous process was repeated. After the second pan-frying, the tea leaves and stems should show a slightly crispy texture. The tea leaves were then evenly spread evenly in a pan over very gentle heat for final drying until the stems are fully dried. The cooled, dry tea leaves were then packed in airtight aluminum bags or other containers for further studies. [17]

Preparation of green tea infusions

0.5 to 1gm processed green tea leaves were brewed in 10 ml boiling water for 2-3 minutes, then cooled and filtered with Whatman 1 filter paper. The volume was made to 10 ml with water. This filtrate was used for further analysis. [18]

Antioxidant assays

ABTS radical decolorization assay

The ABTS assay was performed using a previously described procedure. [19] ABTS⁺⁺, the oxidant, was generated by persulfate oxidation of 2,2’-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid. This solution was diluted with phosphate buffer (pH 7.4) until the absorbance reached 0.7 to 0.8 at 734 nm in a Systronics spectrophotometer (model – 2202). The oxidant solution was mixed with the sample solutions in such a way that total volume of the solution reached 1 ml. The absorbance was read at room temperature, 4 minutes after mixing. The concentration that causes a decrease in the absorbance of initial oxidants by 50% is defined as IC₅₀ of the samples. Gallic acid was used as positive control and comparing with its’ IC₅₀ and the results were expressed as Gallic acid equivalents (µM/gm fresh leaves).
**DPPH radical decolorization assay**

The DPPH assay was performed using a previously described procedure.\[^{19}\] 1 ml DPPH solution (3 mg in 25 ml ethanol) was mixed with 0.5 ml sample solution and the decrease in absorbance of the mixture after 20 minutes of incubation in the dark was monitored at 517 nm in a Systronics spectrophotometer (model – 2202). The concentration that causes a decrease in the absorbance of initial oxidants by 50% is defined as IC\(_{50}\) of the samples. Gallic acid was used as positive control and comparing with its’ IC\(_{50}\) and the results were expressed as Gallic acid equivalents (\(\mu\)M/gm fresh leaves).

**Estimation of total phenolics content**

Total phenolic compound contents were determined by the Folin-Ciocalteau method.\[^{20}\] The samples (0.5 ml) were mixed with Folin-Ciocalteau reagent (5 ml, of 1:10 diluted sample with distilled water) for 5 min and aqueous sodium carbonate (4 ml, 1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm with a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of \(\mu\)g gallic acid equivalent/gm fresh leaves.

**Estimation of tannin content**

Content of Tannins was determined by Folin-Denis method.\[^{21}\] Briefly, 0.5 ml methanol extracted sample solution was taken and diluted 1:10 (v/v) with water. Folin-Denis reagent was added to it. Further 0.5 ml saturated sodium carbonate solution was mixed with it. The volume was made up to 5 ml by addition of 3.75 ml of water. Then the mixture was incubated in the room temperature for 30 minutes. The absorbance of the reaction mixture was then measured at 700 nm with a UV-Vis spectrophotometer (model – Systronics 2202). Tannic acid was used as standard. The results are expressed in terms of tannic acid equivalent (\(\mu\)M/gm fresh leaves).

**Estimation of total flavonoids content**

Total flavonoid content was determined according to a published colorimetric method\[^{21}\] with some modification. Briefly 0.5 ml sample was mixed with 2 ml of distilled water and 0.15 ml of aqueous sodium nitrite solution (NaNO\(_2\), 5% w/v), allowed to stand for 6 min, 0.15 ml aqueous aluminium trichloride solution (AlCl\(_3\), 10% w/v) was added and allowed to stand again for 6 min, followed by addition of 2 ml of aqueous sodium hydroxide (NaOH, 4% w/v) solution. The final volume was made up to 5 ml by distilled water. The reaction mixture was mixed thoroughly and allowed to stand for another 15 min. The absorbance of the reaction mixture was then measured at 510 nm with a UV-Vis spectrophotometer (model – Systronics...
Quercetin was used as standard. The results are expressed in terms of quercetin equivalent (µM/gm fresh leaves).

**Estimation of ascorbic acid content**
The assay was done following a published procedure. Stock iodine solution was standardized by titrimetric method using standard (N/100) sodium thiosulphate solution followed by addition of 1% starch solution, when the solution turned blue and continue the titration until the blue color just discharged. The infusions were then titrated with the standard iodine solution. In a similar procedure, standard ascorbic acid solution was also titrated. Comparing the titers, the results were expressed as mg ascorbic acid/gm fresh sample.

**Statistical Analyses**
Experimental results are expressed as mean ± SD of three individual samples. The statistical analysis was done by using the software the software ‘Prism 4.0’ (GraphPad Inc., USA).

**RESULTS**

**ABTS radical decolorization assay**
The results of this assay indicated that the antioxidant properties of tea leaves were decreasing with the increasing altitudes. The first three samples, collected from higher altitudes (viz. 6900, 5800 and 4500 feet, respectively) showed gallic acid equivalent values of 103.29±13.62, 223.48±21.53 and 342.23 ±12.59 µM/gm processed leaves respectively, whereas the three samples collected from lower altitudes (viz. 3600, 2500 and 500 feet, respectively) showed gallic acid equivalent values of 318.04 ±18.75, 466.50±31.14 and 404.23±19.64 µM/gm of leaves respectively. If one goes carefully through the values, then it can be easily said that there was a trend of increase of radical scavenging activity as the altitude decreases and the three samples collected from lower altitudes showed greater radical scavenging abilities than the samples obtained from higher altitudes (Fig 1).

**DPPH radical decolorization assay**
The results of this assay also indicated that the antioxidant properties of tea leaves were decreasing with the increasing altitudes. The first three samples, collected from higher altitudes (viz. 6900, 5800 and 4500 feet, respectively) showed gallic acid equivalent values of 43.07±0.92, 30.90±1.49 and 33.83 ±3.56 µM/gm processed leaves respectively, whereas the three samples collected from lower altitudes (viz. 3600, 2500 and 500 feet, respectively) showed gallic acid equivalent values of 20.76 ±1.89, 59.69±1.85 and 62.49±2.09 µM/gm of leaves respectively.
leaves respectively. If one again goes carefully through the values, then it can be easily said that although the values were not discrete, there was a trend of increase of radical scavenging activity as the altitude decreases and the two samples collected from lower altitudes showed greater radical scavenging abilities than the samples obtained from higher altitudes (Fig 2).

**Estimation of total polyphenolics content**

The results of this assay indicated that total phenolics were higher, albeit non-significantly, in the higher altitudes (Fig 3). The first samples collected from higher altitudes (viz. 6900, 5800 and 4500 feet, respectively) showed gallic acid equivalent values of 20.92±0.84, 22.40±1.65 and 20.30 ±1.92 μM/gm processed leaves respectively, whereas the three samples collected from lower altitudes (viz. 3600, 2500 and 500 feet, respectively) showed gallic acid equivalent values of 19.82 ±0.91, 20.06±0.98 and 18.25±1.09 μM/gm of leaves respectively. The results indicate that the plants grown in higher altitudes have a tendency to produce more phenolics than the rest.

**Estimation of tannin content**

The results of this assay showed that altitude variation did not affect the tannin content of green tea significantly (Fig 4). It was observed that tannin content was maximum in the green tea prepared from leaves collected at 5800 feet, which was 21.99±1.73 μM tannic acid equivalent/gm processed leaves. The other 5 altitude regions showed more or less similar type of tannin contents, ranging from 16.40±1.61 μM tannic acid equivalent to 19.09±1.59 μM tannic acid equivalent/gm processed leaves.

**Estimation of total flavonoids content**

The results of this assay indicated that flavonoid contents of tea leaves were decreasing with the decreasing altitudes (Fig 5). The two samples, collected from higher altitudes (viz. 6900 and 5800 feet, respectively) showed quercetin equivalent values of 444.93±23.52 and 421.34±19.57 μM/gm processed leaves respectively. The other four samples collected from lower altitudes (viz. 4500, 3600, 2500 and 500 feet, respectively) showed quercetin equivalent values of 264.01±17.82, 272.87±26.43, 297.27±30.88 and 197.64±11.60 μM/gm of leaves respectively, which were significantly lower. From the above data, it can be easily said that although the values were not discrete, there was a trend of decrease in flavonoids content as the altitude decreases and the four samples collected from lower altitudes showed diminished values than the leaves obtained from higher altitudes.
Estimation of ascorbic acid content

The results of this assay indicated a mixed response in the levels of ascorbic acid in the green tea. It was found that the infusion prepared from the leaves collected from the highest altitude (viz. 6900 feet) possessed ascorbic acid content of 105.94±14.13 mg/100gm of processed leaves. Ascorbic acid content gradually decreased with decline in altitude up to 3600 feet. However, ascorbic acid content again increased in the two lower altitudes, viz. 2500 and 500 feet. The contents were 161.77±7.38 mg/100 gm of sample and 179.74±7.65 mg/100 gm of sample (Fig 6).

Figure 1. Comparative ABTS radical scavenging activities of non commercially processed tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [GAE = Gallic acid equivalent (µM gallic acid equivalent/gm processed leaves)].

Figure 2. Comparative DPPH radical scavenging activities of non commercially processed tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [GAE = Gallic acid equivalent (µM gallic acid equivalent/gm processed leaves)].
Figure 3. Total polyphenolics contents of non-commercially processed tea (Camellia sinensis) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [GAE = Gallic acid equivalent (µM gallic acid equivalent/gm processed leaves)].

Figure 4: Tannin contents of non-commercially processed tea (Camellia sinensis) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [TAE = Tannic acid equivalent (µM tannic acid equivalent/gm processed leaves)].
Figure 5: Total flavonoid contents of non commercially processed tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [QE = Quercetin equivalent (µM quercetin equivalent/gm processed leaves)].

Figure 6. Ascorbic acid contents of non commercially processed tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. Results are expressed as mg ascorbic acid present/100 gm processed leaves.

**DISCUSSION**

Tea in its purest natural form has influenced human health by its vast content of antioxidative bioactives from time immemorial. A plethora of evidences suggest that tea phytochemicals like the bioflavonoids are effective in scavenging free radicals thereby producing marked
therapeutic effect on human health when consumed in the form of infusion. However, tea plants are cultivated at different altitudes and there could be some variation in the contents of bioactives as well as their radical scavenging abilities.\cite{16} In the hilly areas of Darjeeling, tea plants are cultivated from 500 feet height to an altitude of around 7200 feet. Since bioflavonoids like catechin and its derivatives have remarkable antioxidant potential and since tea has reported health promoting benefits\cite{23}, the present study was undertaken specifically to observe the effect of altitude on antioxidant potential and some bioactives in tea infusions prepared with the leaves processed as green tea. To decipher the above hypothesis, six assays were chosen specifically as a single assay would not be sufficient for such assessment for a natural product.\cite{24}

The in vitro radical scavenging activities like ABTS assay and DPPH assay are generally used to indicate antioxidant potential of plant extracts. However, ABTS assay is performed in aqueous medium, whereas DPPH assay is based on non-aqueous less polar medium.\cite{19} Since tea leaves contain both polar flavonoids like quercetin, kaempherol, myricetin and rutin and non-polar components like flavones (e.g. apigenin, vitexin)\cite{11}, the above two assays were performed specifically in the present study. Both the assays indicated that radical scavenging abilities deteriorate with increasing altitudes, probably due to onslaught of UV radiations on both polar and non-polar bioflavonoids.

Phenolic compounds of plants having one or more aromatic rings with one or more hydroxyl groups can potentially quench free radicals by forming resonance-stabilized phenoxy radicals which play a role in their antioxidant properties.\cite{25} The present study revealed that the phenolic content was marginally higher in higher altitudes, probably due to production of antioxidative biomolecules by the tea plants to cope up with onslaught of UV radiations or cold stress. This is in clear accordance with the flavonoid contents of the samples, as it was observed that flavonoids were in greater amounts in the samples grown in higher altitudes. However, polyphenolics and tannin contents remained almost same in the six samples, which indicated that the taste and activities of the infusions would remain unaltered despite their different bioactive contents. The higher ascorbic acid contents in the samples collected from lower altitudes might suggest that this bioactive is also vulnerable towards the natural onslaughts.

The bioactives commonly present in tea leaves were reported to be effective against various types of toxic oxidants. However, it was not studied previously whether the effectiveness of the
tea infusions prepared from leaves collected from different altitudes of a single geographical zone could protect against the harmful radicals in the same way. In this context, the study might probably be the first to indicate that altitude variation does change the antioxidant profile but does not affect tea quality significantly.

CONCLUSION
The foremost conclusion arising out of the present study was that, tea plants grown at greater altitude might face enhanced oxidative stress, which was reflected in their *in vitro* radical scavenging abilities. It was observed that ABTS and DPPH radical scavenging abilities of the infusions prepared from green tea leaves were reduced with increasing altitude, probably suggesting depletion of the antioxidative bioactives on exposure to extreme climatic conditions as well as elevated UV radiations. This was substantiated by total polyphenolics and flavonoid contents, as their amounts were higher in the leaves gathered from higher altitudes. Tannin content remained unaltered, suggesting that probably other bioactive molecules and/or antioxidant enzymes might be responsible for their protection from the environmental onslaught, which ultimately retains their tastes. The results indicated that although the plant tries to cope up with extreme climatic conditions, its beverage value remained almost unchanged with variations in altitude, where the plants are cultivated.

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