

Evaluation of in vitro antioxidant potential of Tea (*Camelia sinensis*) leaves obtained 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. Tea plants grown at higher altitude are subject to enhanced oxidative stress due to high UV radiation, climatic changes, and soil conditions, compared to places at low altitude. The present study was undertaken to find out whether the antioxidant activity and the content of bioactives change with the variations of altitudes in a district of Darjeeling, where tea is cultivated most. The samples were collected from six different altitudes of Darjeeling hill, viz. 6900, 5800, 4500, 3600, 2500 and 500 feet. The assays performed included ABTS radical cation decolorization assay, DPPH radical decolorization assay, reducing power assay, total polyphenols content, tannin content and total flavonoid content. It was observed that ABTS and DPPH radical scavenging abilities were reduced with increasing altitude, suggesting probable depletion of the antioxidant bioactives on exposure to extreme climatic conditions as well as elevated UV radiations. However, changes in the major bioactives of tea like tannins and flavonoids with altitude were non-significant, suggesting that although the plant tries to cope up with extreme climatic conditions, its medicinal value remains almost unchanged with altitude.

INTRODUCTION

Tea is one of the most popular beverages worldwide and second most consumed beverage well ahead of coffee, soft drinks, beer or wine (Costa et al., 2002; Rietveld and Wiseman, 2003). The economic and social interest of tea is vast and its consumption is part of daily routine of several populations as an everyday drink and as a therapeutic aid in many illnesses (Cabrera et al., 2006). It is cultivated in more than 30 countries, however mainly in China, India, Japan and Sri Lanka (Gramza et al., 2005). 78% of the total amount of tea produced and consumed in the world is black tea, 20% is green and 2% is oolong tea. Black tea is consumed primarily 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 (Chan et al., 2011).

However, tea cultivated and processed in Darjeeling hill in India is one of the most popular teas & mainly acclaimed universally for its high aroma and also high therapeutic potential (Bhattacharya and Sen Mandi, 2011). Two botanical variants have been identified for tea- the original Chinese variant, *Camelia sinensis* and the Indian variant, *Camelia assamica* (Gramza et al., 2005). Tea beverage is an infusion of the dried leaves of both of the above shrubs, members of Theaceae family. Green tea is prepared from the fresh tea leaf and widely consumed in Japan, China, Korea and Morocco. Western cultures favour black tea which is prepared through the oxidation, curing process of maceration and exposure to atmospheric oxygen. Oolong tea is popular in China and Taiwan (Wu and Wei, 2002; Zuo et al., 2002). 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 (Kodama et al., 2010). Green tea is characterized by its high flavonoid content, mainly catechins (20-30% of the dry weight).

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The major catechins are (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (ECG), (–)-epicatechin gallate (ECG), (–)-gallocatechin, and (+)-catechin. Condensed tannins are also an important component of tea which are transformed products of flavan-3-ols or flavan-3,4-diols (Ashok and Upadhyay, 2012). The other important bioactives are – xanthic bases like caffeine and theophylline, typical amino acids like teanine, proteins and carbohydrates (Cabrera *et al.*, 2006). The leaves have known to possess anti-carcinogenic, anti-obesity, anti-diabetic, anti-bacterial, anti-arthritis, anti-allergic and anti-caries effects. Recent studies revealed that it might possess synergistic effect with antibiotics, anti-Alzheimer, anti-Parkinsonism and anti-viral effects (Jigisha *et al.*, 2012). However, 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 (Chacko *et al.*, 2010).

Plants grown at high altitude are subjects to enhanced oxidative stress due to high exposures to UV radiations (Lesser, 1996; Balakrishnan *et al.*, 2005). To combat such stress, plants have evolved effective cell protective mechanisms that retard cell damage, thus enabling plants to survive (Bhattacharya and Sen Mandi, 2011). In this context, flavonoids were found to be most relevant (Stapleton and Walbot, 1994). These compounds maintain antioxidant potential in cells due to the phenol-quinone tautomerism in the side chain of the flavonoid ring system that serves as electron acceptors from reactive oxygen species (ROS). It has also been noted that UV onslaught is higher in the subtropical regions during the months of April to July (Turunen and Latola, 2005). During this period, tea plants of Darjeeling and Assam produce the first flush of leaves that produces best tea each year (Bhattacharya and Sen Mandi, 2011). This means that the tea plants might be producing greater amounts of antioxidants in this session.

The present study was conducted to find out the relationship between altitude variations and changes of antioxidant profile of raw green tea leaves, which were collected from six different heights of Darjeeling Hill, West Bengal district of India, viz. 6900, 5800, 4500, 3600, 2500 and 500 feet. As we know tea as a popular beverage, this study will provide information about the polyphenolics, especially tannins, in different tea plants situated in 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-picrylhydrazyl (DPPH) were obtained from Himedia, India. Folin-Denis reagent was a kind gift from Dr. Tapan Kumar Pal. 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) (Bhattacharya and Sen Mandi, 2011). 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.

Methanol extraction of fresh leaves

Methanol extracts of the samples were obtained by a reported procedure with minor modifications. 1 gm sample was extracted with 20 ml aqueous methanol (70%, v/v) for 30 min in an orbital shaker at 70°C in the dark. The mixture was filtered through Whatman No.1 and the volume was made up again to 20 ml with aqueous methanol (70%, v/v). All the antioxidant assays were done with the extractive. The whole process was carried out in triplicate (Erol *et al.*, 2009).

Antioxidant assays

ABTS radical decolorization assay

The ABTS assay was performed using a previously described procedure (Chakraborty and Bhattacharyya, 2014). 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 (Chakraborty and Bhattacharyya, 2014). 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 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).

Table 1: Antioxidant potential of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal.

Altitudes (feet)	ABTS assay (GAE)	DPPH assay (GAE)	Reducing power assay (AAE)	Total Phenolics contents (GAE)	Tannin content (TAE)	Flavonoids Content (QE)
6900	186.94±2.83	28.23±1.39	70.69±7.27	11.48±1.12	10.73±0.97	40.33±0.83
5800	182.03±4.83	37.77±1.47	115.90±5.79	12.00±1.58	11.53±1.50	41.96±1.09
4500	176.90±3.97	25.85±2.26	142.31±4.96	11.76±1.31	12.71±1.49	43.26±1.29
3600	267.69±4.61	44.20±0.91	131.94±2.68	12.90±1.60	13.54±0.39	50.65±1.10
2500	301.33±2.90	54.29±2.29	122.98±7.50	13.23±0.62	13.95±1.65	65.88±2.28
500	290.28±3.34	50.15±3.81	113.08±7.17	12.97±0.58	13.16±1.45	64.55±2.31

Data are Mean ± SD (n=3), GAE = gallic acid equivalent (μM gallic acid equivalent/gm fresh leaves); AAE = Ascorbic acid equivalent (μM ascorbic acid equivalent/gm fresh leaves); TAE = Tannic acid equivalent (μM tannic acid equivalent/gm fresh leaves); QE = Quercetin equivalent (μM quercetin equivalent/gm fresh leaves)

Reducing Power assay

The assay was performed using a previously described procedure (Shib *et al.*, 2014). Briefly, 0.5 ml of sample solutions was mixed with phosphate buffer (pH 7.4, 2.5 ml) and aqueous potassium ferricyanide solution (2.5 ml). This mixture was kept at $50\pm 2^\circ\text{C}$ in water bath for 20 minutes. After cooling, 2.5 ml of 10% (w/v) trichloroacetic acid was added and centrifuged at 3000 rpm for 5 min. 2.5 ml of the supernatant was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm in a Systronics spectrophotometer (model – 2202). Control was prepared in similar manner excluding samples. Gallic acid was used as positive control and comparing with its IC_{50} and the results were expressed as gallic acid equivalents (mM/gm fresh leaves).

Estimation of total phenolics content

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Sarkar *et al.*, 2014). The samples (0.5 ml) were mixed with Folin-Ciocalteu 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 μg gallic acid equivalent/gm fresh leaves.

Estimation of tannin content

Content of Tannins was determined by Folin-Denis method (Pal *et al.*, 2014). 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 (μM /gm fresh leaves).

Estimation of total flavonoids content

Total flavonoid content was determined according to a published colorimetric method (Pal *et al.*, 2014) 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 2202). Quercetin was used as standard. The results are expressed in terms of quercetin equivalent (μM /gm fresh leaves).

Statistical Analyses

Experimental results are expressed as mean \pm SD of three individual samples. The statistical analysis was done by using the software the software 'Prism 4.0' (GraphPad Inc., USA).

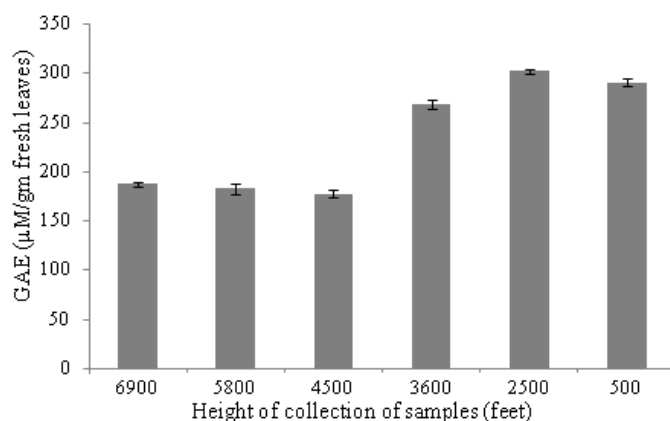


Fig. 1: Comparative ABTS radical scavenging potential of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [GAE = Gallic acid equivalent (μM gallic acid equivalent/gm fresh leaves)]

RESULTS

ABTS radical decolorization assay

The results of this assay indicated that the antioxidant properties of tea leaves were increasing with the decreasing altitudes. The first three samples, collected from higher altitudes (viz. 6900, 5800 and 4500 feet, respectively) showed gallic acid equivalent values of 186.94±2.83, 182.03±4.83 and 176.90 ±3.97 $\mu\text{M}/\text{gm}$ fresh leaves respectively, whereas the three samples collected from lower altitudes (viz. 3600, 2500 and 500 feet,

respectively) showed gallic acid equivalent values of 267.69 ± 4.61 , 301.33 ± 2.90 and 290.28 ± 3.34 $\mu\text{M/gm}$ fresh leaves respectively, which were significantly higher (Table 1). 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 indicated that the antioxidant properties of tea leaves were increasing with the decreasing altitudes. The first three samples, collected from higher altitudes (viz. 6900, 5800 and 4500 feet, respectively) showed gallic acid equivalent values of 28.23 ± 1.39 , 37.77 ± 1.47 and 25.85 ± 2.26 $\mu\text{M/gm}$ fresh leaves respectively, whereas the three samples collected from lower altitudes (viz. 3600, 2500 and 500 feet, respectively) showed gallic acid equivalent values of 44.20 ± 0.91 , 54.29 ± 2.29 and 50.15 ± 3.81 $\mu\text{M/gm}$ fresh leaves respectively, which were significantly higher (Table 1). If one 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 three samples collected from lower altitudes showed greater radical scavenging abilities than the samples obtained from higher altitudes (Fig. 2).

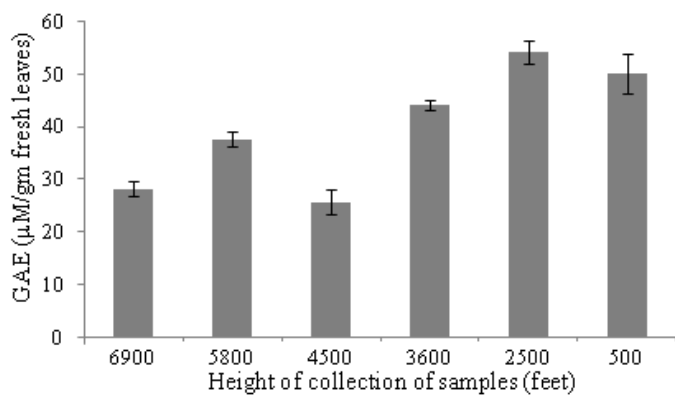


Fig. 2: Comparative DPPH radical scavenging potential of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [GAE = Gallic acid equivalent (μM gallic acid equivalent/gm fresh leaves)]

Reducing power assay

The results of this assay indicated that reducing power of tea leaves were increasing with the decreasing altitudes, although the results were not discrete. As the altitudes gradually decrease (viz. 6900, 5800, 4500, 3600, 2500 and 500 feet, respectively), ascorbic acid equivalent values of 70.69 ± 1.27 , 115.90 ± 0.79 , 142.31 ± 0.96 , 131.94 ± 0.68 , 122.98 ± 1.50 and 113.08 ± 1.17 $\mu\text{M/gm}$ fresh leaves, respectively, were obtained (Table 1). The values indicated that although there is a tendency of the values going upwards as the altitude decreases, no significant differences existed (Fig. 3), indicating preservation of reducing potential i.e. integrity of the bioactives.

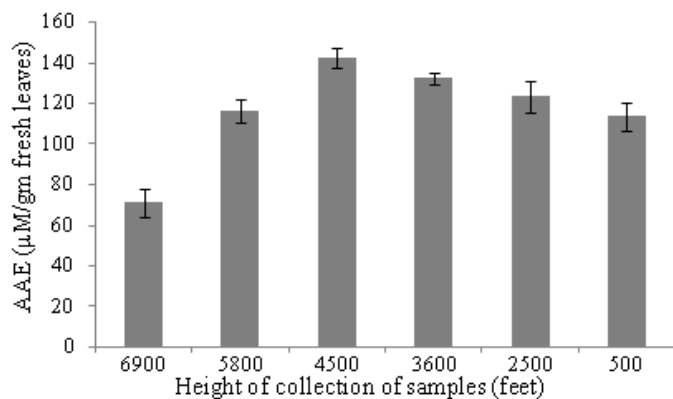


Fig. 3: Comparative reducing power of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [AAE = Ascorbic acid equivalent (μM ascorbic acid equivalent/gm fresh leaves)]

Estimation of total polyphenolics content

The results of this assay indicated that altitude variation does not affect much the polyphenolic content of tea (Table 1). As the altitudes gradually decrease (viz. 6900, 5800, 4500, 3600, 2500 and 500 feet, respectively), gallic acid equivalent values of 11.48 ± 1.12 , 12.00 ± 1.58 , 11.76 ± 1.31 , 12.90 ± 1.60 , 13.23 ± 0.62 and 12.97 ± 0.58 $\mu\text{M/gm}$ fresh leaves were obtained, which indicated that although there was a slight tendency of increment of polyphenolic contents with decreasing height (Fig. 4), there was no as such significant variation.

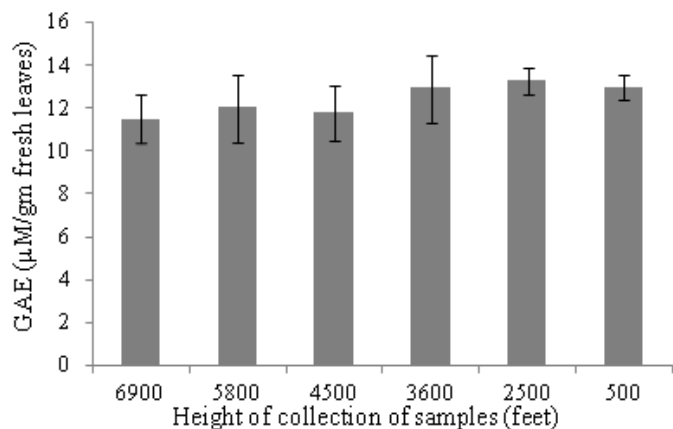


Fig. 4: Comparative total polyphenolics content of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [GAE = Gallic acid equivalent (μM gallic acid equivalent/gm fresh leaves)]

Estimation of tannin content

The results of this assay indicated that altitude variation does not affect much the tannin content of tea (Table 1). As the altitudes gradually decrease (viz. 6900, 5800, 4500, 3600, 2500 and 500 feet, respectively), tannic acid equivalent values of 10.73 ± 0.97 , 11.53 ± 1.50 , 12.71 ± 1.49 , 13.54 ± 0.39 , 13.95 ± 1.65 and 13.16 ± 1.45 $\mu\text{M/gm}$ fresh leaves were obtained, which indicated that although there was a slight tendency of increment of tannin contents with decreasing height (Fig. 5), there was no as such significant variation.

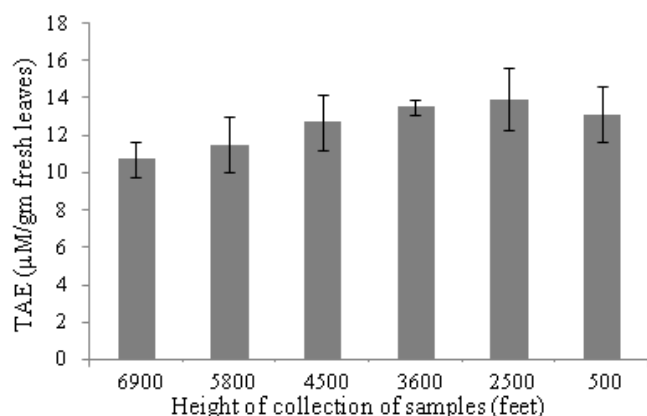


Fig. 5: Comparative tannin content of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [TAE = Tannic acid equivalent (µM tannic acid equivalent/gm fresh leaves)]

Estimation of total flavonoids content

The results of this assay indicated that flavonoid contents of tea leaves were increasing with the decreasing altitudes. The first three samples, collected from higher altitudes (viz. 6900, 5800 and 4500 feet, respectively) showed quercetin equivalent values of 40.33 ± 0.83 , 41.96 ± 1.09 and 43.26 ± 1.29 µM/gm fresh leaves respectively, whereas the three samples collected from lower altitudes (viz. 3600, 2500 and 500 feet, respectively) showed quercetin equivalent values of 50.65 ± 1.10 , 65.88 ± 2.28 and 64.55 ± 2.31 µM/gm fresh leaves respectively, which were significantly higher (Table 1). From the above data, it can be easily said that although the values were not discrete, there was a trend of increase in flavonoids content as the altitude decreases and the three samples collected from lower altitudes showed greater values than the samples obtained from higher altitudes (Fig. 6).

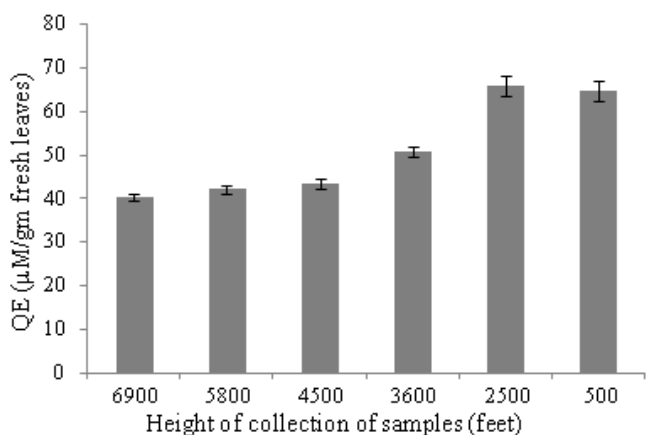


Fig. 6: Comparative total flavonoids content of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal. [QE = Quercetin equivalent (µM quercetin equivalent/gm fresh leaves)]

Per cent reduction of antioxidant potential and bioactive contents from the highest mean considering the lowest mean as basal level of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal, was calculated and given in Fig. 7. It has been observed that reduction power falls sharply between the highest and the lowest mean with the lowest

mean shown by the plants cultivated at higher altitudes, as evident by radical scavenging assays like ABTS and DPPH assays and also in reducing power assay. However, contents of bioactives like polyphenolics and tannins were not altered much in comparison to the reducing power, suggesting that the quality of the leaves would remain same with altitude variation.

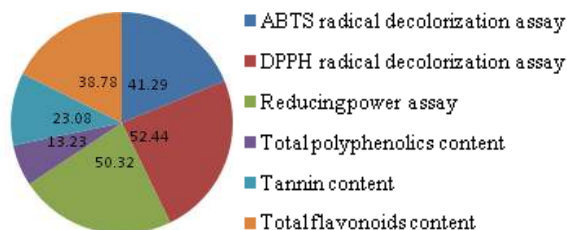


Fig. 7: Per cent reduction of antioxidant potential and bioactive contents from the highest mean considering the lowest mean as basal level of fresh tea (*Camellia sinensis*) leaves collected from different altitudes of Darjeeling Hill, West Bengal.

DISCUSSION

Tea in its purest natural form has always influenced human health by its vast content of antioxidative bioactives throughout the world from time immemorial. A plethora of evidences suggest that tea components like the bioflavonoids are highly effective in scavenging free radicals which would produce marked 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. In the hilly areas of Darjeeling, tea plants are cultivated from 500 feet height to an altitude of 6900 feet. Since bioflavonoids like catechin and its derivatives have remarkable antioxidant potential and since tea has reported health promoting benefits (Yashin *et al.*, 2011), the present study was undertaken specifically to observe the effect of altitude on antioxidant potential and some bioactives in tea leaves. To answer the above objective, six assays were chosen specifically as a single assay would not be sufficient for such assessment for a natural product (Sreeramulu *et al.*, 2009). 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 (Chakraborty and Bhattacharyya, 2014). Since tea leaves contain both water soluble flavonoids like quercetin, kaempferol, myricetin and rutin and non-aqueous soluble components like flavones (e.g. apigenin, vitexin) (Jigisha *et al.*, 2012), the above two assays were performed in the present study. Both the assays indicated that radical scavenging abilities deteriorate on increasing altitude, 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 phenoxyl radicals which play a role in their antioxidant properties (Shib *et al.*, 2014). The present study revealed that the phenolic

content did not change significantly with variations in altitude, clearly suggesting that the quality of the tea was not compromised with altitude difference as most of the bioactives responsible for taste, aroma as well as therapeutic potential of tea are polyphenolics. This was also substantiated by the reducing capacities of the samples, which were non-significantly different. Amongst the tea polyphenolics, flavonoids and tannins are most important. The study revealed that all these bioactives were unaltered with respect to the altitude variations, clearly suggesting no loss of quality of the tea grown in different heights of Darjeeling Hill. 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 leaves against the most harmful ROS would be the same irrespective of the height of collection of the tea leaves from a single geographical zone. In this context, the study would probably be the first to indicate that altitude variation does change the antioxidant profile but does not affect tea quality significantly.

CONCLUSION

The present study concludes 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 were reduced with increasing altitude, probably suggesting depletion of the antioxidative bioactives on exposure to extreme climatic conditions as well as elevated UV radiations. However the contents of total polyphenolics as well as bioactive components like tannins and flavonoids remained unaltered in the tea leaves, suggesting that probably other bioactive molecules and/or antioxidant enzymes might be responsible for their protection from the environmental onslaught thereby sparing them. The results indicated that although the plant tries to cope up with extreme climatic conditions, its medicinal value remained almost unchanged with variations in altitude, where the plants are cultivated.

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