Research Article

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Antioxidant attributes of tea in North Bengal, India: Relation with its principal constituents and properties of soil

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Abstract

This study was performed in 18 tea gardens in North Bengal, India, from 2012 to 2017. The data were pooled to investigate the relationship with soil physico-chemical properties, phyto-constituents, antioxidant attributes and age of the tea bushes and principal component analysis (PCA). PCA and dendro-hit maps were also performed with each region. The 28 principal components were chosen based on their eigen values, explaining the total data variance for tea in Dooars, Terai and Darjeeling hill. In almost all cases, composite soil physico-chemical attributes were heavily loaded on the second principal component and clustered, as visual evidenced by the dendro-hit map. Different attributes were significantly correlated each other in case of Terai *i.e.* (value of "r'' at $P<0.01$ level) clay fraction (0.778), electrical conductivity (0.618), N (0.777), S (0.748), P (0.514 ppm), flavour index (0.918), total polyphenol (0.687) DPPH (0.794), nitric oxide (0.913), anti-lipid peroxidation (0.717) and metal chelating (0.665). In Dooars region, attributes were significantly correlated with silt (0.718), pH (0.875), P (0.615), chloride (0.858), TP (0.776), flavonol (0.923), quinone (0.666), tannins (0.865), DPPH (0.536), superoxide (0.576), ABTS (0.520) and MC (0.777) and in the case of Darjeeling hills, attributes were highly correlated with clay (0.812), sand (0.818), silt fraction (0.974), K (0.932), S (0.999), MC of soil (0.671), TP (0.853), tannins (0.912), DPPH (0.624), ABTS (0.661) and MC (0.633) repectively.

Keywords: Cluster heat map, PCA, phyto-constituents, soil properties, tea antioxidants

Introduction

Thea or tea [*Camellia sinensis* (L.) O. Kuntze; Family: Theaceae] is an acid-loving long shrub or small tree with alternate evergreen leaves, indigenously grown in Eastern Asia. But, in 1823, Major R. Bruce introduced tea plants in North East India. Tea has become one of the most powerful commodities of commercial value during the colonial period. Only luxurious and rich people could afford tea as a beverage at that time. Tea has become the world's cheapest and most widely used drink other than water. It has become a beverage of international fellowship, a bond that brings people together. Tea is an extremely valuable source of much-needed foreign exchange and obviously plays a crucial role in the Indian economy. It is extensively cultivated in the sub-tropical belt slopes of hills and plateaus at an altitude varying from 10 to 2400 meters above sea level. In India, there are three distinctly different tea-growing regions, which are geographically separated, producing three entirely different teas in style and taste/flavour. The three regions of tea production areas are Darjeeling (North-Eastern India), Assam (far North-East India) and Nilgiri (South India). The cool and moist climate, the soil, the rainfall and the sloping terrain combine to give Darjeeling its unique "Muscatel" flavour and exquisite bouquet. The combination of

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natural factors gives Darjeeling tea its uniqueness. Hence this finest and most delicately flavoured of all teas has acquired a reputation as "*The Champagne of the Teas*" over the years.

The main medicinal property ascribed to this plant is its anti-oxidative nature, reported in the many scientific literatures worldwide (Mabe *et al.*, 1999; Hitchon and Gabalawy, 2004; Pajohk *et al.*, 2006). In therapeutics, various tea matrices obtained from different grades/varieties of tea are most often employed for prophylactic purposes and in the formulation of plant-derived elixir to protect against human diseases (Misra *et al.*, 2003; Carol and Hani, 2004). Scientists and medical practitioners have generated maximum data for claiming the health benefits of tea consumption, and much scientific experimental evidence supports these claims.

The quantity and composition of secondary metabolites as antioxidants in tea mainly change according to edaphic composition (Misra *et al.*, 2016a). Several other factors like genotype, bushes age, growing region, climate and cultural practices also affect the external and internal qualities of tea. It has been noticed that several geographical and environmental factors influence the concentration of bioactive phytochemicals in the same plants collected from different regions and seasons (Misra *et al.*, 2016a).

Documentation of the antioxidant quality and phyto-constituents of tea and soil nutrient responses of tea grown in North Bengal has not been reported. So, it is very difficult to visualise how different factors are correlated with each other. Therefore, the objective of this study was to visualise the changes in antioxidants of tea and its secondary metabolites with soil characteristics and age of the bushes from different regions of North Bengal by using principal component analysis and cluster heat map analysis.

Materials and methods

The proposed work was done by collecting season-wise data from different aged tea bushes and

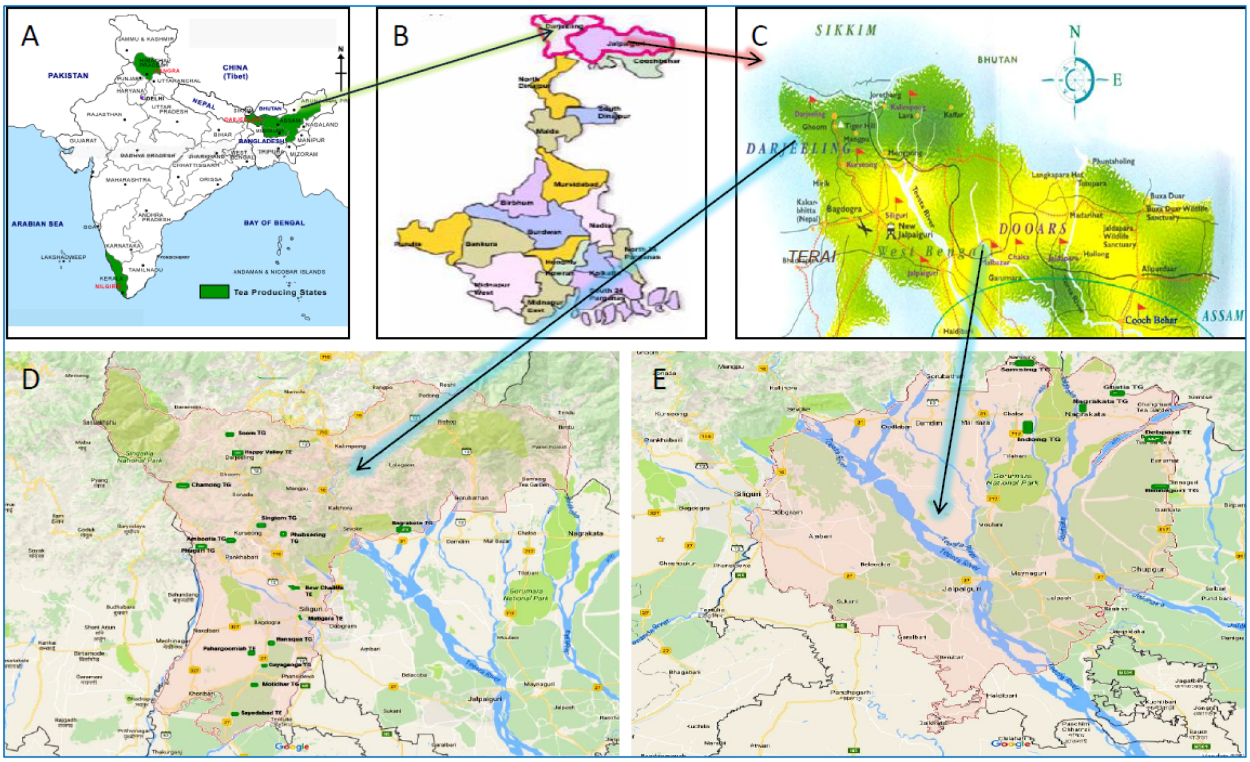


Fig. 1. Map of study area under Darjeeling Hills, Terai and Dooars regions of North Bengal. A. Map of India, green colour showing tea cultivating area. B. Map of West Bengal, pink colour border showing tea cultivated area. C. Map showing Darjeeling, Terai and Dooars regions. D & E. Experimental tea garden/ Estate (Green colour spot with name) of Darjeeling, Terai and Dooars. (*Source : Google map modified*)

soil samples from selected tea gardens situated at Dooars, Terai, and Darjeeling Hills of North Bengal.

Study area

The study area comprised six selected tea gardens from the Terai, Dooars and Darjeeling hills of West Bengal (Fig. 1). In a geographic area of 5.19 lakh hectares, 1,15,000 ha produces tea, including Terai, Dooars and Darjeeling Hills, stretching between 26°12'N to 27°07'N latitude and between 88°05'E to 89°21'E longitude. The altitude of this area varies from the mean sea level to the highest mountain ranges (4580 ft to 140 ft). It is a range and diversity of climate, flora and fauna, with a few parallels in the world. North Bengal presents a paradox of containing the station with the highest mean annual rainfall in the state, moist weather in Dooars and Hills and comparatively dry weather in the Terai area.

Plant materials

At first, the agro-climatic regions were classified as Darjeeling Himalaya, Terai and Dooars regions. A pilot survey has been conducted from the data sources of the tea gardens to document the distribution of tea plant varieties and their age (20 to 50 years). The plant specimens [different selected varieties of *Camellia sinensis* (L.) O. Kuntze], *i.e.,* fresh tea leaves were collected from the different cultivated sections of the tea garden of different agro-climatic areas of North Bengal to assess antioxidant attributes and phyto-constituents.

Soil Samples

Soil samples were collected as per the methods described by Ranganathan and Natesan (1987) from different agro-climatic areas of North Bengal (top and sub soil with 0-22.5 cm and 22.5-45.0 cm depth, respectively), and composite soil was

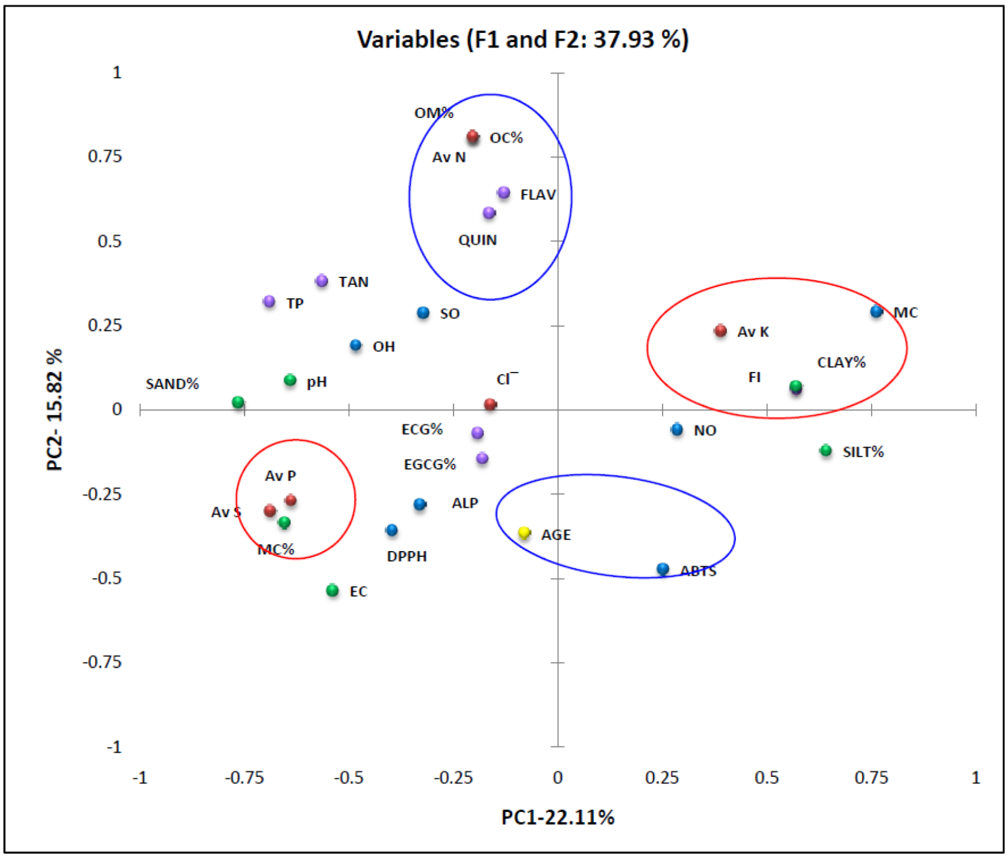
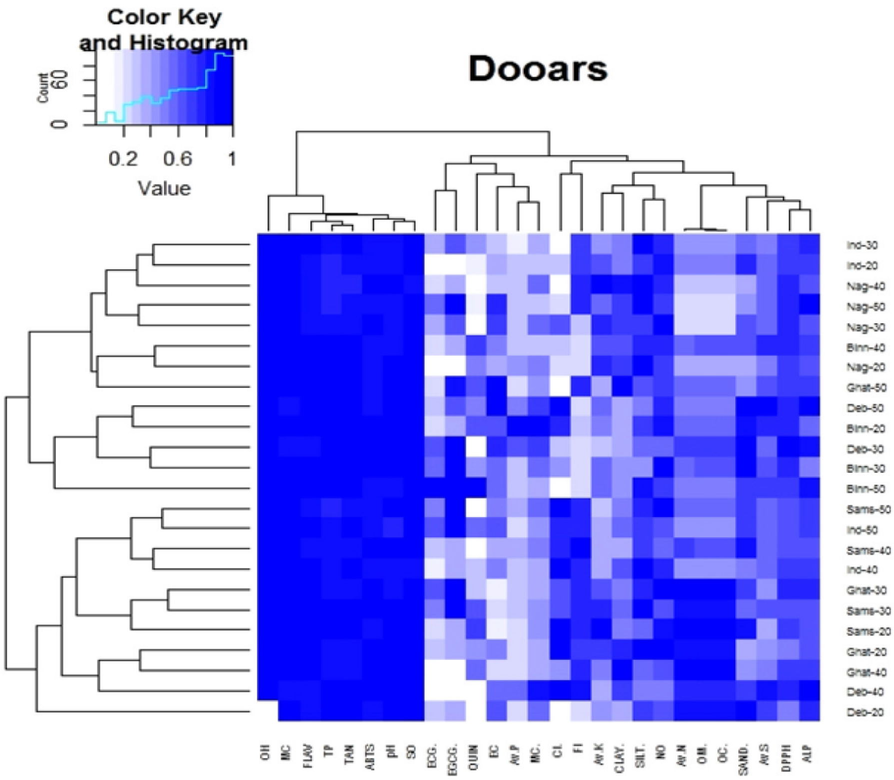


Fig. 2. Plotting (PC1 x PC2) of scores and loadings for the PCA of edaphic factors, phytoconstituents and antioxidant attributes tea for Tea Garden of Dooars region



ABBREVIATIONS USED (Vertical):
20, **30**, **40** and **50** at the back of parenthesis indicates age of the bush in year;
Ind: Indong Tea Estate
Nag: Nagracata Tea Estate
Bin: Binnaguri Tea Estate
Deb: Debpara Tea Estate
Ghat: Ghatia Tea Estate
Sams: Samsing Tea Estate

Fig. 3. Dendrogram for Dooars tea (collected three main seasons) depicted relation among edaphic factors, age of the bush, phytoconstituents and antioxidant activity. Deep blue colour showing attributes with high impact. [ABBREVIATIONS USED (Horizontal):OH: Hydroxyl radical scavenging; MC: Metal chelating; FLAV: Flavonol content; ABTS: ABTS radical scavenging ; pH: Soil pH; SO: Superoxide radical scavenging; ECG: Epicatechin gallate; EGCG: Epigallocatechin gallate; QUIN: Quinone; EC: Soil electrical conductivity; MC%= Soil moisture content; Cl: Soil chloride; FI: Flavour index: Av. K: Soil available potassium; CLAY: Soil clay fraction; SILT: Soil silt fraction; NO: Nitric oxide radical scavenging; Av. N: Soil available nitrogen; OM: Soil organic matter; OC: Soil organic carbon; SAND: Soil sand fraction; Av. S: Soil available sulphur; DPPH: DPPH radical scavenging; ALP: Anti-lipid peroxidation activity]

prepared as per the method of Misra *et al*., 2009. The samples were processed for physicochemical analysis.

Analysis of antioxidant attributes and phyto-constituents in tea

Preparation of methanolic plant extracts

Fresh tea leaves (two leaves and a bud) were crushed with mortar and pestle with methanol. The methanol was completely removed by a vacuum rotary evaporator at 50 °C. One part of these crude extracts were used for experiments, and the other parts were freeze-dried. The powder was stored at 4 °C and used for further investigation.

The radical scavenging activity of plant extracts against stable DPPH (2,2-diphenyl-1- picrylhydrazyl) was determined spectrophotometrically as per the standard method proposed by Blois (1958). The percentage inhibition activity was calculated by the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample}) / A_{control} \times 100]$$

Where, $A_{control}$ is the initial concentration of the stable DPPH radical without the test compound, and A_{sample} is the absorbance of the remaining concentration of DPPH in the presence of methanol. IC_{50} values (mg mL^{-1}) were determined from a plotted graph of scavenging activity against the concentrations of the standard extracts, where IC_{50} is defined as the total amount of antioxidants necessary to decrease the initial DPPH radical concentration by 50 per cent.

The scavenging capacities of tea extracts on superoxide anion were determined according to Mccord and Fridowich (1969) and Nishikimi *et al.* (1972). Percentage inhibition and IC_{50} value were calculated using the same formula mentioned above.

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci *et al.*, 1994; Sreejayan and Rao, 1997). The IC_{50} value was calculated by the same procedure mentioned above.

Hydroxyl radical scavenging potency of tea extracts would be assayed according to Jung *et al.* (2008) and Jainu and Devi (2005). The IC_{50} value was calculated by the same process mentioned above.

The anti-lipid peroxidation (ALP) activity of the sample extracts was determined by the standard method followed by slight modification with the goat liver homogenate (Bauchet and Barrier, 1998; Ohkawa *et al.*, 1979; Sreejayan *et al.*, 1994). The supernatant was taken for absorbance at 535 nm. ALP per cent was calculated by using the following formula:

$$\text{ALP per cent} = \frac{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{Abs. of sample}}{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{Abs. of control}} \times 100$$

The free radical-scavenging activity was determined by the 2,2 Èazinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS radical cation decolourisation assay described by Re *et al.* (1999). The IC_{50} value was calculated by the same procedure mentioned above.

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al.* (1994) with slight modification.

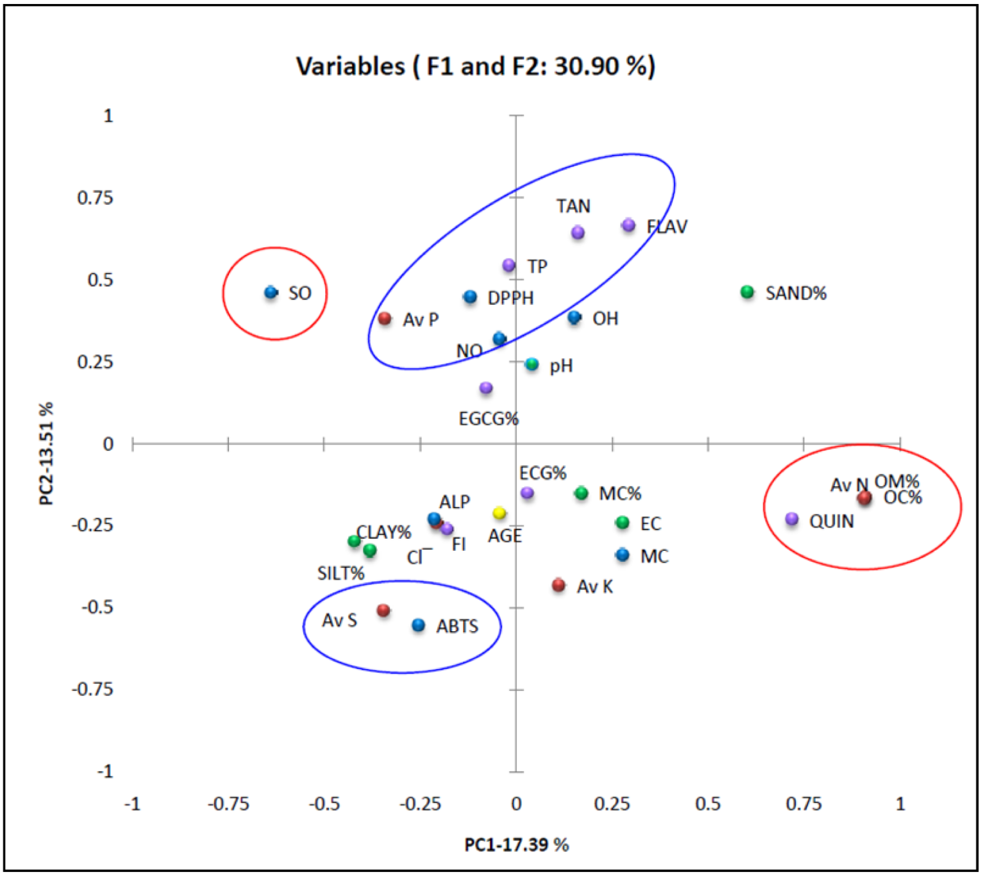
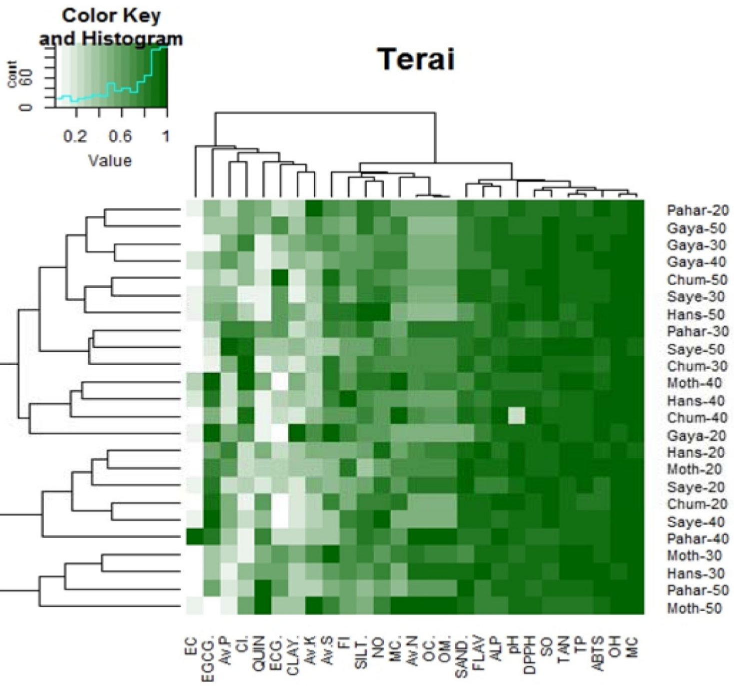


Fig. 4. Plotting (PC1×PC2) of scores and loadings for the PCA of soil physicochemical properties, phytoconstituents and antioxidant attributes of Tea garden of Terai



ABBREVIATIONS USED (Vertical):
20, **30**, **40** and **50** at the back of parenthesis indicates age of the bush in year;
Pahar: Pahargunia Tea Estate
Gaya: Gayaganga Tea Estate
Chum: New Chumta Tea Estate
Hans: Hansqua Tea Estate
Saye: Sayedabad Tea Estate
Moth: Mothidhar Tea Estate

Fig. 5. Dendrogram for Terai tea (collected three main seasons) depicted relation among edaphic factors, age of the bush, phytoconstituents and antioxidant activity. Deep green colour showing attributes with high impact. [ABBREVIATIONS USED (Horizontal):OH: Hydroxyl radical scavenging; MC: Metal chelating; FLAV: Flavonol content; ABTS: ABTS radical scavenging ; pH: Soil pH; SO: Superoxide radical scavenging; ECG: Epicatechin gallate; EGCG: Epigallocatechin gallate; QUIN: Quinone; EC: Soil electrical conductivity; MC%= Soil moisture content; Cl: Soil chloride; FI: Flavour index: Av. K: Soil available potassium; CLAY: Soil clay fraction; SILT: Soil silt fraction; NO: Nitric oxide radical scavenging; Av. N: Soil available nitrogen; OM: Soil organic matter; OC: Soil organic carbon; SAND: Soil sand fraction; Av. S: Soil available sulphur; DPPH: DPPH radical scavenging; ALP: Anti-lipid peroxidation activity]

The chelating activity of the extract for Fe^{2+} was calculated using the same formula mentioned above. The total phenolic compounds of sample extracts were determined by the Folin-Ciocalteu reagent method (Folin and Ciocalteu, 1927; Bray and Thrope, 1954; Malick and Singh, 1980). Spectro-photometric aluminium chloride method was used to determine flavonoids (Sultana *et al*., 2009, Swain and Hills, 1959; Mahadevan and Sridhar, 1986).

Quinone was determined (spectro-photometrically) according to the method described by Mahadevan and Sridhar (1986). Determination of tannins was done by the method of Sadasivam and Manickam (1996), while ortho-dihydric phenol was determined by Mahadevan and Sridhar (1986). Determination of flavour index (FI) was done by the method of Masoud *et al*. (2006).

The analytical determinations of EGCG and ECG were carried out using reverse-phase- high-performance liquid chromatography in isocratic mode as per the method prescribed by Misra *et al*. (2016b). The Waters HPLC system was equipped with an automated gradient controller, 510 pumps, U6K injector, 481 detectors, 746 data modules and Waters µ-bondapak C18 column (3.9 x 300 mm) for the analysis. Elution was carried out at an ambient temperature between 24 to 28 °C using water:methanol:acetic acid (70: 30: 0.5) as a mobile phase at a flow rate of 1.0 mL min^{-1}. All extracts were prepared in triplicate. The UV detection was carried out at 280 nm.

A standard solution of EGCG was prepared by dissolving 4.72 mg of EGCG in 50 mL methanol. The standard plot for HPLC analysis was prepared by injecting in triplicate a constant volume of 5 µL of serially diluted concentrations containing 9.85, 19.70, 39.40, 78.80, 157.60 and 315.20 ng per 5 µL of EGCG and noting AUC corresponding to each concentration. The standard solution of ECG was prepared by dissolving 3.02 mg of ECG in 10 mL methanol. The stock solution was diluted to make 9.40, 18.80, 37.60, 75.20, 150.40 and 300.8 ng per 5 µL dilutions of ECG. A constant volume of 5 µL of each concentration was injected in triplicate. The standard plot was prepared as described for EGCG, and the same analysis conditions were used for the two catechins.

Linear regression was obtained by plotting the peak area versus concentration of a series of dilutions for each phenolic compound. The regression lines, expressed as correlation coefficients, were linear (r^2 =1 and 0.9998 for EGCG and ECG, respectively) in the experimental range. Sensitivity (defined as the lowest measurable concentration of a compound in the sample) was determined as that concentration which generated a peak at least three times higher than the baseline noise range.

Analysis of physico-chemical properties of soil

The soil was analysed as per the methods of Jackson (1973) and Vogel (1962) according to the International Society of Soil Science 1929 and Baruah and Barthakur (1997). pH and electrical conductivity of the sample suspension (soil:water @ 1:2.5) were analysed using calibrated pH and conductivity meter.

The moisture content of the sample was estimated by the method of Baruah and Barthakur (1997).

The particle size distributions of the soil were evaluated according to the hydrometric method described by Bouyoucos (1927) and Day (1965).

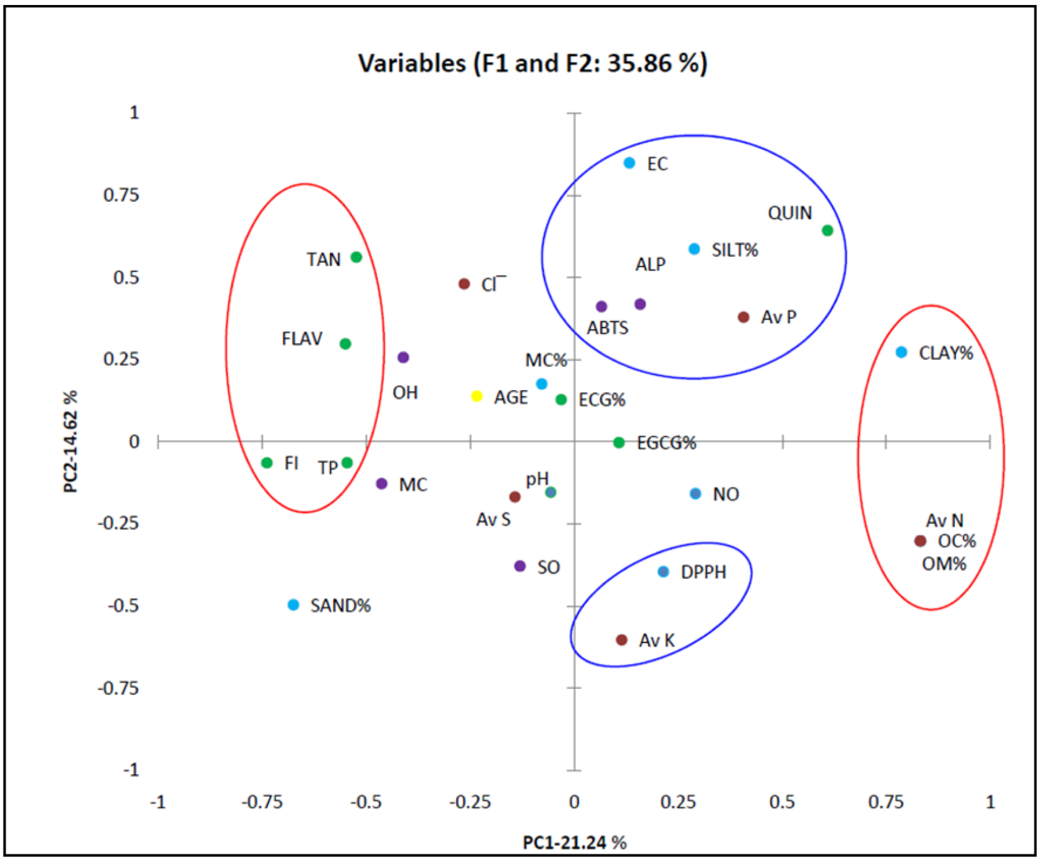


Fig. 6. Plotting (PC1 × PC2) of scores and loadings for the PCA of soil physicochemical properties, phytoconstituents and antioxidant attributes of Tea Garden of Darjeeling hills

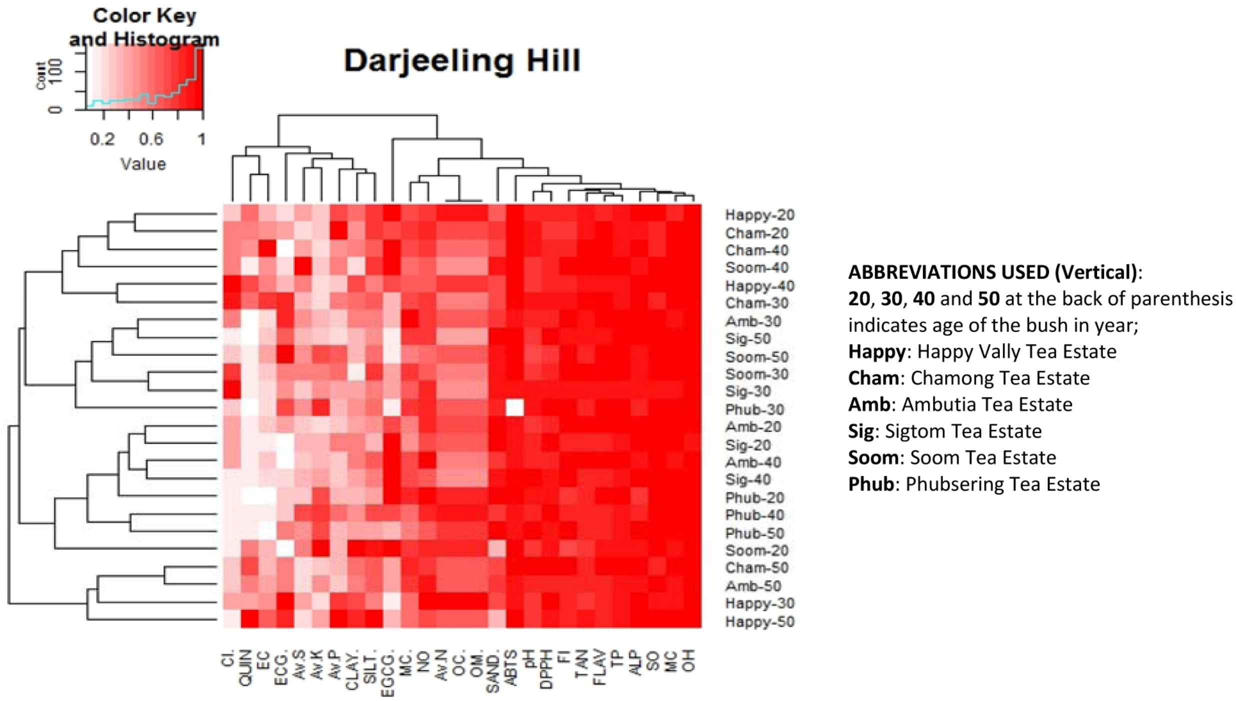


Fig. 7. Dendrogram for Darjeeling Hill tea (collected three main seasons) depicted relation among edaphic factors, age of the bush, phytoconstituents and antioxidant activity. Blood red colour showing attributes with high impact. [ABBREVIATIONS USED (Horizontal):OH: Hydroxyl radical scavenging; MC: Metal chelating; FLAV: Flavonol content; ABTS: ABTS radical scavenging ; pH: Soil pH; SO: Superoxide radical scavenging; ECG: Epicatechin gallate; EGCG: Epigallocatechin gallate; QUIN: Quinone; EC: Soil electrical conductivity; MC%= Soil moisture content; Cl: Soil chloride; FI: Flavour index: Av. K: Soil available potassium; CLAY: Soil clay fraction; SILT: Soil silt fraction; NO: Nitric oxide radical scavenging; Av. N: Soil available nitrogen; OM: Soil organic matter; OC: Soil organic carbon; SAND: Soil sand fraction; Av. S: Soil available sulphur; DPPH: DPPH radical scavenging; ALP: Anti-lipid peroxidation activity]

Organic carbon, organic matter and available nitrogen (N %) of the air dried and sieved (80 mesh) soil sample were determined by the wet digestion volumetric method of Walkley and Black (1934). Available forms of phosphorus (P) were estimated by the colourimetric method of Bray and Kurtz (1945) and Dickman and Bray (1940). The available form of potassium (K) was determined by the flame photometric method described by Stanford and English (1949), while the available form of sulfur (S) was determined by the colourimetric method of Palaskar *et al*. (1981). The chloride content of the sample was determined by the volumetric methods of Baruah and Barthakur (1997).

Statistical analysis

The data were pooled in triplicate to examine and visualise relationships between different phytochemicals and antioxidant traits. A principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1). The Cluster heat map, a graphical illustration of data, was analysed as per methods designed by Kinney in 1991. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used to analyse other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE).

Results and discussion

North Bengal is a land of wide-scale climatic and seasonal variations, affording scope for much diversity in tea cultivation. Seasonal impacts of climatic factors in North Bengal are important because of their fluctuating behaviours, which influence the polyphenol content, soil quality and antioxidant profile of tea leaves (Misra *et al.*, 2022).

In the heat map, the individual values are represented as colours. In this case, the rows (columns) of the tiling are ordered in such a way that the similar rows (columns) are nearer to each other. On the other hand, hierarchical cluster trees were on the vertical and horizontal margins of the tiling.

To understand more about the relationship between soil physicochemical profile phytoconstituents, antioxidant attributes and age of the plantation, PCA was performed in each region of North Bengal. The 28 principal components were chosen based on their eigenvalues, explaining the total data variance for Dooars, Terai and Darjeeling hill. In almost all cases, composite soil physicochemical attributes were heavily loaded on the second principle component (PC2) and clustered, as evidenced by the dendro-hit map. In the case of Dooars (Fig. 2 & 3), metal chelating activity and anti-lipid peroxidation were clustered in opposite coordinates, which indicated that these antioxidant attributes were controlled by soil nutrients. Still, K level and free radical scavenging attributes were loaded on PC1 and clustered in opposite domains, indicating that enhancement of soil potassium level is important for reducing IC_{50} values of these scavenging components. Similar observations were also found by Wu *et al*. (2013) in *Zizyphus jujuba,* who stated that potassium supplementation improved the accumulation of bioactive phenolics and strengthened antioxidant activity. In the case of all six gardens of Dooars, the PCA graphs show that soil S and P levels were placed very nearly to DPPH and ALP, both of which were loaded on PC2, indicating that soil S and P actually increased IC_{50} values or otherwise interfere the activity of scavenging DPPH and ALP free radicals. Soil N, organic carbon and organic matter were clustered together, but these attributes are not such significant contributors to antioxidant activity as revealed from PCA. In *Lebisia pumila*, enhancement of nitrate content through organic fertiliser application in soil improved secondary metabolites production and antioxidant activity (Ibrahim *et al*. 2013). In case of Dooars (Fig. 2&3), different attributes were significantly correlated with each other (Value of "r" at $P < 0.01$ level) *i.e.*, silt (0.718), pH (0.875), P (0.615), Cl^- (0.858), TP (0.776), Flav (0.923), Q (0.666), T (0.865), DPPH (0.536), SO (0.576), ABTS (0.520) and MC (0.777). In the case of Terai (Fig. 4&5), first principle components (PC1), *i.e.*, soil K, moisture, EC and organic N clustered with the metal chelating ability and for PC2 as evidenced by the dendro-hit map; ABTS, ALP, SO, NO, DPPH (IC_{50}) clustered with the available soil S and P. Different attributes were significantly correlated each other *i.e.*, clay fraction (0.778), EC (0.618), N (0.777), S (0.748), P (0.514), FI (0.918), TP (0.687), DPPH (0.794), NO (0.913), ALP (0.717) and MC (0.665) at $P < 0.01$ level. In the case of Darjeeling hills (Fig. 6 &7), in PC1, available soil N, K, P, EC and EGCG were clustered with ALP and ABTS. For PC2, available soil S, pH and MC per cent were clustered with tea phytochemical attributes like TP, FI, ECG and antioxidant attributes like SO and ABTS.

Following attributes were highly correlated with clay (0.812), sand (0.818), silt fraction (0.974), K (0.932), S (0.999), MC of soil (0.671), TP (0.853), tannins (0.912), DPPH (0.624), ABTS (0.661) and MC (0.633) at $P<0.01$ level.

Conclusion

It can be concluded that soil nutrient profile, particularly N, P, K and S, has changed with age variation, which ultimately influences the accumulation of bioactive secondary metabolites and antioxidants in natural vegetation. So, to elicit bioactive metabolites and high-quality nutraceuticals in tea of Dooars, Terai and Darjeeling hills, the application of NPK is required to improve the cultivation technique.

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