Impact of Altitude, Seasonal Variation, and Processing Parameters on Bioactive Compounds and Antioxidant Potential of Darjeeling Tea

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Abstract

The study aims to explore the influence of processing parameters, altitude, seasonal variations, and soil conditions on the metabolite composition and antioxidant activity of Darjeeling tea. The *Camellia sinensis var. sinensis* were sampled from different processing stages of two Darjeeling tea gardens situated at different altitudes representing different environmental conditions and investigated for targeted metabolomics using high-performance thin layer chromatography (HPTLC) techniques where eight key bioactive compounds were chosen for the analysis. The antioxidant activity demonstrated a positive association with altitude, with elevated levels of total phenol (25.7 µg GAE/g), total flavonoid (4253.09 µg QEA/g), DPPH (94.09%), and H_2O_2 (95.94%) observed in the final processed tea from the higher elevation garden. Conversely, seasonal transitions exhibited a detrimental effect on antioxidant activity, with a decline from 94.09 to 78.65% (DPPH) and 95.94 to 84.98% (H_2O_2) during the shift from spring to summer and autumn in the higher elevation garden. Soil analyses unveiled significant positive correlations between pH (r= +0.98) and total flavonoid, carbon (r = +0.85), and potash (r = +0.99) with H_2O_2, and sulfur (r = +0.98) with total phenol. The study highlights the complex interplay of altitude, seasonality, processing parameters, and soil characteristics in shaping the accumulation of bioactive compounds in tea.

Highlights

- HPTLC fingerprinting was employed for targeted metabolomics.
- Cup characteristics and antioxidant potential were evaluated by quantitative estimation of TF, TR, caffeine, TPC, TFC, and DPPH radical scavenging activity.
- Soil parameters of the gardens at various seasons were evaluated to understand the correlation between the antioxidant potential and soil health.
- The HPTLC revealed the presence of various marker metabolites and the higher-altitude garden showed better antioxidant potential compared to the lower-altitude garden.
- The study revealed an intricate relationship between altitude, season, processing parameters, and soil in the accumulation of metabolites in Darjeeling tea.

Keywords: HPTLC, Black tea, Metabolites, Antioxidant, Altitude.

Introduction

Plants, as stationary organisms, have evolved mechanisms to produce a diverse array of specialized metabolites. These compounds enable them to adapt to dynamic environmental conditions, thrive in diverse ecological habitats, and defend against various biotic and abiotic stresses. Primary metabolites play indispensable roles in plant growth, development, and various physiological processes. Conversely, secondary metabolites constitute a broad category of specialized molecules derived from primary metabolism. Secondary metabolites may not directly aid in the growth, development, or reproduction of plants but they possess significant pharmacological, toxicological, and ecological significance (Sharma and Bhagwan,1996). The quality assessment of plants, especially those with medicinal or economic value, encompasses a multifaceted evaluation of their nutritional content and sensory attributes. This evaluation entails examining the levels of phytochemicals, minerals, and different metabolites that influence various aspects of biological activity, longevity, and organoleptic characteristics such as color, aroma, flavor, texture, and appearance (Ahmed and Stepp, 2016; Mattos *et al.,* 2014).

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The tea plant, scientifically known as *Camellia sinensis* (L.) Kuntze offers an exceptional model for research due to its rich repository of nearly 4000 specialized metabolites, distinctive organoleptic characteristics, bioactive properties, and associated health advantages. As one of the most preferred non-alcoholic

drinks globally (Kumar *et al.,*2011), tea cultivation spans over 50 countries (FAOSTAT,2016). India stands out as one of the top producers of black tea worldwide, with Darjeeling tea being a particularly renowned product on the market overseas.

Black and green tea stand as the two predominant variants, celebrated for their antioxidant properties believed to stem from their abundance in polyphenolic content (Vinson and Dabbagh,1998). These tea polyphenols encompass flavonol aglycones such as 3,4,5,7-tetrahydroxyflavonol, 3,4',5,7-tetrahydroxyflavone, and 3,3',4',5,5',7-hexahydroxyflavone as well as catechin groups and theaflavins. (Del Rio *et al.,*2004).

The quality of tea is profoundly shaped by its constituent compounds, notably flavon-3-ols, bisflavanols, flavonol aglycones, methylxanthines, and volatile oils, complemented by carbohydrates and amino acids (Scharbert *et al.,*2004; Drewnowski and Gomez-Carneros,2000). These compounds not only define the flavor and visual appeal of tea but also confer a spectrum of health benefits, including cardioprotective, neuroprotective, anti-cancer, antimicrobial, and anti-inflammatory effects (Clement, 2009; Lin *et al.,*2003; Trevisanato and Kim 2000). The collective influence of these diverse compounds renders tea an esteemed beverage for its multifaceted applications extending beyond mere refreshment.

The concentration of metabolites in tea is subject to fluctuation, contingent upon numerous factors including altitude, environmental conditions encompassing temperature, precipitation, relative humidity, and ultraviolet radiation exposure, soil properties, clonal variations, harvesting seasons (commonly referred to as flushes), and manufacturing methodologies (Wijeratne, 1996). Notably, tea quality is profoundly shaped by the manufacturing techniques, which traditionally involve four key stages aiming for moisture loss through withering, shaping the leaves through rolling, formation of key components through oxidation, and increasing shelf life when the leaves are dried. These manufacturing steps give rise to distinct categories of tea-white, green, oolong, and black-each characterized by their unique flavor profile and biochemical composition (Chow and Kramer, 1990).

The fluctuation in altitudes induces alterations in various environmental parameters, consequently giving rise to diverse stressors such as UV exposure, drought conditions, and cold temperatures. Studies indicate that tea plantations situated at higher elevations typically yield superior-quality tea due to a heightened accumulation of flavonoids, polyphenols, caffeine, and volatile oils. One possible explanation for these observations is that elevated altitudes entail increased oxidative stress and other environmental pressures, thereby stimulating the synthesis of a greater array of specialized metabolites (Abeywickrama *et al.,* 2011; Abeywickrama *et al.,* 2010; Akhlas, 2003).

Darjeeling tea, recognized for its distinctive flavor and captivating aroma, is meticulously cultivated in the hills of Darjeeling under West Bengal, India. This renowned tea variety is grown in the tranquil Himalayan foothills, spanning varying altitudes, contributing to its exceptional quality and diverse flavor profile. Darjeeling tea is susceptible to adulteration, fraudulent practices often involve misrepresenting the tea's origin or mislabeling its brand, substituting premium tea with cheaper alternatives for financial gain. To address these challenges, analytical methods like mass spectrometry and NMR spectroscopy are hugely employed for their sensitivity, stability, and metabolic coverage (Gromski *et al.,*2015). However, these methods have limitations, often necessitating a combination of approaches with hyphenated analytical tools. HPTLC has emerged as a valuable tool for chemical profiling, either independently or in conjunction with other instruments. Its benefits include versatile chemical profiling capabilities, rapid analysis, simultaneous assessment of multiple samples, and enhanced detection facilitated by various post-analytical chemical reagents. HPTLC has also demonstrated compatibility with NMR spectroscopy and is recognized as a high-throughput metabolomics technique, particularly in research of plant biology, owing to its automation and straightforward interpretation using multivariate data analysis (Salomé-Abarca *et al.,*2021).

The primary objective of this study is to investigate how altitude, season and processing factors affect the chemical composition, antioxidant potential, and overall quality of Darjeeling tea by characterizing key metabolites and evaluating physicochemical properties and antioxidant potential. The use of a fast effect-directed, multi-imaging HPTLC profiling technique offers a novel, precise, and high-throughput approach to identifying and characterizing key metabolites in tea. This method improves the accuracy and efficiency of chemical analysis compared to traditional approaches. This study uniquely focuses on the impact of varying altitudes and multiple harvest seasons on the quality of Darjeeling tea, which has been underexplored compared to other factors thus providing a comprehensive insight of the interplay between altitude, chemical composition, and tea quality, contributing valuable knowledge to tea science and industry practices.

MATERIALS

Study Site
The research was conducted in two gardens (27°04'10.65" N 88°17'53.63" E and 26°51'35.89" N 88°15'41.32" E) in the Darjeeling hills of West Bengal, India. These estates are with elevations ranging from 1247 to 1500 meters and 664 to 1096 meters from sea level, respectively.

Collection of Plant Material
Three China hybrid varieties of *C. sinensis* (L.) Kuntze, namely-Runglee Rungliot 4/5 (RR- 4/5), Phoobsering 312 (P-312), and Ambari Vegetative 2(AV-2) (Fig.1) in equal proportions, were sampled from the three flushes and various stages of tea processing, as well as from freshly plucked tea leaves and the final tea product after sorting, grading and curing. Sampling was done in April, June, and November from both gardens. Plant material identification was conducted by the BSI in Howrah, India. Subsequently, a voucher specimen was placed at the CNH of BSI.

Collection of Soil Samples
Soil samples were retrieved using a soil auger from both the gardens in pre-monsoon and post-monsoon periods. Topsoil and subsoil samples were obtained from the immediate vicinity

Fig. 1: The three different clones of *C. sinensis var. sinensis*

of four bushes. Following collection, the soil samples underwent airdrying and sieving to remove debris, after which 500 g of each sample preceded for further analysis. The gardens do not apply chemical fertilizers or pesticides.

Methods

HPTLC analysis

The phytochemical analysis of tea extracts was conducted via HPTLC, following the protocol of Ashraf *et al.,* 2021and Baishya *et al.,* 2023. Methanolic extracts of finely powdered leaf samples from various processing stages, along with standard compounds (1-mg/mL), were introduced to pre-coated silica gel 60 F_{254} HPTLC plates as 8-mm bands using a 100 µL HPTLC syringe (Hamilton, Bondauz, Switzerland). The injection speed was maintained at 150 nl/s by employing compressed air, with the injections positioned 10 mm apart from the lower edge, 8 mm from the bottom, and at least 15 mm from the plate side, utilizing the Linomat 5 applicator (CAMAG, Muttenz, Switzerland).

Plate development with authentic compounds and tea extracts occurred in a pre-saturated TLC chamber with a mobile phase comprising chloroform, ethyl acetate, and formic acid in a ratio of 5:4:1 v/v/v for the compounds caffeine (CAF), chlorogenic acid (CA), kaempferol (KMF), gallic acid (GA), quercetin (QR), and toluene, acetone, and formic acid in a ratio of 4.5:4.5:1 v/v/v for epigallocatechin gallate (EGCG), gallocatechin (GC), and theaflavin (TF) within a Twin Trough Chamber (CAMAG). The migration distance was set at 7 cm, with the development at 25 ± 5ºC.

Following drying, chromatogram documentation was performed using a TLC UV Cabinet 4 (CAMAG), utilizing various wavelengths including 254, 366, and 416 nm with a TLC scanner 4 operated by vision CATS software (CAMAG; slit width 6 × 0.45 mm) for chromatogram evaluation.

Chemical derivatization

The derivatization process was conducted utilizing a CAMAG derivatizer equipped with an automatic spraying mechanism, which applies the derivatizing reagent onto the plates utilizing innovative "microdroplet" spraying technology. Various types of nozzles are employed depending on the specific derivatizing reagents utilized.

To prepare the reagent for derivatization, 140 mg of fast blue salt B was dissolved in a solution comprising 10 mL of water and 140 mL of MeOH. Over 50 mL of Dichloromethane was added

to it. The resulting mixture was prepared freshly and sprayed onto the HPTLC plate using the derivatizer followed by heating the plate at 100°C for 2 minutes.

Chlorophyll content

Chlorophyll content was quantified by following the method of Ni *et al.,*2009 with few adjustments. Green leaves were finely ground and weighed. Subsequently, 0.4 g finely powdered sample was combined with 80% 5 mL acetone, followed by mixing for 5 minutes and stored in darkness at 4°C for 15 minutes. The content was then centrifuged for 15 minutes at 3,000 rpm, and the resulting aliquot was kept in darkness. The whole process was done twice, and the aliquots obtained were then combined to achieve a total volume of 25 mL using 80% acetone.

The whole content was shaken thoroughly, and the measurements as absorbance were taken at 663 and 645 nm, against blank. The following formula expresses the chlorophyll content:

Chlorophyll a(mg/g) = $[12.7 \times A663 - 2.69 \times A645] \times V / 1000 \times W$

Chlorophyll b(mg/g) = $[22.9 \times A645 - 4.86 \times A663] \times V / 1000 \times W$

Chlorophyll a+b (mg/g) = $[8.02 \times A663 + 20.20 \times A645] \times V / 1000 \times W$

Where V = volume of the extract (mL); W = Weights of fresh leaves (g); A_{645}: absorbance at 645 nm; A_{663}: absorbance at 663 nm.

Reducing sugars

Reducing sugar was evaluated using the procedure outlined by Miller,1959. In summary, 1-mL of tea extract was added with 2 mL of DNS and vortexed. The content was heated for 5 minutes and subsequently allowed to cool. Absorbance readings were obtained at 540 nm. Dextrose was used as a standard to construct the calibration curve.

Caffeine content

The caffeine content was estimated by using the method of Harrison *et al.,* 2010. The standard curve was prepared by using a 1000 ppm stock solution. Over 0.25 g of tea samples were weighed, to 20 mL of dist. water was added. The content was placed in a 250 mL flask, following the addition of 10 mL of 0.01 M/L HCL and 2 mL of $Pb(C_2H_3O_2)_2$ solution. The entire content was then diluted to a final volume of 250 mL with dist. H_2O, thoroughly shaken followed by filtration to clarify. From the resulting filtrate, 50 mL was taken into another flask of 100 mL following the addition of 0.2 mL of 4.5 M/L H_2SO_4, and the

volume was adjusted to 100 mL with dist. H_2O. After thorough agitation and subsequent filtration, the spectral measurements were taken at 274 nm.

Estimation of Theaflavins (TFs), Thearubigins (TRs) & Theabrownins (TBs) in black tea

The percentage of TFs, TRs, and TBs were analyzed following the methods of Jiang *et al.,* 2018, and Akuli *et al.,* 2016 with some modifications. An extract of tea samples was prepared by infusing 5 g of plant material with 100 mL of boiled water following filtration. 50 mL of EtOAc was added to 50 mL of cooled filtrate, and mixed vigorously using a liquid partitioner.

   Sol^n A: In 25 mL of sol^n A was prepared by adding 4 mL of EtOAc layer with 21 mL of MeOH.

   Sol^n B: In 25 mL of Sol^n B was prepared by partitioning equal amounts of the remaining EtOAc with 2.5% (W/V) $NaHCO_3$ following pipetting of 4 mL of the organic layer with the addition of 21 mL MeOH.

Sol^n C: About 25 mL Sol^n C was prepared by, mixing 2 mL of water layer from the first separation with the same amount of saturated $C_2H_2O_4$ following dilution with 6 mL of H_2O and 15 mL MeOH.

   Sol^n D: Over 25 mL Sol^n D was prepared by partitioning 15 mL of plant extract with an equal amount of 1-butanol following mixing 2 mL of the water layer from the separation with the same amount of saturated $C_2H_2O_4$ and diluting the solution with 6 mL of H_2O and 15 mL MeOH.

   The absorbances of Sol^n A, B, C, D as AA, AB, AC, and AD were recorded at 380 nm against blank. The percentages of TFs, TRs, and TBs were expressed by the formulas-

TFs (%)- $2.25\ x\ AA$

TRs (%)- $7.06\ x\ (2AA + 2AC - AB - 2AD)$

TBs (%)- $2\ x\ AD\ x\ 7.06$

Antioxidant activities

Total phenol content (TPC)

 Quantification of TPC was done using the FCR method outlined by Sadasivam *et al.,* 2008 with gallic acid serving as an authentic standard. To 3 mL of sample extract, 0.5 mL of 1(N) Folin-Ciocalteu reagent was added and allowed for 5 minutes of reaction time following the addition of 2 mL of 20% Na_2CO_3. The whole sample mixture was vortexed following heating for 1-minute. The spectral measurements were recorded at 650 nm against a blank.

Total flavonoid content (TFC)

TFC was estimated following a modified protocol by Kim *et al.,* 2003 with quercetin serving as a standard reference. To 1 mL sample extract 4 mL of dist. H_2O was added following the addition of 0.3 mL of 5% $NaNO_2$ and allowed for a 5-minute reaction time. Following this 0.3 mL $AlCl_3$ (10%) was added and kept for 1-minute. The whole content was combined with 2 mL NaOH (1 M) following dilution with 2.4 mL of dist. H_2O. The spectral measurements were recorded at 510 nm.

DPPH radical scavenging activity

activity The DPPH radical scavenging activity was determined using the method described by Braca et al., 2001. To conduct the assay, 0.1 mL of the methanolic sample solution was combined with 3 mL of a 0.004% (v/v) DPPH solution. After incubating the mixture for 30 minutes, the absorbance was recorded at 517 nm, and the inhibition was computed using the formula-

$[(A0 - Ae) / A0]\ x\ 100$, where A_0 = spectral measurement without sample; A_e = spectral measurement with sample.

H_2O_2 radical scavenging activity

Hydroxyl radical scavenging activity was evaluated using the method described by Smirnoff and Cumbes, 1989. The reaction mixture included 500 µL of 1.5 mM $FeSO_4$, 150 µL of 20 mM $C_7H_5O_3Na$, 500 µL of the sample extract, and 350 µL of 6 mM H_2O_2. The content was kept at 37°C for 1-hour in a water bath. Following incubation, the absorbance was measured at 562 nm. The percentage of inhibition was calculated using the formula-

$$\%inhibition = [\ 1 - (A1 - A2)]\ /\ A0 * 100$$

Where,
A1= spectral measurement of tea samples
A2= spectral measurement without sodium salicylate
A0= spectral measurement of H_2O_2+ $FeSO_4$+ sodium salicylate

Reducing power

The reducing power assay was conducted according to the procedure described by Yen et al.,1995. To 500 µL of sample extract, 1.25 mL of PBS (0.2 M, pH 6.6) and 1.25 mL of 1% $K_3[Fe(CN)_6]$ solution was added. The mixture was incubated at 50°C for 20 minutes following the addition of 1.25 mL of 10% TCA and centrifugation at 3000 rpm for 10 minutes. A 1.25 mL aliquot of the resulting supernatant was taken and diluted with 1.25 mL of H_2O followed by a subsequent addition of 0.25 mL of 0.1% $FeCl_3$.The absorbance was recorded at 700 nm.

Soil Analysis

pH

The pH analysis of the soil samples was conducted following the procedure of Baruah and Barthakur,1997. Before taking the readings, the pH meter was calibrated with standard buffer solutions to maintain precision.

Carbon Content

The carbon content of the samples was quantified by using the titration method as described by Walkley, 1947.

Nitrogen, Available Phosphorus, Potassium, and sulfur content

The nitrogen content within the samples was estimated by using the Kjeldahl technique as described by Jackson, 1973. In order to assess the phosphorus and potassium of soil, a diacid digestion technique was implemented. The soil available phosphorus was measured colorimetrically, following the approach described by Bray and Kurtz in 1945, with minor modifications. Potash was quantified in the samples using a modified methodology presented by Chapman and Pratt in 1961. Additionally, sulfur was evaluated as sulfate through a modified methodology described by Ensminger in 1954.

RESULTS AND DISCUSSIONS

Identification of marker metabolites *via* HPTLC

The targeted metabolite fingerprinting of 36 tea samples across all processing stages (withering, rolling, fermenting, and drying), along with fresh and fully processed leaves, was investigated using HPTLC. Chromatographic conditions were established based on isocratic separation, employing different solvent combinations of Chloroform:Ethyl acetate:Formic acid in a ratio of 5:4:1 for phenolic compounds and alkaloids, and Toluene:Acetone:Formic acid in a ratio of 4.5:4.5:1 for flavan-3-ols. The stationary phase utilized pre-coated aluminum silica plates F_{254}.

Eight bioactive compounds were chosen as reference standards for tea profiling, including the primary purine alkaloid (caffeine), two flavonol aglycones (quercetin, kaempferol), two polyphenols (gallic acid, chlorogenic acid), two flavan-3-ols (catechins - GC, EGCG), and the main bis flavanol, theaflavin. Prior to derivatization, the developed plates were examined under normal light along with shorter and broader wavelengths of UV light. Visual inspection at 254 nm wavelength (Fig 2.a-c and 3.a-c) revealed darker regions against a backdrop of green fluorescence, indicative of aromatic compounds in the black tea extracts (BTE), although these specific aromatic compounds remained unidentified.

The presence of phenolic compounds was inferred from pinkish-violet and light blue regions observed in the chromatograms. Bands visible under both natural light and at UV light (366 nm) (Fig. 2 a-c and 3.a-c) exhibited violet, blue, red, grey, or green colors, corresponding to phenolic compounds, isoprenoids, carbohydrates, and steroids, respectively, with Fast blue salt B serving as the derivatizing agent (Lim *et al.*, 2021). Prolonged color development was achieved by heating the plates for 2 minutes at 100°C to evaporate excess reagent.

Analysis of the 36 Black tea extracts revealed the presence of the majority of marker metabolites used for identification, as well as compounds like terpenoids, sugars, and steroids (Fig. 2 a-c and 3.a-c).

Qualitative analysis of Eight Reference Standards

Chemical derivatization confirmed the existence of the marker metabolites as well as some other unknown phytoconstituents. A validated HPTLC method was used to identify and quantify the eight marker metabolites. The HPTLC profiling reveals the R_f values of the marker metabolites i.e. CAF(R_f:0.47), CA (R_f:0.04), KMF (R_f:0.64), GA (R_f:0.31), QR (R_f:0.56), EGCG (R_f:0.43), GC (R_f:0.50) and TF (R_f:0.62). The chromatogram scans at 254 nm of the different BTE, specifically the final processed tea of the garden at a higher altitude (1247–1500 mts) represented in Fig.4.a-f and the final processed tea of the garden at a lower altitude (664–1096 mts) represented in Fig. 5a-f. The presence of CAF, CA, KMF, GA, QR, EGCG, and GC & TF was confirmed in the black tea extracts by comparing them with their corresponding R_f values.

Physicochemical Properties and Cup Characteristics

Chlorophyll content

Chlorophyll is the primary and fundamental plant pigment directly involved in photosynthesis, facilitating the accumulation of growth substances and exerting a vital role in the absorption, transmission, and transformation of light energy. The composition of photosynthetic pigments in plants can indicate their ability to adapt to changes in environmental conditions (Vasileva and Ilieva, 2017; Smirnova *et al.*, 2013; Titova, 2010; Nurmakova, 2003). Chlorophyll levels, which are unique to each plant species or variant, can serve as bioindicators of the plant's activity, biomass yield, vitality, resilience to adverse environments, and response to factors such as habitat, weather, and human activity (Zielewicz *et al.*, 2020; Li *et al.*, 2018). Fig. 6. a. illustrates the variations in total chlorophyll content in fresh leaves of tea plants at different altitudes across each flush. The garden at a higher altitude (HA) exhibits the highest chlorophyll content during the first flush (plucking season late February-April), with a total chlorophyll (chl a+b) content of 0.47 mg/g. In comparison, the second and third flushes show a decrease in chlorophyll content, with total chlorophyll content of 0.38 and 0.36 mg/g, respectively (Fig. 6 a). Comparing the gardens at two different altitudes, the garden at the lower elevation shows total chlorophyll content values of 0.44, 0.30, and 0.24 mg/g for the spring (first), summer (second), and autumn (third) flushes, respectively (Fig. 6 a). A similar trend is observed for chlorophyll a. However, for chlorophyll b, the third flush of the garden at a lower altitude has a higher chlorophyll b content (0.07 mg/g) compared to the third flush of the garden at a higher altitude (0.05 mg/g). Photosynthetic pigments are vulnerable to environmental stressors such as drought, intense light, and high temperatures, which can decrease chlorophyll content, potentially damage chloroplast structures, or slow synthesis. Some herbaceous species, such as buttonweed (Adams *et al.*, 2006; Adams *et al.*, 2004; Adams *et al.*, 2002; Adams *et al.*, 2001; Verhoeven *et al.*, 1999), winter cereals (Hurry *et al.*, 1995a; Hurry *et al.*, 1995b; Hurry *et al.*,1995c), and spinach (Adams *et al.*,1995; Holaday *et al.*,1992;), have exhibited enhanced photosynthetic activity as a response to cold stress. The effect of partial pressure of CO_2 in the air on photosynthesis has been a focal point for researchers of plant science and ecology. Numerous studies have explored this impact (Bowman *et al.*,1999; Körner and Diemer,1987; Billings *et al.*,1961). Addressing the altitudinal gradients from 200 m to 1100 m, Friend *et al.*,1989 observed an augmented net rate of photosynthesis in European blueberry and Matgrass. Their findings indicated an increase in the net rate of photosynthesis in both species with rising altitude, potentially due to an increase in the nitrogen in leaves per unit of the leaf area.

Reducing Sugars

The primary component responsible for imparting sweetness to tea is reducing sugar. Fig.6. b. shows that the BTE (black tea extract) of six processing stages in the first flush of the garden at HA contains higher amounts of reducing sugar: 31.15 mg/g (fresh leaves), 25.65 mg/g (withering), 19.46 mg/g (fermentation), 21 mg/g (final processed), 18.58 mg/g (drying), and 9.16 mg/g (rolling). In comparison, the first flush of the garden at LA shows lower amounts: 28.26 mg/g (fresh leaves), 16.6 mg/g (withering), 10.98 mg/g (fermentation), 11.2 mg/g (final processed), 9.97 mg/g (drying), and 7.81 mg/g (rolling).
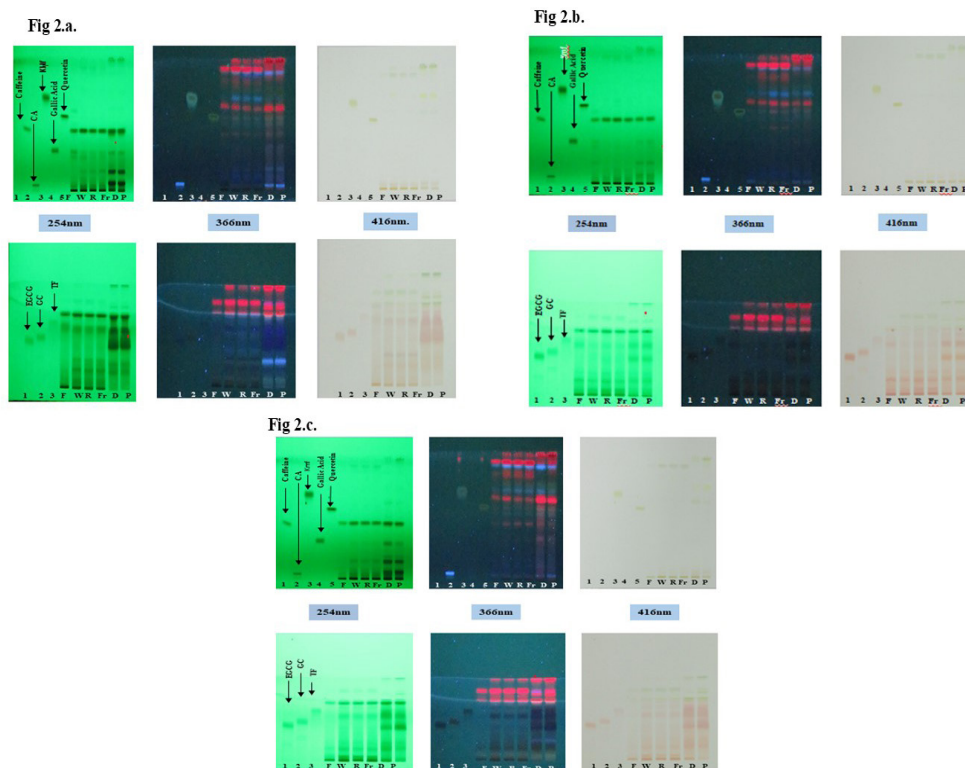
Fig. 2(a-c): HPTLC fingerprinting of first, second, and third flush orthodox tea of each processing step (altitude- 1247–1500 mt)
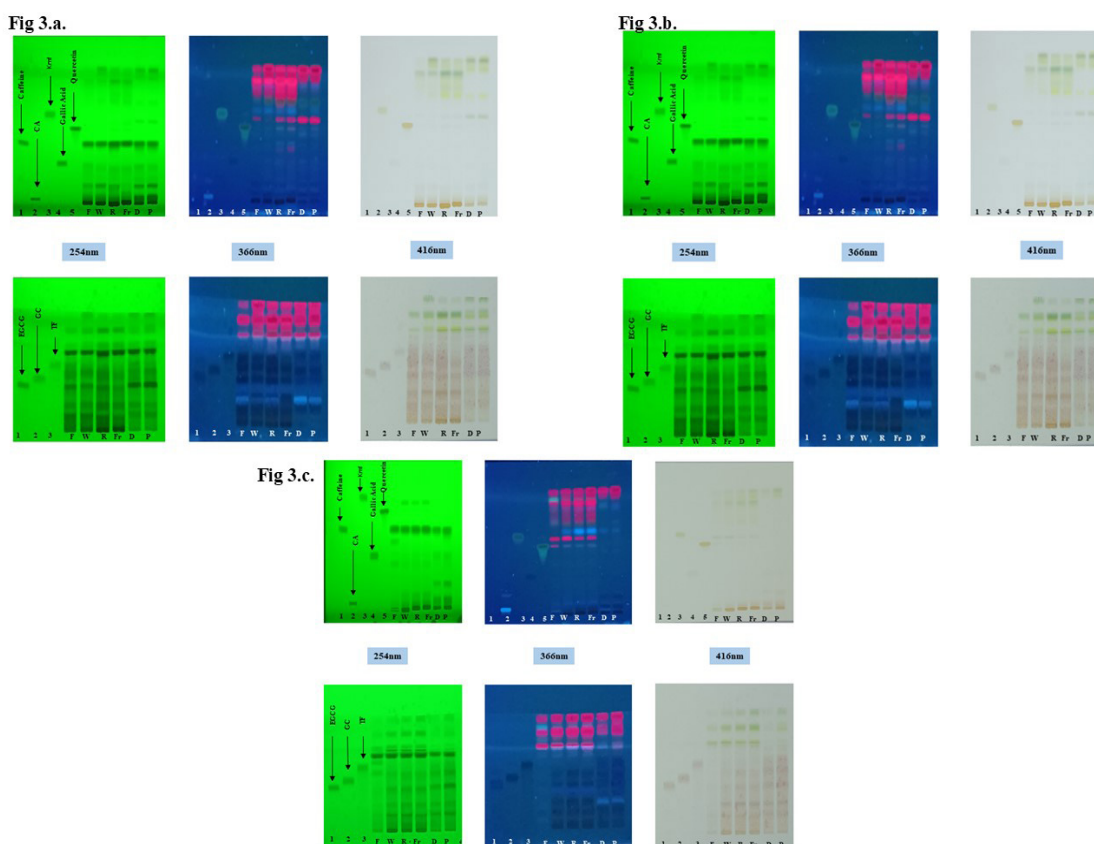

Fig. 3(a-c): HPTLC fingerprinting of first, second & third flush orthodox tea of each processing step (altitude- 664–1096 mt)
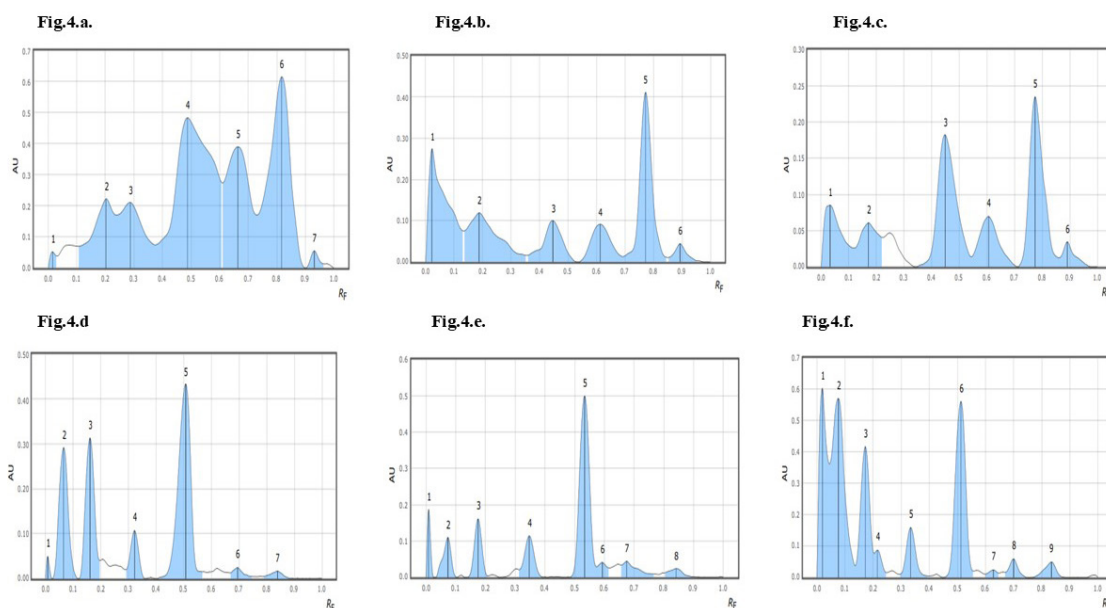
Fig. 4(a-f): Chromatograms of first, second & third flushes of the final processed tea from the high-altitude garden at 254 nm to identify EGCG, GC, TF, CAF, CA, KMF, GA & QR
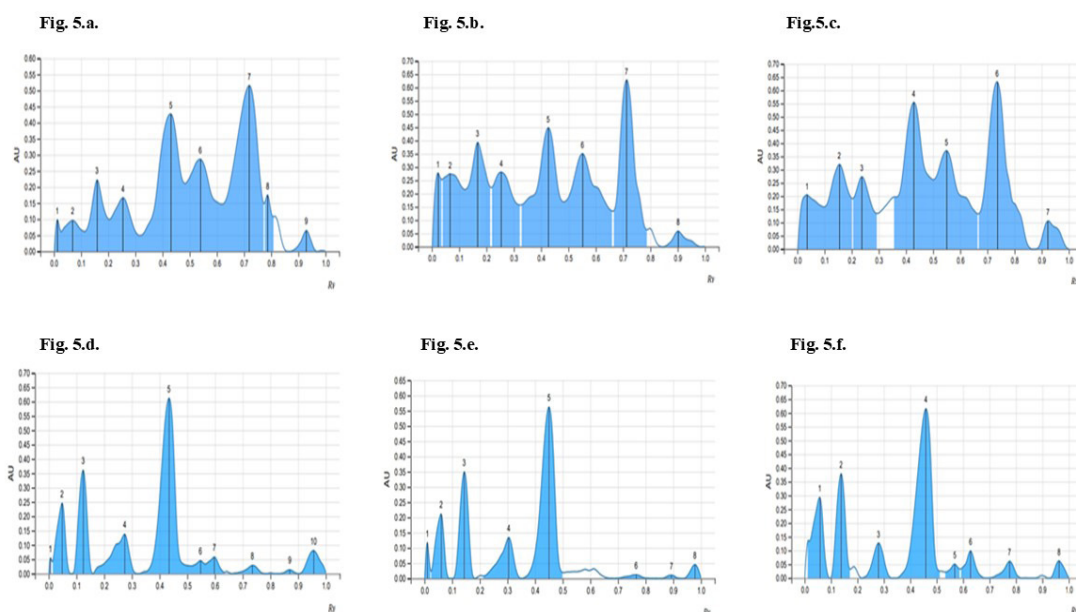

Fig. 5(a-f): Chromatogram of first, second & third flushes of the final processed tea from the lower-altitude garden at 254 nm to identify EGCG, GC, TF, CAF, CA, KMF, GA & QR.

Both gardens show a decrease in reducing sugar content in the second flush, with the fresh tea leaves from HA having 23.05 mg/g and from LA having 18.37 mg/g. In the third flush, the reducing sugar content is 30.78 mg/g and 24 mg/g in fresh tea leaves of HA and LA gardens, respectively.

A notable reduction in the amount of reducing sugars was observed from freshly harvested to final processed tea leaves in the course of black tea processing. This decline is likely attributed to the generation of browning compounds or by-products of the Maillard reaction, commonly acrylamide. These compounds emerge during the drying of green leaves at temperatures

exceeding 120°C during (Mizukami *et al.*, 2006). The Maillard reaction is a complex non-enzymatic process that occurs when reducing sugars and amino acids interact at high temperatures, leading to the formation of brown pigments and a caramelized flavor (Wang *et al.*, 2023).

The reduction in sugar levels occurs due to the degradation of monosaccharides as a consequence of respiration, as harvested leaves cannot produce sugar through photosynthesis. Consequently, amounts of simple sugars like glucose and fructose significantly decline in the withered leaves, followed by rolled ones. Numerous studies have demonstrated that
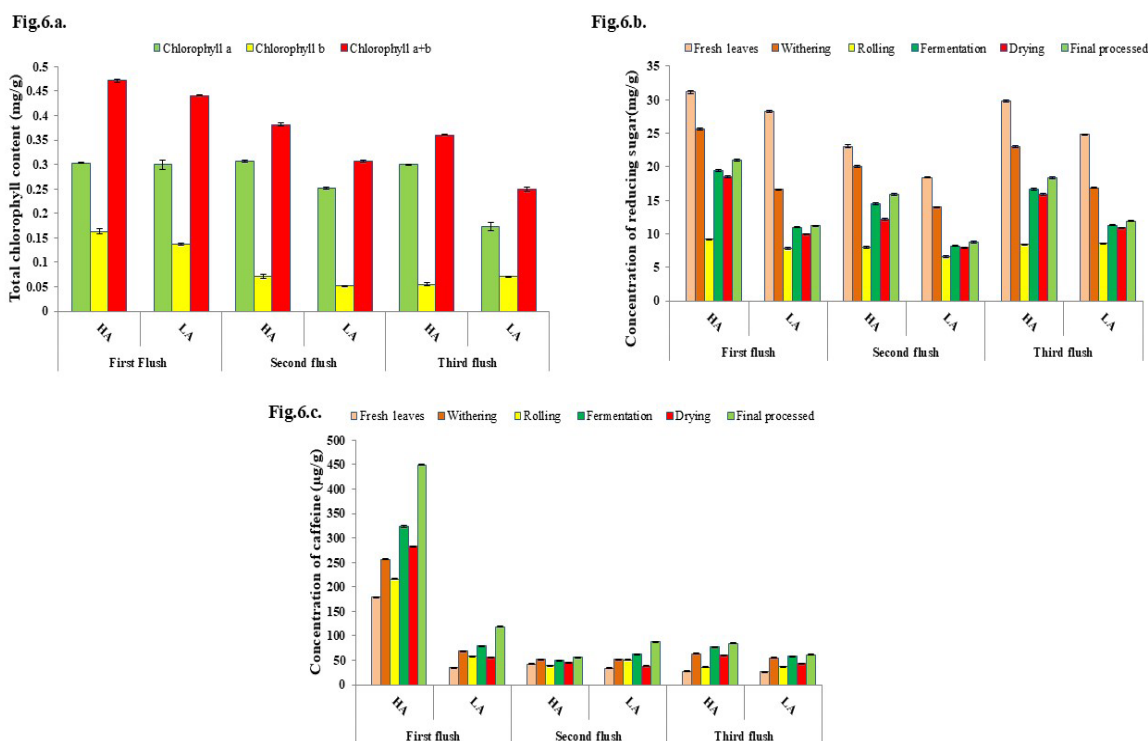
Fig.6.a.

Fig.6.b.

Fig.6.c.

Fig. 6a-c: The chlorophyll content, reducing sugar content, and caffeine content of black tea samples of HA and LA gardens across different flushes

sugars are essential to a plant's response mechanism against various abiotic (heat, cold, drought, UV) and biotic stress factors (pathogens, insects) (Keunen *et al.,* 2013; Chen *et al.,* 2010; Nakagawa, 1975). As altitude increases, plants encounter more abiotic stress factors like cold and UV stress. Acclimation to cold exposure necessitates changes in carbohydrate metabolism, involving the accumulation of soluble sugars and starch hydrolysis in chloroplasts. Orzechowski *et al.,*2021 examined the early response of potato plant leaves to cold stress, noting increased activities of glucan phosphorylase, amylase, and invertase, which led to an accumulation of soluble sugars. This suggests that the higher amount of reduced sugar in the HA garden may be due to the various abiotic stress factors it experiences. Additionally, the first flush is plucked after three months of winter dormancy, which supports the accumulation of sugar compounds. The presence of sugars in tea leaves can also be influenced by seasonal variations. This study investigated the effects of wet (second flush) and dry seasons (first and third flush) on the inherent levels of reduced sugar in black tea (Ansari *et al.,* 2011).

The findings revealed a notable difference in the amounts of reduced sugar in black tea between the dry and wet periods. It was observed that the inherent sugar levels of the tea extracts from both gardens followed a similar pattern: sugar levels were higher during the dry season compared to the wet season (Lee *et al.,* 2015; Ahmed *et al.,* 2014).

Researchers have shown that higher concentrations of soluble sugars like glucose and fructose can heighten a plant's ability to endure various abiotic stressors such as salinity, heat, drought, and cold. A drought period during the plucking

season also contributes to this effect. Plants typically respond to drought by adjusting their metabolism to manage water stress, often synthesizing soluble sugars like glucose, fructose, and sucrose. These sugars help maintain osmotic potential and water balance within the cells during dry spells (Khaleghi *et al.,*2019; Rosa *et al.,*2009). Additionally, these soluble sugars not only support the biosynthesis of polyphenols in tea but also aid in their transport within the plant, impacting the aroma, flavor, and quality of the tea (Qian *et al.,*2018; Liu *et al.,*2014).

Caffeine Content

Caffeine is one of the most abundant methylated derivatives of purine in tea, contributing to its bitter taste. Our study found that the first flush of the HA garden has the highest caffeine content, with levels increasing from 178.5 µg/g in fresh leaves to 449.52 µg/g in the final tea product. The progression of caffeine content through the stages-rolling (216 µg/g), withering (256.64 µg/g), drying (282.9 µg/g), and fermentation (323.76 µg/g)-is depicted in Fig. 6.c. This increase is due to complex biochemical pathways active during processing. During withering, tea shoots respire, depleting sugar reserves and providing precursors for caffeine biosynthesis, thus increasing caffeine content (Deka *et al.,* 2021; Ullah, 1984). Additionally, amino acid metabolism, which serves as a precursor for many biochemical pathways, may enhance conditions for caffeine synthesis (Deka *et al.,* 2021; Roberts and Sanderson, 1966). Another significant pathway involves the breakdown of nucleic acids during withering, further contributing to the rise in caffeine content (Deka *et al.,* 2021; Sari and Velioglu, 2013).
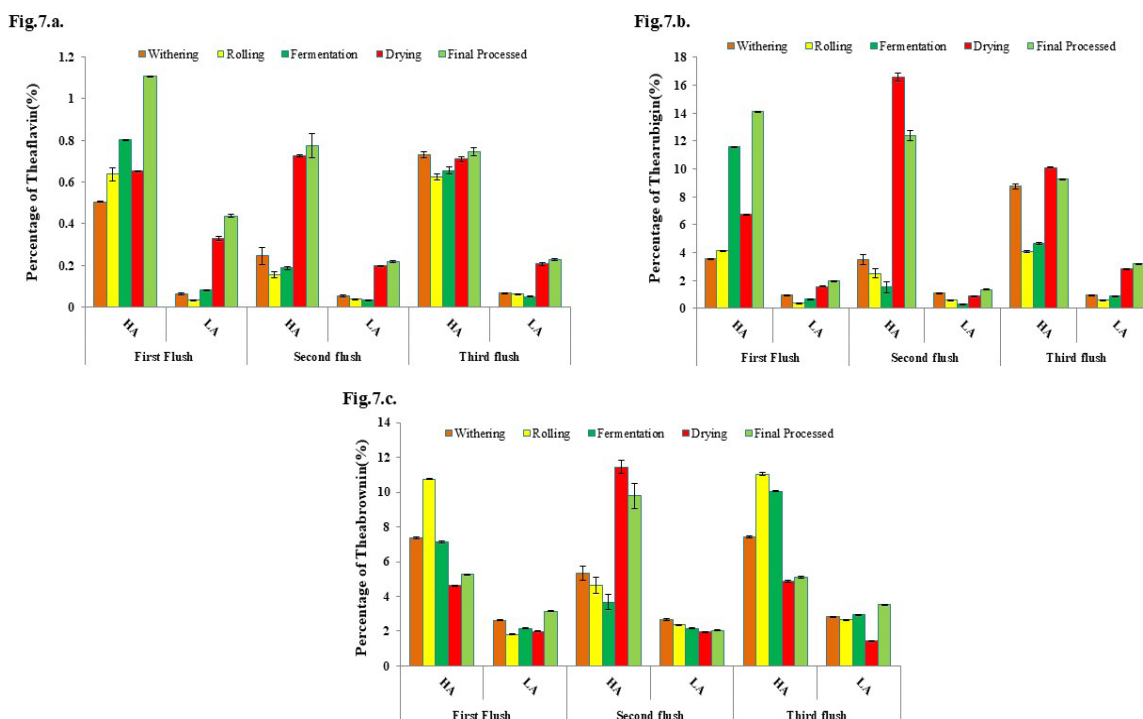
Fig. 7a-c: illustrates the TF, TR, and TB content at various processing stages in both the HA and LA gardens across different flushes

The variations in caffeine content in tea plants due to seasonal factors and elevation are influenced by various environmental and physiological conditions. Research has shown that tea plants grown at higher elevations have higher caffeine levels compared to those at lower elevations. This is because higher-elevation tea plants experience cooler temperatures and direct sunlight, resulting in a slower growth rate and longer maturation period, which leads to the accumulation of higher levels of secondary metabolites like caffeine (Ohno *et al.,* 2011; Abeywickrama *et al.,* 2010; Akhlas, 2003). A study by Lee *et al.,* 2015 on green tea samples indicated a negative correlation between climate and caffeine content, suggesting that lower temperatures stimulate caffeine biosynthesis in tea shoots, whereas higher temperatures result in lower caffeine concentrations. Seasonal changes also affect caffeine levels, with a decrease observed from the spring harvest to the summer, with a shift to the rainy and autumn seasons (Ahmed *et al.,* 2014; Ansari *et al.,* 2011).

TF, TR & TB content

The fermentation process in tea production facilitates the enzymatic oxidation of the tea leaves, where the flavan-3-ols are polymerized into bis flavanols catalyzed by polyphenol oxidase. The resultant products of this transformation are the primary black tea pigments, known as theaflavins and thearubigins (De and Ray, 2022). TFs and TRs contribute to the sweet aroma of malt sugar and the dark brown color of black tea. Apart from caffeine, TF, TR, and TB are crucial in determining the flavor profile of black tea. The final processed tea from the HA garden shows the highest percentage of TF in the first flush at 1.1%, followed by 0.77% in the second flush and 0.74% in the third flush. In contrast, the LA garden shows 0.43, 0.220, and 0.226% in

the spring, summer, and autumn flushes, respectively, illustrated in Fig.7.a. In both gardens, the percentage of TF decreases with each subsequent flush.

Similarly, for TR, the final processed tea from the HA garden shows the highest amount of TR in the first flush, followed by the second and third flushes, with percentages of 14.14, 12.39, and 9.30%, respectively (Fig. 7b). In the LA garden, the TR percentages are 1.97, 1.37, and 3.19% for the spring, summer, and autumn flushes, respectively. The TB content is highest in the second flush of the final processed tea from the HA garden, at 9.79% (Fig. 7c)

The oxidation process of black tea involves a sequence of enzymatic reactions between catechins. Initially, catechins interact with the benzotropolone ring, producing theaflavin and theasinensin. These catechin oxidation products are structurally unstable and prone to further oxidation, leading to the formation of additional oligomeric compounds such as thearubigin and theabrownin (Tan *et al.,* 2016). In our study, it was found that the content of TF, TR, and TB increased notably after fermentation, supporting the hypothesis that these compounds are formed in substantial amounts during oxidation. Additionally, an altitudinal variation was noted, with higher altitude gardens showing greater amounts of TFs, TRs, and TBs in all flushes, likely as a stress response of the plant.

Antioxidative potential

Five antioxidative parameters were considered for evaluating the antioxidant capacity of 36 samples across six processing stages, in three flushes, from two gardens at different altitudes. The final processed tea from the first flush of the HA garden exhibited the highest antioxidant capacity across all assessed

parameters. For TPC, the highest concentrations were found in the tea extracts of the processed tea and the tea samples of the withering stage (25.7 µg/g and 18.16 µg/g, respectively)> rolled leaves (17.59 µg/g)> dried leaves (17.18 µg/g)>green leaves (16.93 µg/g)>oxidized/fermented leaves (15.47 µg/g) (Fig. 8a). In contrast, the first flush of the LA garden showed a total phenol content of 5.71 µg/g in green leaves, 6.59 µg/g in rolled, 7.07 µg/g in fermented, 8.61 µg/g in withered, 11.53 µg/g in dried, and 15.17 µg/g in final processed tea samples.

For the other two flushes, the third flush of both gardens had higher TPC in the final processed tea (17.69 µg/g for HA and 11.13 µg/g for LA) compared to the second flush (7.44 µg/g for HA and 6.66 µg/g for LA), as shown in Fig. 8a.

Fresh and unfermented tea leaves primarily contain flavan-3-ols, phenolic acids, proanthocyanidins, C-glycosyl flavones, O-glycosylated flavonols, and their derivatives. Fermented (oxidized) tea contains theaflavins and thearubigins formed during the fermentation process (Kosińska and Andlauer, 2014). The manufacturing process of tea significantly alters the phenolic compound profile, individual compound content, and the antioxidant capacity of the final tea. Gallic acid and its quinic acid ester, theogallin, are noted as the most prevalent simple polyphenols in tea. The content of gallic acid increases dramatically during black tea production due to the oxidative degradation of phenolic esters (Kosińska and Andlauer, 2014)

Prolonged exposure to intensified UV-B radiation and low-temperature stress, particularly at high altitudes, can cause severe oxidative stress. However, the accumulation of phenolic compounds can effectively counteract this damage, thereby enhancing the plant's adaptive capabilities to withstand adverse conditions and thrive in challenging environments (Zeng et al., 2020; Harborne and Williams, 2000).

For TFC, the third flush of both gardens exhibited the highest TFC, followed by the first and second flushes. In the HA garden, the third flush had the highest TFC, with 4253.09 µg/g in the final processed tea, followed by 3706.3 µg/g in drying, 1887.92 µg/g in withering, 1493.71 µg/g in fermentation, 883.35 µg/g in fresh leaves, and 821.74 µg/g in rolling (Fig. 8b). The third flush of the LA garden showed 1608.15 µg/g TFC in the final processed tea. TFC in the final processed tea samples of the first and second

flushes were 1938.77, 1375.82, 1773.46, and 781.62 µg/g in the HA and LA gardens, respectively.

Flavonoids are crucial in the agricultural and industrial sectors due to their significant medicinal value (Zhou et al., 2021). Plants produce these secondary metabolites as a defense mechanism in response to various stress factors, including UV radiation, drought, and extreme temperatures, helping to protect plant tissues from potential harm (Sun et al., 2021; Walia et al., 2005). The study highlights the differential accumulation of flavonoids across different altitudes, suggesting that high-altitude ecotypes exhibit higher flavonoid levels than low-altitude ecotypes. This indicates a potential adaptive response to environmental conditions at higher altitudes, such as increased UV radiation and cold stress. Flavonoids play a fundamental role in absorbing UV-B rays, which can damage nucleic acids, proteins, and cell membranes. Gardens at higher altitudes are more susceptible to UV radiation due to reduced atmospheric filtration and various stressors like cold and drought. Research has shown that prolonged exposure to light, especially under low UV-B conditions and dry spells, can stimulate flavonoid biosynthesis in tea (Bhattacharya and Sen, 2011). This is an adaptive response to enhance plant tolerance against various stressors.

DPPH is an organic stable free radical frequently utilized for assessing the ROS scavenging potential of antioxidants. The final processed tea from the HA garden exhibited the highest DPPH radical scavenging activity at 94.09%, followed by the final processed tea from the third flush of the LA garden at 83.52%. The first flush final processed tea from the LA garden showed an inhibition of 82.24%, while the third flush final processed tea from the HA garden demonstrated a scavenging activity of 78.65% (Fig. 9a). Both gardens showed a decrease in scavenging activity in the second flush samples, which aligns with the trends observed in other parameters.

The hydroxyl radical, a type of ROS generated through the Fenton reaction, was also assessed. The first flush samples of the HA garden showed the highest activity, with 83.6, 85.93, 86.48, 94.5, and 95.94% in fresh, withered, rolled, dried, and final processed samples, respectively, except for fermentation at 37.65%. The second and third flush samples of the HA garden
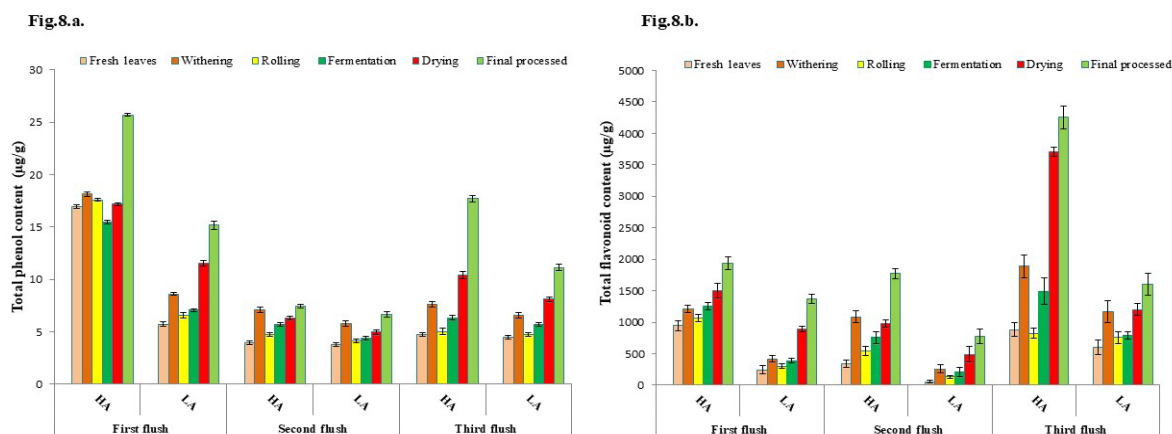
Fig.8.a.

Fig.8.b.

Fig. 8 a-b: represents TPC and TFC of the black tea samples of HA & LA garden through the flushes

exhibited scavenging activities of 95.48 and 84.98% in the final processed tea, respectively (Fig. 9b). The lower altitude garden showed the highest scavenging activity in the third flush samples, with 42.78% (fresh leaves), 56.84% (withering), 47.53% (rolling), 52.76% (fermentation), 67.98% (drying), and 77.26% (final processed), followed by the first and second flush sample. Coming to reducing power assay, the antioxidants from the sample extract react with Fe^{3+} to form Fe^{2+} which is the ferrocyanide form which in turn reacts with $FeCl_3$ to form Fe^{3+}/ Fe^{2+} complex that gives absorbance at 700 nm. By analyzing the absorption values at 700 nm, the degree of reduction is ascertained (Fig. 9.c.). The final processed tea infusions of all flushes from both gardens exhibited the strongest reducing power activity compared to other processing stages. Regarding altitude, a similar pattern was observed: the first flush tea samples from the HA garden displayed the strongest reducing power compared to those from the LA garden. For the LA garden, the third flush samples had the strongest reducing power, followed by the first and second flushes. In the HA garden, the third flush samples showed greater reducing power than the second flush samples.

The study revealed that the TPC, TFC, and antioxidant potential (measured through DPPH, H_2O_2, and reducing power assays) of tea samples significantly increased with both altitude and the various stages of processing. These findings indicate that higher altitudes enhance the accumulation and improvement of phenolic, flavonoid, and alkaloid compounds, as well as the antioxidant activity in *C. sinensis var. sinensis*.

Soil analysis

The Tea Board of India recommends that the pH of tea plantations should be within the range of 4.5 to 5.5. The soil organic carbon, a quantifiable element of soil organic matter, consists of products derived from decayed plant and animal tissues as well as non-mineral substances present in the soil. According to Tea Board standards, soil with 2% organic carbon is recommended, while levels below 1% are considered low. The recommended nitrogen content is between 0.1 to 2.0%. Soil samples from both gardens were collected during two periods, pre-monsoon and post-monsoon, to better understand the relationship between soil and phyto constituents. A range of soil factors like pH, electrical conductivity (EC), organic carbon (OC), nitrogen (N), potash (K), sulfur (S), and phosphorus (P), were analyzed at different study sites of *C. sinensis var. sinensis*.

The pH of the soil samples obtained from the study areas (HA & LA) in both pre-monsoons is 4.80 (top), 4.90 (sub) & 4.88 (top), 5.00 (sub) and 4.59 (top), 4.63 (sub) & 4.62 (top), 4.73 (sub) in post-monsoon (Table-1) respectively which is within the recommended range (4.5–5.5). The organic carbon of the study area (HA & LA) falls within the recommended range i.e., 2.309 & 1.680% in pre-monsoon whereas 3.259 & 3.209% (Table-1) in post-monsoon. In both cases, the higher altitude garden shows richer carbon content. The nitrogen content of the study area (HA & LA) as estimated in both top (0.184, 0.156%) and sub (0.129, 0.120%) in pre-monsoon & top (0.265, 0.288%) and sub (0.217, 0.267) (Table-1) in post monsoon was within the recommended range (0.1–2.0%) but is higher in the garden at HA in pre-monsoon and post-monsoon it is higher in the LA garden. The potassium, phosphorus, and sulfur status of the soil samples indicated that the lower-altitude garden was richer in phosphorus and sulfur compared to the higher-altitude garden (Table 1)

Various soil elements alongside pH are effective in the synthesis of various phytochemicals. Studies have underscored the influence of soil and nutrient profiles on the synthesis of flavan-3-ols and phenolics (Ruan *et al.,* 2013; Ruan *et al.,* 2010; Ruan *et al.,* 2007a; Hu *et al.,* 2003), the internal defense mechanisms (Yang *et al.,* 2014; Mukhopadhyay *et al.,* 2012), and caffeine (Jayaganesh
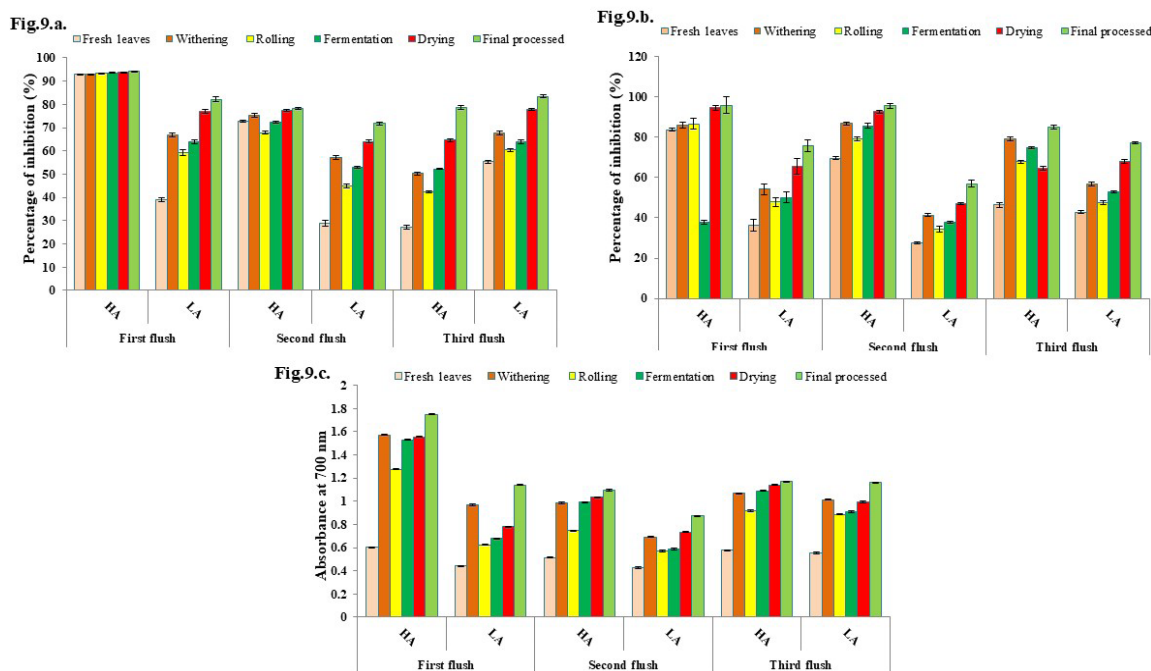

Fig.9.a.

Fig.9.b.

Fig.9.c.

Fig. 9a-c: represents DPPH radical scavenging activity, Hydroxyl radical scavenging activity, and reducing power assay respectively of the processing stages of HA & LA garden through the flushes

Table 1: Various soil parameters estimated in both top and subsoil in both the gardens in pre-monsoon & post-monsoon period

HA Garden (1247-1500mts)			pH	Electrical Conductance (μS/cm)	Organic carbon (%)	Total Nitrogen (%)	K_2O (PPM)	S (PPM)	P_2O_3 (PPM)
	Pre-monsoon soil	Topsoil	4.8	0054	2.309	0.184	136	77	24
		Subsoil	4.9	0044	1.616	0.129	170	63	18
	Post- monsoon soil	Topsoil	4.59	0311	3.259	0.265	246	42	34
		Subsoil	4.63	0239	2.61	0.217	189	55	26
LA Garden (664-1094 mts)	Pre-monsoon soil	Topsoil	4.88	0078	1.680	0.156	177	79	28
		Subsoil	5.00	0073	1.252	0.120	194	93	21
	Post- monsoon soil	Topsoil	4.62	0243	3.209	0.288	184	88	26
		Subsoil	4.73	0192	3.202	0.267	145	65	35

	Reducing Sugar	Caffeine	TF	TR	TB	TPC	TFC	DPPH	H2O2	Reducing Power	pH	EC	Carbon	Nitrogen	Potash	Sulphur	Phosphorus
Reducing Sugar		0.40443	0.18406	0.28806	0.0031425	0.33882	0.97066	0.94123	0.43891	0.61586	0.19463	0.068138	0.2158	0.20457	0.0051891	0.01514	0.18955
Caffeine	0.20935		0.043823	0.21607	0.93367	8.97E-07	0.82655	0.001229	0.63101	0.00012055	0.76056	0.69742	0.91196	0.97046	0.81012	0.64483	0.84204
TF	-0.3279	0.47996		5.21E-06	0.0020477	0.039674	0.021153	0.28749	0.37906	6.56E-05	0.44771	0.30236	0.68323	0.63733	0.28996	0.17943	0.70603
TR	-0.26492	0.3065	0.85823		0.020129	0.21383	0.044136	0.26652	0.3888	0.0097274	0.46354	0.43959	0.76962	0.71249	0.63854	0.49545	0.84859
TB	-0.65553	0.021131	0.67656	0.54208		0.97073	0.77962	0.88323	0.34192	0.066801	0.5104	0.29409	0.59728	0.57695	0.091975	0.054092	0.55798
TPC	0.23933	0.88757	0.48853	0.30793	-0.009318		0.12728	0.000598	0.26229	0.00062776	0.4821	0.65953	0.69631	0.65627	0.89948	0.98754	0.81285
TFC	0.0093396	0.055601	0.53843	0.47934	0.070966	0.37309		0.81455	0.75271	0.15659	0.98138	0.84029	0.72186	0.77369	0.79344	0.63404	0.68618
DPPH	0.018721	0.69967	0.26522	0.2766	0.037285	0.72903	0.05951		0.06156	0.014398	0.043944	0.060444	0.19249	0.15902	0.29276	0.24674	0.24572
H2O2	-0.19466	0.12151	0.2206	0.21623	0.23784	0.27896	0.079878	0.44907		0.31677	0.55938	0.64694	0.85615	0.8021	0.89004	0.85303	0.96624
Reducing Power	-0.12689	0.78334	0.80069	0.59146	0.44124	0.72717	0.34835	0.56575	0.25014		0.88682	0.68163	0.87768	0.9202	0.52231	0.39538	0.8773
pH	0.80537	-0.23944	-0.55229	-0.53646	-0.4896	0.5179	-0.01863	-0.95606	0.44062	-0.11318		0.037331	0.055599	0.037796	0.25715	0.26175	0.09088
EC	-0.93186	0.30258	0.69764	0.56041	0.70591	-0.34047	0.15971	0.93956	-0.35306	0.31837	-0.96267		0.099689	0.0806	0.10973	0.10673	0.11337
Carbon	-0.7842	-0.088043	0.31677	0.23038	0.40272	-0.30369	-0.27814	0.80751	-0.14385	-0.12232	-0.9444	0.90031		0.0017544	0.2634	0.32439	0.0072323
Nitrogen	-0.79543	-0.029543	0.36267	0.28751	0.42305	-0.34373	-0.22631	0.84098	-0.1979	-0.079801	-0.9622	0.9194	0.99825		0.25514	0.30646	0.013962
Potash	-0.99481	0.18988	0.71004	0.36146	0.90803	0.10052	0.20656	0.70724	-0.00996	0.47769	-0.74285	0.89027	0.7366	0.74486		0.014903	0.22615
Sulphur	0.98486	-0.35517	-0.82057	-0.50455	-0.94561	-0.012458	-0.36696	-0.75326	0.14697	-0.60462	0.73825	-0.89327	-0.67561	-0.69354	-0.9851		0.29978
Phosphorus	-0.81045	-0.15796	0.29397	0.15141	0.44202	-0.18715	-0.31382	0.75428	-0.033757	-0.1227	-0.90912	0.88663	0.99277	0.98604	0.77385	-0.70022	

Fig. 10a: Heat map of Pearson's correlation coefficients for the high-altitude (HA) garden, illustrating the relationships between various soil parameters, physicochemical properties, and antioxidant activities of Darjeeling tea. The results, which are replicates of three samples ± SD, are color-coded to represent correlations from positive (+1) to negative (-1), with green signifying positive and red signifying negative correlations

et al., 2011). Lower pH levels facilitate aluminum accumulation, stimulating tea plant growth (Duan *et al.,* 2012; Chen *et al.,* 2011) and enhancing concentrations of phytochemicals linked with the production of greater-quality tea (Lin *et al.,* 2012) Research by Duan *et al.,* 2012 and Sae-Lee *et al.,* 2012 revealed that increased aluminum levels correlate with elevated caffeine, amino acids, and polyphenol content in tea leaves. So, based on the previous works it can be said that the greater antioxidant potential and higher number of phytochemicals in the samples of the HA garden is not only an effect of various stress factors but also an effect of soil parameters that promote tea plant growth of and positively influence the synthesis of certain essential compounds.

Relation among soil parameters, cup characteristics, and antioxidant activity of tea

The study analyzed soil factors like pH, EC, OC, total nitrogen, potash, sulfur, and phosphorus across different sites of *C. sinensis var. sinensis*. Their relationship with reducing sugar, caffeine, theaflavins (TF), thearubigins (TR), theabrownins (TB), total phenols, total flavonoids, DPPH, H_2O_2, and reducing power was tested using Pearson's correlation coefficient and illustrated as heat maps (Fig. 10.a-b.).

In the high-altitude (HA) garden, a correlation was observed between soil pH and TFC (+0.98), reducing power (+0.88), and caffeine (+0.76) (Fig.10. a.). In the low-altitude (LA) garden, a correlation was observed between soil pH and TB (+0.99), TF (+0.96), caffeine (+0.86), DPPH (+0.94), and H_2O_2 (+0.94) (Fig.10b).

Soil organic carbon also demonstrated a favorable correlation with caffeine (+0.91), TR (+0.76), TFC (+0.72), H_2O_2 (+0.85), and reducing power (+0.87) in the HA garden (Fig.10. a.). In the LA garden, it showed strong positive correlations with TF (+0.93), TR (+0.89), TB (+0.82), TFC (+0.95), and H_2O_2 (+0.88) (Fig.10b)

Soil nitrogen, potash, sulfur, and phosphorus positively correlated with the physicochemical properties and antioxidant activity in the LA garden. In the HA garden, nitrogen, phosphorus, and potash positively influenced tea quality. Overall, the correlation coefficients suggest that soil organic carbon, nitrogen, phosphorus, potash, and sulfur positively affect caffeine content, total flavonoids, and antioxidant parameters, indicating that increased levels of these soil parameters enhance the synthesis of secondary metabolites. Conversely, pH, EC, carbon, and nitrogen negatively correlated with reducing sugar content in both gardens, suggesting these parameters negatively impact the synthesis of reducing sugar.

	Reducing Sugar	Caffeine	TF	TR	TB	TPC	TFC	DPPH	H2O2	Reducing Power	pH	EC	Carbon	Nitrogen	Potash	Sulphur	Phosphorus
Reducing Sugar		0.12459	0.12433	0.15299	0.0045381	0.58515	0.60013	0.10061	0.28449	0.19553	0.34958	0.16702	0.2665	0.26132	0.80328	0.98321	0.85282
Caffeine	-0.37555		0.2141	0.12709	0.012706	0.0016454	0.1347	0.017874	0.017359	0.024441	0.86333	0.63218	0.61611	0.67202	0.58935	0.74726	0.75034
TF	-0.37579	0.30775		4.63E-09	0.077667	0.0061721	0.0001979	0.0002312	5.26E-05	0.0014472	0.9638	0.76679	0.93427	0.93058	0.92777	0.56401	0.58869
TR	-0.3512	0.37326	0.94309		0.019844	0.0011337	2.78E-05	2.92E-05	2.24E-06	5.87E-05	0.70225	0.9704	0.89761	0.86227	0.93051	0.79408	0.7412
TB	-0.63616	0.57417	0.42636	0.54312		0.056332	0.035907	0.0032067	0.012886	0.00027264	0.99519	0.7234	0.82547	0.84683	0.98087	0.81133	0.82101
TPC	-0.13795	0.6867	0.6189	0.70313	0.45738		0.0008826	0.0002054	2.92E-06	0.0014027	0.52599	0.7842	0.67743	0.65519	0.71201	0.59983	0.5284
TFC	-0.13253	0.36648	0.76797	0.82261	0.49693	0.7136		9.02E-06	5.29E-08	1.31E-06	0.71992	0.97651	0.95261	0.90136	0.90326	0.96562	0.90051
DPPH	-0.39937	0.55066	0.7629	0.82142	0.6545	0.76676	0.84747		6.97E-10	1.39E-06	0.94523	0.6769	0.76785	0.79209	0.95975	0.86774	0.88277
H2O2	-0.26682	0.55274	0.8066	0.87321	0.57323	0.86869	0.92218	0.95529		6.14E-07	0.9444	0.78168	0.88657	0.90804	0.94386	0.77917	0.779
Reducing Power	-0.31996	0.52757	0.69248	0.80366	0.75738	0.69387	0.8819	0.88089	0.893		0.55258	0.81294	0.6904	0.67402	0.67229	0.55608	0.49441
pH	0.65042	-0.13667	0.036196	0.29775	-0.00481	0.47401	0.28008	-0.054773	0.055597	0.44742		0.037939	0.045275	0.022126	0.58113	0.65962	0.47824
EC	-0.83298	0.36782	0.23321	-0.029602	0.2766	-0.2158	-0.023495	0.3231	0.21832	-0.18706	-0.96206		0.040569	0.022038	0.62244	0.74425	0.56546
Carbon	-0.7335	0.38389	0.065731	-0.10239	0.17453	-0.047393	0.23215	0.11343	-0.3096	-0.95472	-0.95943	0.95943		0.005184	0.38202	0.48253	0.32917
Nitrogen	-0.73868	0.32798	0.069421	-0.13773	0.15317	-0.34481	-0.098641	0.20791	0.091958	-0.32598	-0.97787	0.97796	0.99482		0.45975	0.55971	0.39389
Potash	0.19672	-0.41065	0.27223	0.069493	0.019127	0.28799	-0.09674	-0.040248	0.056136	0.32771	0.41887	-0.37756	-0.61798	-0.54025		0.017475	0.020907
Sulphur	0.016789	-0.25274	0.43599	0.20592	0.18867	0.40017	0.034377	0.13226	0.22083	0.44392	0.34038	-0.25575	-0.51747	-0.44029	0.98252		0.020298
Phosphorus	-0.14718	0.24966	-0.41131	-0.2588	-0.17899	-0.4716	-0.09949	-0.11723	-0.221	-0.50559	-0.52176	0.43454	0.67083	0.60611	-0.97909	-0.9797	

Fig. 10b: Heat map of Pearson's correlation coefficients for the low-altitude (LA) garden, illustrating the relationships between various soil parameters, physicochemical properties, and antioxidant activities of Darjeeling tea. The results, which are replicates of three samples ± SD, are color-coded to represent correlations from positive (+1) to negative (-1), with green signifying positive and red signifying negative correlations.

Conclusion

In conclusion, this study aimed to provide an in-depth investigation into the key aspects of Darjeeling tea, focusing specifically on its physical properties, metabolite composition, and antioxidant activity. The research was conducted across three distinct seasons—spring, summer, and autumn—allowing for a comprehensive understanding of how seasonal variations affect the tea's characteristics. Additionally, two different altitudes were considered—lower and higher elevations—recognizing the potential influence of altitude on tea quality and composition.

The study focused on the identification of several major components within the tea, which are critical for assessing its overall value and health benefits. The HPTLC method was employed, a sophisticated analytical technique that enabled the precise detection of targeted metabolites in the black tea samples. This method confirmed the presence of these metabolites, contributing valuable data to the existing body of knowledge on Darjeeling tea and its unique properties.

The study primarily also focused on examining how *C. sinensis var. sinensis* responds to variations in altitude, as well as seasonal changes. It compared the characteristics of tea harvested during the first flush in spring with those from the Second Flush, which occurs between summer and the rainy season, and the Third Flush, harvested during autumn. The findings revealed a seasonal decline in metabolite concentrations and antioxidant activity as the harvesting progressed from the First Flush.

Interestingly, higher altitudes were associated with increased levels of key bioactive compounds, including phenolic compounds, flavonoids, caffeine, theaflavins (TF), and thearubigins (TR). Additionally, antioxidant capacity was significantly enhanced at elevated altitudes. These changes are likely influenced by environmental stressors commonly found at higher elevations, such as increased UV radiation, lower temperatures, and reduced water availability, which may stimulate the production of these beneficial compounds.

The comparative analysis between the two tea gardens, located at different altitudes and evaluated across various seasons, demonstrated that the garden situated at a higher altitude exhibited superior antioxidant potential and a greater concentration of phytonutrients. Among the seasonal harvests, the First Flush, conducted in the spring, consistently produced the highest-quality tea, characterized by its enhanced biochemical profile and superior antioxidant properties.

Author Contributions

Ms. Dwaipee De conducted sample collection, sample preparation, method validation, biochemical assays, and experimental analysis of the samples and also wrote the original draft. Ms. Gouhar Jahan Ashraf and Dr. Ranabir Sahu performed and analyzed HPTLC reports. Dr. Sonali Ray conceptualized and designed the research study, and contributed to drafting the article. All authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no potential conflicts of interest related to their work.

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