



***In vitro* validation of the antioxidant potential of tea infusions and study of their simulated gastrointestinal digestion**

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Abstract. Tea is a popular non-alcoholic beverage that managed to secure second position in terms of global consumption. Having tea has become part of the daily routine in Indian households, where CTC tea, orthodox tea, green tea, and CTC tea infused with additives are generally preferred. Current research focuses on the total phenol and total flavonoid estimation of each infused tea cup, followed by the evaluation of *in vitro* antioxidant activities (DPPH, FRP, and lipid peroxidation inhibition). The highest total phenol content (2.077 ± 0.060 mg GAE/mL) and total flavonoid content (35.54 ± 0.643 µg RE/mL) were revealed by green tea and CTC tea respectively. All the studied tea infusions showed more than 80% of DPPH scavenging power. However, CTC tea infused with lemon, ginger, and black pepper reported higher lipid peroxidation inhibition potential, which corresponded to the total phenol content of tea infusions. Further, an *in vitro* gastrointestinal digestion assay was conducted to evaluate the digestibility of each cup, which revealed a significant decrease in the total phenol, total flavonoid, and DPPH scavenging activity of tea infusions along the gastrointestinal tract, confirming the bioaccessibility of the bioactive compounds present in tea samples. Moreover, the infusions of green tea, Broken Pekoe, and CTC tea infused with lemon were the most effective ones.

Keywords and phrases: total phenol content, green tea, bioactive compounds, antioxidant, gastrointestinal digestion

1. Introduction

Globally, tea is a much-appreciated non-alcoholic beverage made from the young leaves of *Camellia sinensis* (L.) O. Kuntze (family *Theaceae*). Over 30 countries worldwide cultivate tea on a commercial scale, with estimated figures of around 78%, 20%, and 2% for black tea, green tea, and oolong tea respectively (Abudurehman *et al.*, 2022). A large proportion of the population in India and other parts of the world habitually drinks tea, attracted by its characteristic aroma, taste, and flavour. Volatile compounds such as terpenes, aldehydes, alcohols, and esters are major contributors to the unique aroma of tea (Zhai *et al.*, 2022), with polyphenols (phenolics and flavonoids), alkaloids, tannins, coumarin, terpenoids, etc. being its key components. These compounds are known to have numerous health benefits, including antioxidant, anti-inflammatory, antidiabetic, anticancer, and cardioprotective properties (Cabrera *et al.*, 2006; Tang *et al.*, 2019). Tea drinkers are particularly interested in the anticipated “antioxidant” property of different tea types because of the perceived health benefits associated with habitual consumption. Therefore, “tea” has gained popularity in both national and international markets due to its associated health benefits (Huda *et al.*, 2024).

In tea, polyphenols alone constitute 30-35% of the dry weight of tea leaves (Graham, 1992). Besides caffeine, amino acids, vitamins (vitamin C, vitamin B-complex), and minerals such as potassium, magnesium, fluoride, and manganese are also minor contributors to the biochemical profile of tea (Yamamoto *et al.*, 1997). Consequently, to determine the sensory and market value of tea, it is important to know the complete biochemical profile of manufactured tea. Based on the degree of fermentation of tea leaves during the manufacturing process, three types of tea are recognized; black, green, and oolong tea (Salman *et al.*, 2022). Fermentation usually allows enzymatic oxidation of the cellular components of tea leaves. During the fermentation of catechins, the major tea flavonoids are converted to theaflavins and thearubigins (Abudurehman *et al.*, 2022), which are characteristic of black tea. Theaflavins and thearubigins are well known to have antioxidant properties, and reports suggest that the regular consumption of black tea can significantly reduce LDL cholesterol and support cardiovascular health (Vermeer *et al.*, 2008; Imran *et al.*, 2018). On the contrary, partial to no oxidation of tea leaves occurs in oolong tea and green tea. During green tea processing, catechins remain intact, and some common catechins present in green tea are epigallocatechin gallate (EGCG), epicatechin (EC), epigallocatechin (EGC), and epicatechin gallate (ECG).

Green tea promotes human health in multiple ways because of its antioxidant, anti-inflammatory, cardioprotective, antidiabetic, and anticarcinogenic properties (Zhao, 2003; Ohishi et al., 2016; Radeva-Ilieva et al., 2025).

Apart from being a healthy drink, tea is deeply integrated into Indian culture. It serves as a symbol of warmth and hospitality in every social gathering. Every Indian starts their day with a cup of tea in the morning. Emerging from vast cultural and regional differences, people in our country have adopted different tea drinking habits over the past. Common black teas popular in Indian households are orthodox tea and crush-tear-curl (CTC) tea, which are brewed in water and consumed with milk and sugar. In addition, some may add spices like cardamom, ginger, lemon, black pepper, cinnamon, and cloves, resulting in the beverage that has come to be known as “Masala chai”. On the other hand, green tea is prepared by simply steeping the tea leaves in hot water for 2 to 3 min at a few degrees below boiling temperature. In CTC tea, the spices are generally added as flavouring agents. However, CTC tea with added spices has been found to have certain health benefits (Ochanda et al., 2015; Sunarharum et al., 2022). Therefore, the current study was designed to carry out a comparative evaluation of antioxidant properties of some common tea types and tea grades. Furthermore, the bioaccessibility of antioxidant molecules of tea along the gastrointestinal (GI) tract was evaluated in post-gastric and post-intestinal phases of digestion to affirm their nutritional efficacy.

2. Materials and methods

Collection of tea samples

Tea samples used for this study were collected from the market and a tea factory. Three tea samples, i.e. green tea (GT), black orthodox tea (OT), and crush-tear-curl (CTC) tea, were purchased from a local tea shop in Shivmandir, Siliguri, West Bengal, 734011. Also, six freshly manufactured CTC tea grades: Broken Orange Pekoe (BOP), Broken Pekoe (BP), Broken Orange Pekoe Small (BOPSM), Orange Fannings (OF), Pekoe Dust (PD), and Dust (D) were collected from a tea factory (latitude 26°43′ 53″ and longitude 26°43′53″) in Hasimara, District Alipurduar, West Bengal, India. Lemon, ginger, and black pepper were purchased from a nearby market.

Preparation of tea infusion

While preparing tea infusion, maintaining an optimum temperature, infusion time, and tea-to-water ratio is critical. This defines the overall aroma and flavour of tea. It also allows the careful extraction of different phytochemicals responsible

for the overall health benefits of tea. In the present study, a mass of $m = 2.50$ g of tea samples was added to 100 mL of boiled, double-distilled water after allowing it to cool to 80-85 °C. Tea infusions prepared during this study are presented in *Table 1*. CTC tea was infused with two different concentrations of lemon, ginger, and black pepper, as shown in *Table 1*, for comparative evaluation with CTC tea, OT, GT, and different tea grades used in this research. All experiments were performed using freshly prepared tea infusions.

Table 1. Tea type/grade, sample codes, and proportions of different components used for tea infusions

Tea type/grade	Additive	Sample code	Infusion (100 mL)	
			Tea	Additive
Crush-tear-curl	–	CTCbg	2.5 g	–
Orthodox	–	OT	2.5 g	–
Green tea	–	GT	2.5 g	–
Crush-tear-curl	Lemon	CTCL.5	2.5 g	500 µL
Crush-tear-curl	Lemon	CTCL1	2.5 g	1,000 µL
Crush-tear-curl	Ginger	CTCG.5	2.5 g	500 mg
Crush-tear-curl	Ginger	CTCG1	2.5 g	1,000 mg
Crush-tear-curl	Black pepper	CTCBP.25	2.5 g	250 mg
Crush-tear-curl	Black pepper	CTCBP.5	2.5 g	500 mg
CTC-Broken Pekoe	–	BP	2.5 g	–
CTC-Broken Orange Pekoe	–	BOP	2.5 g	–
CTC-Broken Orange Pekoe Small	–	BOPSM	2.5 g	–
CTC-Orange Fannings	–	OF	2.5 g	–
CTC-Pekoe Dust	–	PD	2.5 g	–
CTC-Dust	–	D	2.5 g	–

Qualitative phytochemical analysis

For the qualitative analysis of phytochemicals, such as phenol, flavonoid, coumarin, tannin, cardiac glycoside, terpenoids, and steroid, protocols previously used by *Labar et al.* (2019) and *Subba et al.* (2024) were followed as given in *Table 2*.

Table 2. Procedures of the qualitative test of phytochemicals

Phytochemical	Procedure
Polyphenols	200 μL of Folin–Ciocâlteu reagent was introduced into 2 mL of tea infusion. Later, 2 mL 20% Na_2CO_3 was added to the reaction mixture. The formation of blue colour suggested the presence of phenol in the tested sample.
Flavonoids	In 1 mL of tea sample, 2-3 drops of FeCl_3 solution were added. The appearance of blue to green colour confirmed the presence of flavonoids.
Terpenoids	An initial 50 μL of the sample was evaporated, and the remaining was dissolved in chloroform. Then, conc. H_2SO_4 was carefully added from the sidewalls of the test tubes. The development of reddish to brown colouration at the base confirmed the presence of terpenoids.
Cardiac glycosides	0.5 mL of each tea infusion was transferred into a test tube and allowed to evaporate. After drying, 1 mL of glacial acetic acid was used to dissolve the sample. Then, 1 drop of 10% FeCl_3 solution was added, followed by 1 mL of conc. H_2SO_4 from the test tube walls. A brown-coloured ring formed at the interface, which indicated the presence of cardiac glycosides.
Coumarins	1 mL of the sample was taken, to which a few drops of NaOH solution were added. A yellow colour was formed at the end of the reaction that confirmed the presence of coumarins in the tested sample.
Tannins	Few drops of HNO_3 were added to 1 mL of each tea infusion. A red-to-yellow colour formation indicated the presence of tannins.
Steroids	2 mL of chloroform was introduced to the previously evaporated tea sample (0.5 mL). Then, 2 mL of conc. H_2SO_4 was carefully added to the test tube containing the dissolved sample. The reaction resulted in a red-coloured ring formation in the middle layer of the solution, confirming the presence of steroids.

Optical density (OD) and transmittance (%T)

Optical density (OD) and transmittance (%T) of tea samples were recorded after 5 and 90 minutes of infusion time using a UV-Vis spectrophotometer at $\lambda = 420$ nm (Das et al., 2020).

Total Phenol Content (TPC) analysis

The total phenol quantification methods used by Mamun et al. (2026) were followed with some modifications. In 200 μL of each infused tea extract, 2,800 μL of double-distilled water was added, followed by 500 μL of FC reagent. Then, 2 mL of 20% Na_2CO_3 was added, and the mixture was incubated at room temperature for 60 minutes. The absorbance was measured at $\lambda = 650$ nm by a

visible spectrophotometer. Standard curve ($y = 0.003x + 0.0256$; $R^2 = 0.9993$) was prepared using gallic acid. Results were recorded in three replicates and expressed in mg GAE/mL of sample (GAE: Gallic Acid Equivalent).

Total Flavonoid Content (TFC) analysis

Infusions of tea grades selected for this study were subjected to TFC analysis following the protocol of *Chakraborty et al.* (2021). To 250 μ L of each infused tea extract, 1.2 mL double-distilled water and 75 μ L of 5% NaNO_2 were added. The reaction mixture was kept at room temperature for 5 minutes. After that, 150 μ L of 10% AlCl_3 was added to it and kept for 6 min at room temperature. Later, 500 μ L of 1 mM NaOH and 275 μ L of double-distilled water were added to the mixture and allowed to incubate for 30 minutes. Then, the absorbance was noted at $\lambda = 510$ nm by a UV-Vis spectrophotometer. Rutin trihydrate was used to prepare the standard curve, $y = 0.0097x + 0.0053$; $R^2 = 0.9988$. Results were recorded in triplicate and expressed as μ g RE/mL (RE: Rutin Trihydrate Equivalent).

In vitro antioxidant assays

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay is the most common and easiest *in vitro* antioxidant assay to determine the radical scavenging power of unknown samples. In this study, procedures used by *Subba et al.* (2025) were followed. In a test tube, 300 μ L of the sample was taken, to which 3 mL of DPPH solution was added. The mixture was allowed to incubate in dark for 20 minutes. After incubation, the absorbance of the tested tea infusions was recorded in a UV-Vis spectrophotometer at $\lambda = 517$ nm. The results were expressed as a percentage of radical scavenging potential by using formula (1):

$$DPPH = 100 \cdot \frac{A_c - A_s}{A_c}, \quad (1)$$

where: DPPH – DPPH scavenging potential (%); A_c – absorbance of control (only DPPH); A_s – absorbance of the sample with DPPH solution. The absorbance of all samples was recorded after 5 and 90 minutes of infusion.

Ferric reducing power (FRP) assay

For determining the ferric reducing power of tea samples, the method described by *Ghosh et al.* (2020) was followed, which is based on the classical reducing

power assay of Oyaizu (1986). In 500 μL of phosphate buffer ($\text{pH} = 7.4$), 200 μL of sample was added, followed by 1% 500 μL potassium ferricyanide. The mixture was incubated for 20 minutes. Later, 500 μL of 10% tri-chloroacetic acid, 1.5 mL double-distilled water, and 300 μL of 0.1% FeCl_3 were added consecutively. Then, the absorbance of sample solutions was taken at $\lambda = 700 \text{ nm}$. Standard curve ($y = 0.0105x + 1.727$; $R^2 = 0.9897$) was obtained using ascorbic acid, and the results were expressed as $\mu\text{g AAE/mL}$ (AAE: Ascorbic Acid Equivalent) of the sample.

Lipid peroxidation inhibition potential (LPIP) assay

The protocol of Rahman et al. (2015) was followed to assess the *in vitro* lipid peroxidation inhibition potential of tea samples. Goat liver was used as a lipid source, which was crushed using a mortar pestle and homogenized in phosphate buffer. The homogenized liver solution was centrifuged at 12,000 rpm for 15 minutes at 4°C to obtain the liposome. Homogenization and filtration were carried out under low temperature conditions. Further, reactions were carried out with the supernatant obtained after centrifugation. In each test tube, 0.5 mL of supernatant was taken, to which 1 mL of 0.15 M KCl and 0.3 mL of tea infusions were added. The peroxidation was initiated by the addition of 300 μL of 0.5 mM FeCl_3 solution. Then, the mixture was incubated at 37°C for 30 minutes. Eventually, 2 mL of cold TCA-HCl-BHT solution was added to the reaction mixture and subjected to hot water bath at 90°C for 60 minutes. The reactants were allowed to cool under low-temperature conditions and were centrifuged at 3,000 rpm for 5 minutes, and then the intensity of pink-coloured complex was measured at $\lambda = 532 \text{ nm}$. The percentage of lipid peroxidation inhibition was calculated by using formula (2):

$$LPIP = 100 \cdot \frac{A_c - A_s}{A_c}, \quad (2)$$

where: LPIP – lipid peroxidation potential (%); A_c – absorbance of control (only the reagents); A_s – the absorbance of the sample.

In vitro Gastrointestinal Digestion (GID) Simulation

For evaluating the bioaccessibility of tea infusions, *in vitro* GID (gastrointestinal digestion) assay was performed following the protocol proposed by Brodtkorb et al. (2019) as “INFOGEST static *in vitro* simulation of gastrointestinal food digestion”. According to the protocol, digestion of tea infusions was simulated in three consecutive phases, i.e. oral phase (OP), gastric phase (GP), and intestinal phase (IP) of the gastrointestinal tract. To maintain all parameters, i.e. electrolytes,

enzymes, bile, dilution, pH, and time of digestion, standard conditions, as described by Minekus *et al.* (2014), were followed.

For the preparation of simulated fluids, i.e. salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), appropriate concentrations of enzymes, electrolyte stock solutions, and calcium dichloride (CaCl_2) were taken as per Shen *et al.* (2023) with slight modulations. The simulation procedure was initiated by oral phase digestion, diluting the tea infusions with SSF in 1:1 (vol/vol) ratio, to which 1,500 U/mL alpha-amylase (SRL, India) and 100 μL 0.3 M CaCl_2 were added. The mixture was allowed to incubate for 2 minutes, maintaining the pH at 7.0. The digested oral mixture (final OP solution) was then diluted with simulated gastric fluid (SGF) in 1:1 (vol/vol) ratio, to which 2,500 U/mL gastric enzyme pepsin (SRL, India) and 17.5 μL CaCl_2 (0.3 M) were introduced. Further, the mixture was incubated at pH 3.0 for 2 h. HCl was used to lower the pH. Later, the digested gastric mixture (final GP solution) was diluted in 1:1 (vol/vol) ratio with simulated intestinal fluid (SIF) containing 10 mM bile salt (SRL, India), where 2,000 U/mL of pancreatic enzyme (pancreatin) (SRL, India) and 100 μL of CaCl_2 (0.3 M) were added. This phase of digestion was incubated for 2 h after adjusting the pH to 6.0 (Calero *et al.*, 2014; Majumder *et al.*, 2024). Throughout the digestion process, the pH of each phase was monitored carefully, and an optimal temperature of 37 °C was maintained. After each phase of simulated digestion, infusion mixtures were collected to evaluate their DPPH scavenging assay, TPC, and TFC. Further, the bioaccessibility index was calculated using the following formula (3):

$$LPIP = 100 \cdot \frac{A_c - A_s}{A_c}, \quad (3)$$

where: BI – Bioaccessibility Index (%); A – the amount of compound (DPPH scavenging activity, TPC, and TFC) in the digested sample; B – the amount of compound (DPPH scavenging activity, TPC, and TFC) in the undigested (crude) sample.

Statistical analysis

All experiments were performed in three replicates, and the standard deviation (SD) of all data was calculated in MS Excel. Principal Component Analysis (PCA) was carried out to represent the data in a precise, reader-friendly form. In this study, TPC, TFC, DPPH at 5 minutes, FRP, and LPIP data were used for correlation analysis (package used: ggplot2, reshape, and corrplot) and Principal Component Analysis (PCA) (package used: tidyverse and factoextra) in R (version 4.5.1).

3. Results and discussion

Qualitative phytochemical analysis

The qualitative assessment of tea infusions for certain biologically active compounds, such as flavonoids, phenols, tannins, coumarins, cardiac glycosides, steroids, and terpenoids, was performed. The degree of colour development was observed after the addition of specific reagents, and the results were recorded in a numerical system as 0 (no reaction), 1 (low intensity), 2 (moderate intensity), and 3 (high intensity). The heat map (*Figure 1*) was generated as per the obtained results, which clearly showcased higher amounts of tested phytochemicals in GT, OT, BP, and CTGbg compared to others. Among the six tea grades, BP stands out in the qualitative analysis, with high flavonoid, glycoside, and terpenoid contents. Tea infusions with additives produced moderate results. Polyphenols, flavonoids, coumarins, steroids, and terpenoids are some of the promising phytochemicals with great health benefits. Therefore, studies indicate that GT, OT, CTC, and BP are some of the superior tea samples. Nevertheless, all tea samples are also considered for subsequent analysis.

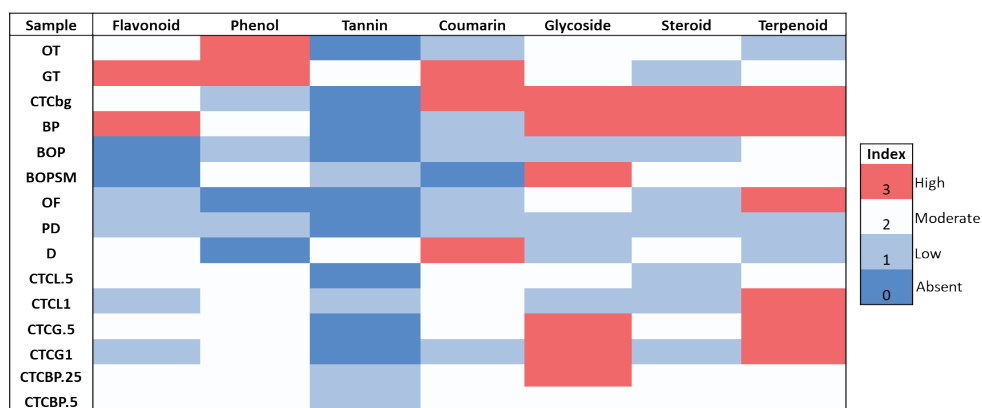


Figure 1. Heat map of qualitative tests for the studied tea infusions [(Red–White–Sky blue–Deep blue) = (High–Moderate–Low–Absent)]

Optical density (OD) and transmittance (%T)

Optical parameters such as OD and % T values are primarily considered for the qualitative assessment of tea. Tea infusion has a characteristic yellow to brown colour, which shows maximum absorbance at $\lambda = 420$ nm, making it an ideal wavelength for assessing tea colour and quality. Infusion time is another important

factor while preparing tea, as it greatly influences the quality of tea. Typical tea infusion times range from 2 to 5 minutes. However, in this experiment, we have intentionally included a 90-minute extraction to obtain the maximum amounts of phytochemicals from the sample under exhaustive extraction conditions. By comparing 5 and 90 minutes, it is understood how extraction changes over time and affects the chemical composition and biological activity of tea. Thus, the 5-minute infusion represents normal tea drinking conditions, while the 90-minute infusion shows the extended procedure adopted by some local tea consumers. Recorded “OD” and “% T” are presented in *Table 3*. OD values of tea infusions ranged from 1.42 (CTCBP.25) to 0.04 (GT) and from 2.007 (CTCBP.5) to 0.543 (GT) in 5 and 90 minutes respectively. All infusions revealed greater OD values when extending infusion time from 5 to 90 minutes (*Table 3*). This finding suggests the enhanced release of biomolecules during prolonged infusion hours, resulting in a darker appearance of the liquid. In a previous study, Takeo (1974) suggested that higher OD values were due to the presence of antioxidant molecules such as theaflavins, thearubigins, and other polyphenols dissolved in tea infusions. In addition, the rise in OD value of CTC tea with additives, such as lemon, ginger, and black pepper, when compared to CTCbg (without additives), indicated that the use of additives significantly enhances the amounts of dissolved tea components, resulting in improved cup quality. The “% T” value measures the amount of light passing through the infusion and allows for an estimation of the concentration of dissolved components in tea infusions. Almost all samples (*Table 3*) showed a sharp decrease in % T values from 5 to 90 minutes, indicating the rapid release of more biomolecules from the tea samples to the solution over time. Further, samples such as OT (3.1 to 0.4), BP (4.02 to 0.4), and CTCBP.5 (3.9 to 0.7) showed only minor variations in transmittance, suggesting limited extraction of essential biomolecules with increasing infusion time.

Table 3. Optical density (OD) and transmittance (% T) of tea infusions at 5 and 90 minutes

Sample	OD (5 minutes)	OD (90 minutes)	% T (5 minutes)	% T (90 minutes)
OT	0.153	0.464	3.1	0.4
GT	0.04	0.453	35.4	9.7
CTCbg	0.495	0.528	22.4	1.4
BP	0.98	1.285	4.02	0.4
BOP	0.646	0.975	86.3	13.2
BOPSM	0.836	0.941	57.2	21.4
OF	0.345	1.448	73.2	3.4

Sample	OD (5 minutes)	OD (90 minutes)	% T (5 minutes)	% T (90 minutes)
PD	0.386	1.137	57.1	7.3
D	0.627	1.645	88.3	2.3
CTCL.5	1.089	1.162	31.7	7.8
CTCL1	0.912	1.093	49.4	11
CTCG.5	1.113	1.234	29.7	6
CTCG1	1.232	1.391	22.3	4.6
CTCBP.25	1.42	1.568	14.9	1.6
CTCBP.5	1.149	2.007	3.9	0.7

Total Phenol Content (TPC)

Phenols are secondary metabolites naturally produced by some plants during various biotic and abiotic stresses. These molecules are potent antioxidants and are able to scavenge cell-damaging free radicals from the cellular system (Tuladhar et al., 2021). Other bioactivities such as antimicrobial, anti-inflammatory, and antiproliferative properties are also reported (Albuquerque et al., 2021) for phenols, which has attracted considerable attention from researchers. Tea is a preferred beverage among Indians as well as worldwide because of its refreshing aroma and taste. Besides, its high antioxidant content makes it a healthy choice. Since polyphenols are the major phytoconstituents in tea, their total phenol quantification can be a key characteristic for determining tea quality. Results for TPC in the studied tea infusions are graphically represented in Figure 2a, according to which GT ranked the highest (2.077 ± 0.060 mg GAE/mL), followed by OT (1.323 ± 0.081 mg GAE/mL). OF ranked the lowest in the row, with 0.099 ± 0.023 mg GAE/mL of total phenol. Interestingly, all CTCs with additives (CTCL.5, CTCL1, CTCG.5, CTCG1, CTCBP.25, CTCBP.5) revealed greater TPC than CTC tea without additives (CTCbg), as shown in Figure 2a. An increase in TPC was observed with increasing additive concentrations, except for lemon-added infusion. Different tea grades revealed lower TPC than the tea types considered for this study. Similar results were obtained in a study conducted by Das et al. (2020).

Total Flavonoid Content (TFC)

Similar to phenols, flavonoids (polyphenolic compounds) are naturally present in tea and are associated with antioxidant properties that may help reduce cardiovascular and inflammatory disorders, and potentially lower cancer risks (García-Lafuente et al., 2009). Bitterness and astringency, the primary sensory properties of tea, are mainly determined by phenols and flavonoids (Soares et

al., 2017). In current TFC analysis, varying results were obtained for the studied tea samples, as depicted in *Figure 2b*. Among all the samples, CTCbg yielded the highest values, i.e. $35.54 \pm 0.643 \mu\text{g RE/mL}$, and BOP yielded the lowest, i.e. $1.93 \pm 0.403 \mu\text{g RE/mL}$ of TFC. The decreasing order of total flavonoids among all infusions were as follows: CTCbg ($35.54 \pm 0.643 \mu\text{g RE/mL}$) > CTCG.5 ($33.37 \pm 0.847 \mu\text{g RE/mL}$) > CTCL.5 ($26.46 \pm 0.960 \mu\text{g RE/mL}$) > CTCBP.5 ($25.74 \pm 0.886 \mu\text{g RE/mL}$) > CTCL1 ($20.48 \pm 0.921 \mu\text{g RE/mL}$) > CTCBP.25 ($15.64 \pm 0.846 \mu\text{g RE/mL}$) > GT ($15.02 \pm 0.887 \mu\text{g RE/mL}$) > OT ($13.89 \pm 0.618 \mu\text{g RE/mL}$) > BP ($13.89 \pm 0.929 \mu\text{g RE/mL}$) > D ($9.45 \pm 0.525 \mu\text{g RE/mL}$) > CTCG1 ($7.08 \pm 0.627 \mu\text{g RE/mL}$) > OF ($4.61 \pm 0.832 \mu\text{g RE/mL}$) > PD ($3.37 \pm 0.355 \mu\text{g RE/mL}$) > BOPSM ($2.65 \pm 0.744 \mu\text{g RE/mL}$) > BOP ($1.93 \pm 0.403 \mu\text{g RE/mL}$). Unlike TPC results, CTC with additives revealed lower TFC than CTCbg, demonstrating that additives have no impact on the flavonoid content of tea infusions. This finding was in alliance with previous findings of *Uduwana et al.* (2023), where the TFC content of green tea was reduced upon the addition of honey and lemon.

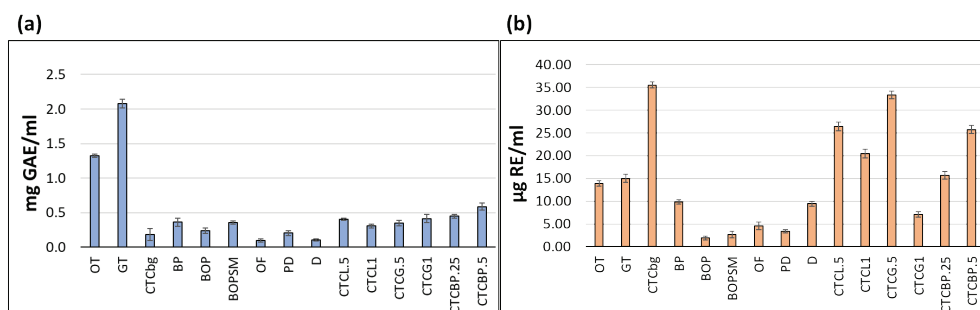


Figure 2. Quantitative analysis: (a) Total phenol content and (b) Total flavonoid content

In vitro assays for antioxidant property

Three simple and effective *in vitro* experiments, namely DPPH scavenging activity assay, FRP assay, and lipid peroxidation inhibition assay, were performed with the aim of analysing the antioxidant properties of the studied tea samples. DPPH scavenging assay measures the free radical scavenging power in samples. Results obtained from this study suggested higher DPPH scavenging power in tea infusions with 90 minutes than with 5 minutes of infusion time [*Figure 3a*]. This provides a clear understanding that radical scavenging potentials of tea infusions can be significantly increased upon extended infusion hours, possibly due to the release of more antioxidant molecules. The highest values were yielded by CTCL1 ($89.17 \pm 0.75\%$), followed by CTCL.5 ($89.03 \pm 0.65\%$) at 5 minutes. At 90 minutes

of infusion, CTCL.5 ranks the highest ($94.72 \pm 0.35\%$), and the second highest is GT ($94.05 \pm 0.55\%$). According to our findings, high phenol and flavonoid levels in tea infusions might be responsible for their outstanding DPPH scavenging power (more than 80%) across all tea infusions. As a result, it may be hypothesised that with extended infusion time, the radical scavenging potential of tea infusions can be enhanced.

FRP assay measures the samples' ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This assay has potential implications for biological systems, as it indirectly reflects the antioxidant ability of the tested samples. Results from this study revealed greater variations in FRP values across different tea infusions [Figure 3b]. Specifically two infusions, namely CTCL.5 and BP, exhibited ferric reducing activity greater than $50 \mu\text{g AAE/mL}$, i.e. $50.67 \pm 0.362 \mu\text{g AAE/mL}$ and $50.48 \pm 0.413 \mu\text{g AAE/mL}$ respectively. The descending order of FRP values was as follows: CTCL.5 ($50.67 \pm 0.362 \mu\text{g AAE/mL}$) > BP ($50.48 \pm 0.413 \mu\text{g AAE/mL}$) > BOP ($49.62 \pm 0.460 \mu\text{g AAE/mL}$) and OF ($49.62 \pm 0.450 \mu\text{g AAE/mL}$) > BOPSM ($47.24 \pm 0.619 \mu\text{g AAE/mL}$) > CTCG1 ($46.38 \pm 0.382 \mu\text{g AAE/mL}$) > PD ($42.67 \pm 0.460 \mu\text{g AAE/mL}$) > CTCL1 ($38.19 \pm 0.286 \mu\text{g AAE/mL}$) > CTCBP.5 ($29.81 \pm 0.469 \mu\text{g AAE/mL}$) > CTCBP.25 ($25.62 \pm 0.407 \mu\text{g AAE/mL}$) > D ($17.90 \pm 17.90 \mu\text{g AAE/mL}$) > OT ($16.95 \pm 0.472 \mu\text{g AAE/mL}$) > CTCbg ($13.05 \pm 0.336 \mu\text{g AAE/mL}$) > GT ($10.57 \pm 0.375 \mu\text{g AAE/mL}$) > CTCG.5 ($6.29 \pm 0.522 \mu\text{g AAE/mL}$). Surprisingly, six tea grades (except "D") showed promising results in FRP analysis. All CTC teas with additives – CTCL.5, CTCG1, CTCL1, CTCBP.5, and CTCBP.25 –, except CTCG.5, revealed greater FRP percentage than CTCbg (without additive). Overall, the findings of this study suggest that additives in CTC teas may enhance FRP activity, supporting the inclusion of spices like lemon, black pepper, and ginger in tea.

Lipid peroxidation inhibition assay helps us to examine the antioxidants' ability to prevent the peroxidation of lipid biomolecules of liver cells, which is also indicative of hepatoprotective activity. Results obtained for this test are shown in Figure 3c. Among all tested tea infusions, GT was determined to have the highest percentage of lipid peroxidation, i.e. $58.27 \pm 0.66\%$, and it also recorded the highest TPC, indicating that phenolic compounds in GT were responsible for minimizing lipid peroxidation. The lowest percentage of anti-lipid peroxidation was revealed by BOPSM, i.e. $7.13 \pm 0.972\%$. FRP of CTCbg was $9.01 \pm 0.524\%$, which was much lower than CTCL1 ($30.61 \pm 0.662\%$), CTCL.5 ($31.86 \pm 0.555\%$), CTCBP.5 ($36.68 \pm 0.821\%$), CTCG.5 ($37.94 \pm 0.816\%$), CTCBP.25 ($36.48 \pm 0.761\%$), and CTG1 ($38.99 \pm 0.960\%$), indicating that added lemon, ginger, and black pepper increased the lipid peroxidation inhibitory activity of CTC tea. Consequently, this study suggests that the antioxidant properties of tea infusions can be enhanced through synergistic interaction with lemon, ginger, and black pepper. The sample OT exhibited a considerable TPC value ($28.93 \pm 0.788\%$), while the concentrations

of TPC varied greatly among the six tea grades selected. The decreasing order of lipid peroxidation inhibition percentages of CTC grades (collected from factory) were as follows: BP ($35.64 \pm 0.948\%$) > D ($33.9 \pm 0.876\%$) > OF ($28.51 \pm 0.764\%$) > PD ($23.90 \pm 0.806\%$) > BOP ($14.47 \pm 0.914\%$) > BOPSM ($7.13 \pm 0.972\%$). BP ranked the highest and also exhibited high TPC and TFC values, together with strong performance in FRP assay, suggesting superior antioxidant properties among the studied tea grades.

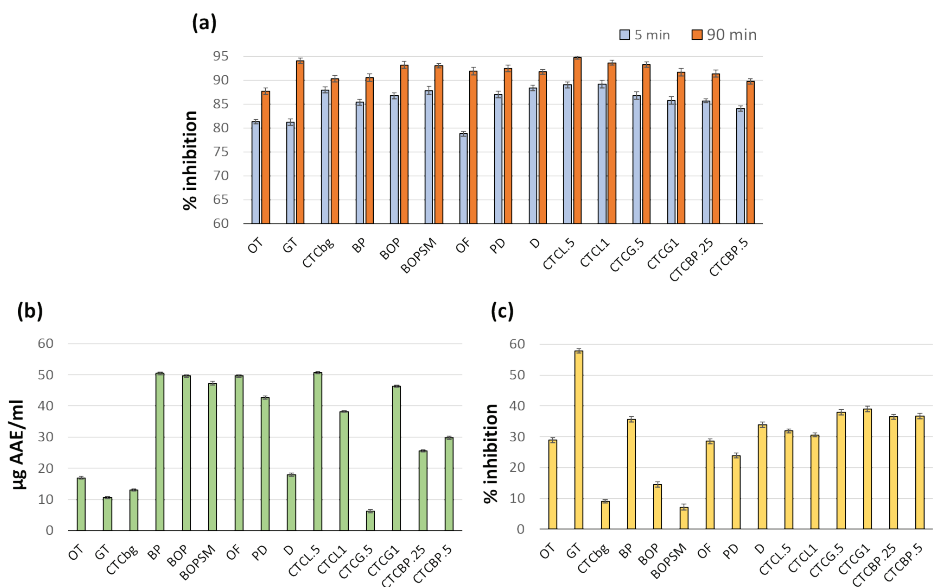


Figure 3. *In vitro* antioxidant assays: (a) DPPH scavenging power, (b) Ferric reducing power, and (c) Lipid peroxidation inhibition power of the studied tea infusions

Correlation and multivariate statistical analysis (PCA)

The heat map provided in *Figure 4* shows an uneven correlation between antioxidant activities and total phenol content or total flavonoid content. The correlation coefficient of $r = 0.58$ was obtained between total phenol content and lipid peroxidation inhibition potential (LPIP), suggesting that phenols have contributed positively to inhibiting the peroxidation of lipid molecules, while the moderate negative correlation of $r = -0.52$ and $r = -0.45$ of DPPH activity and FRP with TPC indicates that antioxidant properties do not depend solely on total phenolics. Rather, the specific composition and structure of phenolic compounds, including free and bound phenolics (Avinash *et al.*, 2024), the presence of non-

phenolic antioxidants such as ascorbic acid, carotenoids (Maqbool et al., 2023), or interactions among phytochemicals produce antagonistic effects on the antioxidant activity of some phenols (Joshi et al., 2022), thereby weakening or reversing the correlation with TPC. Similar observations have also been reported (Al-Laith et al., 2019; Osman et al., 2020). Also, TFC has very little contribution to DPPH activity and LPIP, while no contribution to the FRP of tea infusion given that correlation coefficients of $r = 0.23$, $r = 0.15$, and $r = -0.55$ were obtained respectively. Overall, it is understood that apart from phenol and flavonoid content, antioxidant activities in tea were largely influenced by multiple factors like the presence of other compounds (tannins, coumarins, alkaloids) and possible interactions between these phytochemicals.

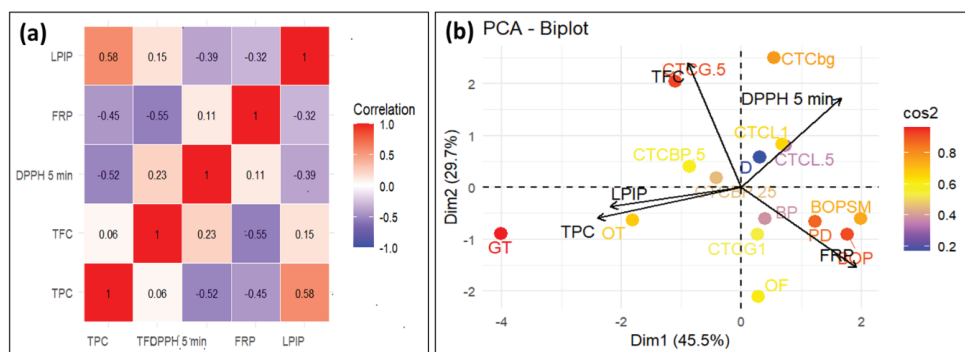


Figure 4. (a) The correlation matrix illustrating the relationships among TPC, TFC, DPPH (5 minutes), FRP, and LPIP, with correlation coefficients (r) ranging from 1 to +1; (b) PCA biplot showing the distribution of 15 samples based on TPC, TFC, DPPH (5 minutes), FRP, and LPIP

Principal Component Analysis (PCA) was performed to examine the variability among the biochemical properties (TPC and TFC) and antioxidant activities (DPPH, FRP, and LPIP) of tea infusions. In the resultant biplot (Figure 4), we can observe a maximum variability of 45.5% and 29.7% by PC1 and PC2, accounting for a total variability of 75.2%. PC1 differentiated the samples based on DPPH at 5 minutes and FRP activities. Ten out of fifteen samples, all belonging to the CTC type (CTCBg, CTCL1, BOP, PD, BOPSM, BP, etc.) were positively loaded on PC1 quadrants. Negative loadings of TPC, LPIP, OT, and GT on PC1 reveal their negative relationship with parameters of PC1. The close association between LPIP and TPC suggests a positive correlation between the two. One significant finding of the PCA analysis in this study was that both GT and OT have a positive correlation to TPC, distinguishing them from all other tea samples and providing insight into their oxidation level and processing methods. Green tea and orthodox tea allow little to moderate oxidation, keeping the polyphenols intact, while CTC tea undergoes

intensive mechanical processing, which may convert polyphenols into other compounds, thereby lowering the measurable TPC. Moreover, the distant placement of GT on the negative side of PC1 corresponds to its high TPC and LPIP, suggesting its biochemical distinctness from the rest of the tea infusions, mainly due to the processing methods involved in making GT. Meanwhile, PC2 differentiates samples based on DPPH activity and TFC, and CTCbg, along with most of the CTC tea with additives – CTCG.5, CTCL1, CTCL.5, CTCBP.5, and CTCBP.25, except CTCG1 –, shows positive loadings on PC2. This indicates that DPPH activity at 5 minutes is dependent on the TFC content of the aforementioned tea, which might be actually influenced by the additives used.

***In vitro* gastrointestinal simulation**

While passing through the gastrointestinal (GI) tract, ingested food/drink is subjected to the action of various digestive enzymes like amylase, pepsin, pancreatin, etc., upon varying pH at different phases of digestion (oral, gastric, and intestinal) (Carbonell-Capella *et al.*, 2014). The enzymes will tend to convert the complex biochemicals in foods/drinks into simple and easily absorbable biomolecules. The measure of the released bioactive compounds (nutrients) from foods/drinks as a result of digestion by enzymes can be termed as its “bioaccessibility” (Mengucci *et al.*, 2020). Therefore, to assess the nutritional value of tea, it is important to evaluate the bioaccessibility of the bioactive compounds present in the digested mixture.

In vitro gastrointestinal simulation is a simple and cost-effective experiment that allows us to predict the percentage of biomolecules that are digested in each phase. Since tea is mostly celebrated for its antioxidant properties (Vishnoi *et al.*, 2018), the nutritional efficacy of tea is tested by estimating the antioxidant activity after each phase of digestion (only gastric and intestinal phases are considered). Results after post-digestion are presented in *Figure 5a–i*, which provides us a graphical representation of the amounts of DPPH scavenging activity, as well as TPC and TFC in post-simulated gastric and intestinal phases of digestion with respect to crude infusion (undigested). From the graphs, a high rate of digestion of antioxidant molecules can be observed in the gastric phase, followed by the intestinal phases of digestion, as indicated by a steep slope. However, for TPC and TFC digestion, variations among different tea infusions were revealed. After comparing TPC and TFC of tea infusions in PSGF and PSIF with crude infusions, the maximum reduction of TPC was observed in GT (from 2.077 ± 0.060 to 0.618 ± 0.069) mg GAE/mL, followed by OT (from 1.323 ± 0.022 to 0.293 ± 0.068) mg GAE/mL in PSGF. Likewise, the maximum reduction of TFC was noticed in CTCbg (from 35.54 ± 0.643 to 14.81 ± 0.95) mg GAE/mL in PSGF. Furthermore, reduction in DPPH activity of infusions CTCL.5, CTCG.5, CTCBP.25, and CTCBP.5 (*Figure 4c*) in PSGF was observed as mainly owing to a reduction in their TFC

(Figure 5i). Similar results were revealed by moringa leaf infusions, Wedang uwuh (traditional beverage), and kodo-based beverages (Martínez-Las Heras et al., 2017; Fauziah et al., 2023; Majumder et al., 2024). The acidic pH and the action of digestive enzymes (pepsin) may have caused instability in the structures of some antioxidant molecules (Moyo et al., 2020; Lou et al., 2021), indicating a decrease in the DPPH activity of tea infusions (post-gastric digestion). Digestion continues in the intestinal phase by the action of pancreatin, leading to decrease in the respective contents.

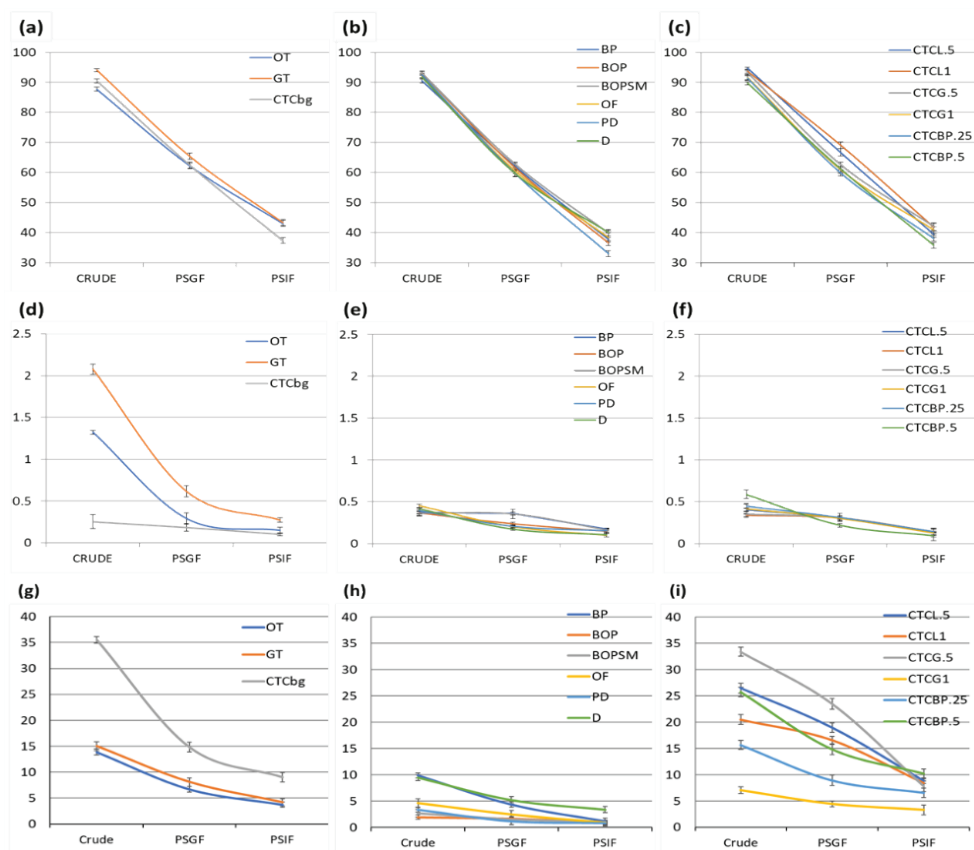


Figure 5. Decrease in DPPH scavenging activity: (a)–(c) total phenol content (TPC); (d)–(f) total flavonoid content (TFC); (g)–(i) of all tea infusions in the crude sample (undigested tea), the post-simulated gastric fluid (PSGF), and the post-simulated intestinal fluid (PSIF)

The bioaccessibility index was also calculated for all tea infusions after the gastric and intestinal phases of simulated digestion, and the obtained results are presented in Figure 6; accordingly, BI (%) for DPPH activity, TPC, and TFC

was much higher in post-simulated gastric fluid (PSGF) than in post-simulated intestinal fluid (PSIF). For DPPH scavenging power, BI (%) for all infusions appears to be increasingly stable in PSGF (over 60%) and PSIF (around 40%). The BI (%) for TPC and TFC showed more variations among tea infusions in both phases considered. Recorded BI (%) was the highest in CTCL1 (110.39%) for TPC in PSGF and the lowest in BP (11.96%) for TFC in PSIF. The higher bioaccessibility of DPPH activity, TPC, and TFC in PSGF than PSIF indicates the greater availability of these biomolecules for absorption after its digestion by gastric and intestinal enzymes (Mosele *et al.*, 2016). This research has delved into the antioxidant activity of studied tea infusions and assessed their nutritional efficacy in the GI tract by way of a simulation study of gastrointestinal digestion. The tea infusions considered in this research were bioaccessible, which thereby confirms the nutritional value of the studied tea within the GI tract.

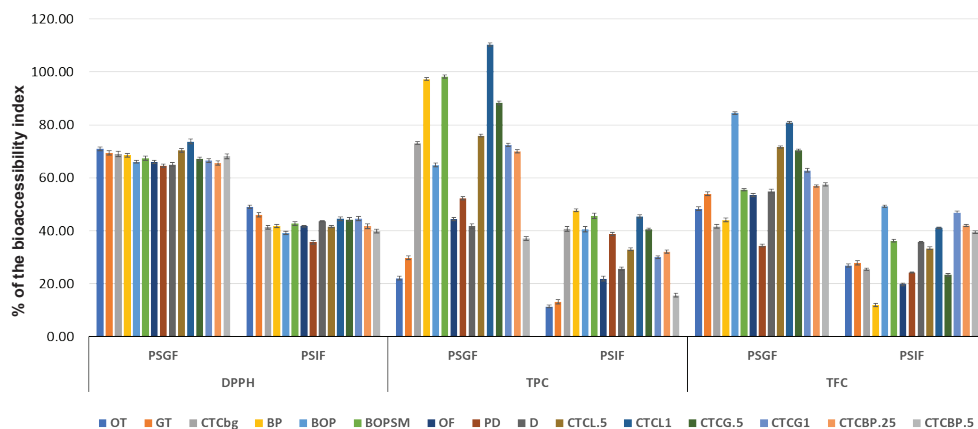


Figure 6. Percentage of the bioaccessibility index of DPPH scavenging activity, total phenol content (TPC) and total flavonoid content (TFC) of studied tea infusions in post-simulated gastric fluid (PSGF) and post-simulated intestinal fluid (PSIF)

4. Conclusions

The comparative evaluation of green tea (GT), orthodox tea (OT), and CTC (crush-tear-curl) tea, along with CTC infused with additives such as ginger, lemon, and black pepper has revealed significant differences in both qualitative and quantitative phytochemical analysis and bioactivities. The presence of key secondary metabolites, including flavonoids, phenols, coumarins, cardiac glycosides, steroids, and terpenoids, ensures the related bioactivities like antioxidant, anti-inflammatory, antidiabetic, and other health-promoting activities in the studied tea infusions.

The highest amount of total phenolic content in GT associated with high FRP and DPPH scavenging activity makes it a better choice over other tea grades when considering its antioxidant properties. The current study supports the inclusion of spices such as lemon, ginger, and black pepper in CTC tea because it significantly enhances total phenol content, lipid peroxidation inhibitory activity and DPPH scavenging activity. Furthermore, extending infusion time has a positive impact on DPPH radical scavenging activity, while the overextraction of biomolecules needs to be controlled, as it might disrupt the delicate flavour and aroma of tea. The high bioaccessibility of tea infusions within the gastrointestinal tract confirmed tea as a nutritionally rich beverage. This study confirms the potential health benefits of tea and encourages regular tea consumption – mainly GT, OT, BP, and CTC tea infused with lemon (CTCL5 and CTCL1). Also, investigations into the structural modifications of antioxidant molecules following the action of digestive enzymes should be carried out in order to properly understand the mechanisms involved.

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